Manipulating PEPC levels in plants

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Received 17 April 2002; Accepted 21 June 2002

Abstract

This review examines the current understanding of the structural, functional and regulatory properties of C4 and C3 forms of higher plant phosphoenolpyruvate carboxylase. The emphasis is on the interactive metabolic and post-translational controls acting on the enzyme in the physiological context of C4 photosynthesis and the anaplerotic pathway. A brief overview is given concerning the recent developments of PEPC-based genetic engineering of C3 plants with the aim of improving photosynthetic performance in normal and limiting environmental conditions. So far, in spite of achieving a considerable increase in PEPC levels, more work needs to be done with respect to the correct dosage and location before that goal is reached. Some unpublished results on the transformation of maize with a sorghum C4 PEPC cDNA are also presented. They show that it is possible to increase photosynthetic PEPC levels in this C4 plant and that the modification in enzyme content has a pleiotropic physiological impact and, notably, an improved water use efficiency when water is limited.

Key words: Genetic engineering, phosphoenolpyruvate carboxylase, signal transduction.

Introduction

Higher plant phosphoenolpyruvate carboxylase (PEPC; E.C. 4.1.1.31) is a multifaceted enzyme involved in various physiological contexts (Andreo et al., 1987). In C4 and CAM photosynthesis, a specific form of the enzyme initiates the pathway (Lepiniec et al., 1994), while in all plant types, a C3 PEPC is branched to glycolysis to replenish Krebs cycle intermediates (the so-called anaplerotic function), thereby providing precursors for amino acid synthesis (Stitt, 1999). Furthermore, in lipid-rich seeds, malate has been reported to be the best precursor for fatty acid synthesis in leucoplasts and a high PEPC activity coincides with seed maturation (Smith et al., 1992). A wealth of data has been gathered about the multigenic family encoding this enzyme, including the structure and function of genes and promoters and phylogenetic relationships (Chollet et al., 1996; Lepiniec et al., 1994). Since the discovery of PEPC by Bandurski and Greiner (1953), many studies have established the allosteric nature of this homotetrameric enzyme that is subject to an opposite and antagonistic effect of metabolites, for example, malate (negative feedback) and sugar-P (positive feedback). Interest concerning this enzyme has been revitalized after it was discovered to be subjected to post-translational control (Nimmo et al., 1984; Chollet et al., 1996; Vidal and Chollet, 1997). All plant PEPCs studied so far contain an N-terminal consensus domain that is phosphorylated on a regulatory serine by a dedicated, Ca2+-independent PEPC kinase (PEPCk). Pharmacological and molecular-based approaches have led to the dissection of the highly complex signalling cascade controlling PEPCk levels in C4 and CAM plants. Now, there are emerging data to support the idea that a similar cascade is also involved in the regulation of C3 plant PEPCk (Vidal and Chollet, 1997; J Vidal, unpublished data). Phosphorylation modulates the metabolic regulation of photosynthetic PEPC in a way that ensures protection against the feedback effect exerted by malate. Recently, the 3-D structure of the E. coli enzyme has been determined by X-ray diffraction thereby making clear the topography of active and inhibitor sites as well as the mechanism of inhibition (Kai et al., 1999).

Genetic manipulations have been undertaken to modify PEPC levels in plants with the goal of clarifying the role of the enzyme in various physiological contexts and to
improve plant productivity. One major approach has been to introduce this key biochemical component of the C4 photosynthetic pathway into C3 plants (Ku et al., 1999; Matsuoka et al., 2001). In this regard, preliminary work has been devoted to solving the problems of targeting the PEPC to the desired location and to achieving high expression levels in the host plants.

This report is designed to summarize data related to the regulatory and functional properties of both the photosynthetic (C4 type) and anaplerotic (C3 type) forms of PEPC. Then, a brief survey of what has been learnt from PEPC genetic manipulations in both C3 and C4 plants will be presented.

**Regulatory properties of the photosynthetic PEPC**

C4 plants display a concentric organization of photosynthetic leaf tissues (mesophyll and bundle sheath) in which enzymes of the photosynthetic pathway, C4 cycle and Benson-Calvin cycle, are distributed (Fig. 1). In the mesophyll cell cytosol, the C4 PEPC is subject to both light-dependent transcriptional and post-translational control (Schäffner and Sheen, 1992; Chollet et al., 1996). The expression of the photosynthetic gene and the accumulation of the corresponding C4 PEPC form in the cytosol of sorghum mesophyll cells are mediated by phytochrome and the regulatory phosphorylation of trans-acting factors during leaf greening (Rydz et al., 2000). These aspects of PEPC regulation are beyond the scope of this review and will not be developed further.

Regulation of C4 PEPC in the mesophyll cell cytosol involves photosynthesis-related metabolite effectors like glucose-6-phosphate (G-6-P; activator) and malate (feedback inhibitor) and a highly complex, light-dependent, reversible phosphorylation process (Chollet et al., 1996). This latter modification changes the functional properties of C4 PEPC, increasing $V_m$ and $K_a$ for G-6-P, and decreasing its sensitivity to malate when measured at suboptimal pH (7.3) and PEP concentration (2.5 mM). The recent determination of the 3-D-structure of *E. coli* PEPC has shed some light on the molecular mechanism of malate inhibition and how phosphorylation can relieve this effect (Kai et al., 1999). In the *E. coli* PEPC, arginine 587, located in a highly conserved glycine-rich loop, is shared by the aspartate binding site and the active site. Upon binding of this effector (equivalent to L-malate in the plant enzyme), the loop is removed from the catalytic site thus perturbing substrate binding and causing a loss of catalytic activity (Kai et al., 1999). It has been suggested that the plant invariant phosphorylated N-terminus (acid-base-X-X-S-I-D-A-Q-L-R) of the enzyme moves closer to the entry of the inhibitor site thus impeding access of malate. Recent mutational analyses based on the structural features of the C4 PEPC from maize have identified putative residues (R183 and R184) involved in the allosteric
activator site binding G-6-P (Terada et al., 2001). Moreover, a study of the enzyme chimera between C₄ and C₃ PEPC isoforms from Flaveria species and site-directed mutagenesis have revealed the crucial role of the residue at position 774 (serine in C₄ and alanine in C₃ enzymes) as a major determinant for the specific properties of each enzyme form (Bläsing et al., 2000).

In vitro, C₄ PEPC activity, phosphorylation and metabolic control are highly sensitive to pH (Echevarria et al., 1994; Gao and Woo, 1996). The increase in pH that has been proposed to occur in the mesophyll cell cytosol upon illumination of the C₄ leaf (between pH 7 and 7.5, see below) is expected to activate the C₄ PEPC and, partially, to promote desensitization of the enzyme towards the effectors, notably, L-malate. Therefore, most of the interactive players acting on the regulation of PEPC (i.e. pHc, G-6-P, phosphorylation) are opposed to the negative feedback exerted by malate.

The light-transduction chain leading to C₄ PEPC phosphorylation by its requisite, Ca²⁺-independent protein kinase has been studied by flow cytometry, confocal microscopy, molecular biology, and cellular pharmacology techniques using mesophyll protoplasts from crabgrass (Digitaria sanguinalis) and pharmacological reagents (Giglioli-Guivarc’h et al., 1996). This has led to a model of the spatio-temporal organization of the cascade in the C₄ leaf as shown in Fig. 2. In this scenario, 3-phosphoglyceric acid, generated during photosynthesis in bundle sheath cell chloroplasts represents the intercellular metabolic message that diffuses into mesophyll cells. In these cells, its subsequent transport into the chloroplasts under the protonated 2⁻ form is expected to cause alkalization of pHc. This early cascade event is followed by activation of a mesophyll cell phospholipase C (PI-PLC) and transient production of the second messengers inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ causes tonoplast calcium channels to open and efflux of calcium into the cytosol while DAG and calcium activate a PKC-like activity. The rapid synthesis of the calcium-independent PEPC kinase leads to the phosphorylation of C₄ PEPC. Question marks indicate the steps that remain to be elucidated.

![Fig. 2. Schematic model for the spatio-temporal organization of the transduction cascade in the C₄ leaf. An increase in 3-phosphoglyceric acid (3-PGA) ensures the intercellular coupling via pH changes in the mesophyll cell cytosol. This is followed by activation of a mesophyll cell phospholipase C (PI-PLC) and transient production of the second messengers inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ causes tonoplast calcium channels to open and efflux of calcium into the cytosol while DAG and calcium activate a PKC-like activity. The rapid synthesis of the calcium-independent PEPC kinase leads to the phosphorylation of C₄ PEPC. Question marks indicate the steps that remain to be elucidated.](image-url)
diacylglycerol (DAG). The ultimate step in the cascade implicates the nucleus and the up-regulation of a PEPCk gene. One consistent hypothesis might be that the Ca2+-dependent protein kinase phosphorylates a transcription factor that increases PEPCk gene expression. This highly complex, light-dependent cascade involves the contribution of various components (pHc, ion channels and signalling enzymes), cell types (bundle sheath and mesophyll) and subcellular compartments (chloroplast, vacuole, nucleus).

In CAM plants, up-regulation of the PEPCk and PEPC phosphorylation occurs during the night and is dependent on a circadian oscillator (Nimmo, 2000). In the CAM species Mesembryanthemum crystallinum, it has been shown that a cascade similar to that found in C4 plants operates in mesophyll cells during the night (Bakrim et al., 2001).

The Ca2+-independent PEPCk gene/cDNA has been cloned recently from CAM, C3 and C4 plants (Nimmo, 2000; Taybi et al., 2000; Tsuchida et al., 2001). This unique Ser/Thr protein kinase exhibits several interesting features. It is the smallest protein kinase known so far with a predicted molecular mass around 31 kDa (274, 279 and 284 amino acids in the case of the Kalanchoë fedtschenkoi, Mesembryanthemum crystallinum and Arabidopsis thaliana enzyme, respectively). Indeed, it is made up of a catalytic domain with minimal or no additions. Although it belongs to the Ca2+-calmodulin-regulated group of protein kinases, it lacks the regulatory auto-inhibitory region and the EF-hands. It displays an alkaline pHc of around 8 (using the recombinant enzyme from Mesembryanthemum crystallinum) and, in reconstituted assays, it specifically phosphorylates the N-terminal regulatory serine of the target PEPC, thereby decreasing the malate sensitivity of the enzyme, as expected. Its activity is not modulated directly by second messengers (such as Ca2+/calmodulin or cyclic nucleotides) or by phosphorylation/dephosphorylation processes, but rather through rapid changes in its turnover rate.

From a physiological point of view, the blocking of PEPCk synthesis in the illuminated C4 leaf led to a marked inhibition of CO2 assimilation (Bakrim et al., 1993). Therefore C4 PEPC phosphorylation appears to be a critical event governing carbon assimilation in the C4 photosynthetic pathway. These complex mechanisms involving the light-dependent control of C4 PEPC at both the transcriptional and post-translational levels allow the adjustment of intercellular carbon flow according to the demand of the Calvin cycle and thus ensure efficient functioning and homeostasis of C4 photosynthesis.

Regulatory phosphorylation of PEPC in the anaplerotic pathway linked to amino acid synthesis

In the C3 plant leaf, PEPC is no longer involved in photosynthesis, but fulfils a variety of physiological roles. In the anaplerotic pathway (Fig. 3), that also occurs in C4 plants, the enzyme contributes to the replenishment of Krebs cycle intermediates when organic acids are directed towards other metabolic pathways such as amino acid (via the GS/GOGAT cycle) and protein synthesis (Stitt, 1999; Champigny and Foyer, 1992). In this respect, PEPC can be considered as a branch of the glycolytic pathway. In relation to the anaplerotic function, PEPC activity also contributes to the homeostasis of cell cytosolic pH (Sakano, 1998) and the chloroplastic OAA/malate shuttle that provides the cytosol with reducing power required by the nitrate reductase (NR) (Oaks, 1994). As nitrogen assimilation proceeds, primary metabolism is reset so that more carbon is diverted to respiratory metabolism by means of a complex co-ordinated regulation of many enzymes and transporters, including signalling networks and metabolites. Intuitively, the concept that PEPC must be protected against malate (via a phosphorylation process) as proposed in C4 and CAM plant photosynthesis, should apply to any system in which the concentration of this metabolite increases, such as anaplerotic C-flow and its interaction with N-metabolism. Indeed, regulatory phosphorylation of C3 PEPC is supported by a number of facts. (1) The presence of the N-terminal phosphorylation domain in all plant PEPCs sequenced so far, whatever the physiological type. (2) The presence of a Ca2+-independent PEPC-kinase in leaves of C3 plants (Vidal and Chollet,
elements identified in C4 mesophyll cells are also key players in the C3 cascade remains poorly documented. Experiments using barley leaf protoplasts have suggested that, while PEPC phosphorylation occurs in situ in the light and is modulated by protein synthesis and calcium, the mechanism leading to up-regulation of the corresponding PEPCk might differ from that found in C4 mesophyll protoplasts (Smith et al., 1996). However, recent results (J Vidal, unpublished data) suggest that, in addition to the PEPCk, the central component of the C4 cascade (namely, a C3 PI-PLC) is present in the C3 plant signalling system. This finding may also indicate that alkalization of pHc is a requisite step of the C3 cascade. In support of this hypothesis are experimental data showing that mesophyll cell pHc increases upon illumination of the C4 plant leaf (Yin et al., 1990). However, this point needs to be characterized further. The rate of C-flux through the anaplerotic PEPC is also modulated by NO3− and/or amino acids via a change in PEPC phosphorylation status (Li et al., 1996; Van Quy and Champigny, 1992; Champigny and Foyer, 1992). Collectively, the data indicate that the C3 leaf PEPCk activity increases in the light and that leaf N-status can influence the regulatory phosphorylation of C3 PEPC.

Transgenic plants

Recent developments in plant genetic engineering have allowed the production of C3 and C4 transgenic plants that are aimed at studying the function of PEPC gene promoters and the impact of over- and under-expression of the enzyme on the physiology and growth of the transformed plants.

C3 plants

C3 photosynthesis suffers from O2 inhibition due to the fact that Rubisco is a bifunctional enzyme with competing oxygenase and carboxylase activities. Oxygenation of RuBP initiates the photorespiratory pathway that subsequently leads to a considerable loss of carbon (and perhaps N). By contrast, C4 plants have evolved a CO2 concentrating mechanism (the C4 cycle) to overcome this wasteful process (see below and Fig. 1). The acquisition of this new photosynthetic strategy by a wide variety of plant species indicates that it has originated independently and on many separate occasions during the evolution of flowering plants (Lepiniec et al., 1994). The important question is: can evolution be mimicked by taking advantage of genetic manipulations to import some C4 photosynthetic characteristics into C3 species? One key enzyme of the C4 cycle is the C4 PEPC that occurs at very high levels in the mesophyll cell cytosol where it very efficiently assimilates CO2 (as the hydrated HCO3− form). Over the last three years, several attempts have been made to transfer C4 photosynthetic traits, with emphasis on PEPC, into C3 plants in order to improve CO2 assimilation rates. It has been found that the C4 PEPC gene promoter (from maize) can drive high level expression of a reporter gene in transgenic rice plants in an organ-specific, mesophyll-cell-specific and light-dependent manner (see Matsuoka et al., 2001). The next experiments used plasmid constructs containing a full-length PEPC cDNA fused to the C4 PEPC gene promoter. In tobacco, a 2–5-fold increase in leaf PEPC activity (maize C4 PEPC) was accompanied by a corresponding increase in malate content. However, these biochemical differences did not produce any significant physiological changes with respect to photosynthetic CO2 assimilation rate and CO2 compensation point (Matsuoka et al., 2001). In transgenic potato overexpressing a bacterial PEPC gene, the CO2 compensation point was found to be lowered, together with an increase in respiration and glucose and starch contents (Gehlen et al., 1996; Häusler et al., 1999). Therefore, these first attempts were successful in terms of PEPC ectopic expression in C3 plants, however, the overexpression levels were generally low.

More recently, the use of the Agrobacterium-mediated transformation of rice with an intact C4 PEPC gene of maize has allowed this problem to be solved. Indeed, a tremendous increase in mesophyll cell PEPC was attained that accounted for about 12% of total leaf soluble protein, a level that exceeds by 2–3-fold the enzyme concentration in C4 leaves (Ku et al., 1999). These plants exhibited reduced O2 inhibition of photosynthesis but photosynthetic rates were comparable to those of untransformed plants. Moreover, the maize PEPC in transgenic rice leaves remained in its dephosphorylated and less active form during illumination (Matsuoka et al., 2001).

Therefore, although progress is being made in manipulating PEPC levels in C3 plants, efficient strategies to obtain the desirable phenotypes are yet to be formulated. As mentioned above, the maize C4 PEPC is not phosphorylated in the leaves of transformed rice and, therefore, it is expected to be weakly active. This raises the question of whether the exogenous enzyme is a poor target for the C3 leaf PEPCk, or alternatively, whether a compensation mechanism is induced to buffer the impact of the genetic modification. Another potential problem is the availability of PEP in the mesophyll cells of C3 plants. Too much...
PEPC is expected to decrease the content of this substrate to a level that would perturb plant metabolism.

In a similar strategy, transgenic plants have been produced using genes or cDNAs encoding pyruvate-Pi dikinase (PPDK) and NADP-malic enzyme (NADP ME) and it has been shown that it is possible to overexpress the corresponding enzymes in C₃ leaf mesophyll cells. However, it was found that PPDK overexpression had no effect on carbon metabolism while NADP-ME had a detrimental effect (Matsuoka et al., 2001). Ideally, the C₄ pathway genes should be expressed at the correct level and ratios in the correct cell compartments of leaf tissues that lack the Kranz anatomy. Interestingly, a recent report has described the existence of the biochemical characteristics of C₄ photosynthesis in the stems and petioles of C₃ plants (Hibberd and Quick, 2002). The presence of the correct biochemical pathways (e.g. decarboxylating enzymes, NAD and NADP-ME; PEP regeneration, PPDK) in the photosynthetic cells surrounding the vascular bundles indicates that the essential biochemical components and regulatory elements controlling the cell specific gene expression required for C₄ photosynthesis are already present in C₃ plants. This may explain the polypheletic evolution of C₄ plants. Understanding how this system has evolved in leaves of C₃ plants to give rise to C₄ plants during evolution may give important clues that would help to manipulate C₃ plants more efficiently. For example, the promoter of an NADP-ME gene from bean has been shown to direct expression in cells around the vascular systems (Schaaf et al., 1995).

C₄ plants

In photosynthetic tissues of the illuminated C₄ leaf, the C₄ cycle acts as a pump increasing CO₂ levels in the vicinity of Rubisco. As a consequence of the elevated ratio of CO₂/O₂, the oxygenase activity of this enzyme and, thus, photorespiration, are markedly reduced with respect to C₃ plants. When grown in their natural environment, often consisting of hot climates with sporadic rainfalls, C₄ plants have a selective advantage over C₃ plants. They show higher photosynthetic rates, biomass productivity and water and nitrogen use efficiencies. Within the last century, the use of classical plant breeding methods has led to a doubling of the yield of maize. This was essentially due to an increase in the leaf surface and not to an improved performance of the photosynthetic apparatus. Because the CO₂ pumping system overcomes O₂ inhibition of photosynthesis and causes the CO₂ compensation point to be very low, C₄ photosynthesis is near saturated at atmospheric CO₂ levels. Therefore, it is believed that engineering higher PEPC levels in C₄ plants should not lead to a significant improvement in photosynthetic efficiency. In addition, the metabolic and post-translational regulation of the C₄ PEPC may afford compensation effects counteracting the quantitative modification. However, when maize is subjected to a water stress, photosynthesis is depressed, although C₄ PEPC content is slightly increased (Rodriguez-Penagos and Munoz-Clares, 1999). The impact of water stress is quite complex and, in essence, pleiotropic, notably altering photosynthesis following an increase in stomatal closure (mild progressive stress) (Cornic, 2000) and/or irreversible damages to the photosynthetic apparatus (e.g. ATP-synthase) (Tezara et al., 1999) leading to lower ATP contents and CO₂ assimilation rates. However, within the range of relative water content occurring in natural conditions, it is likely that stomatal closure plays the major role in the decrease in leaf photosynthesis while the photosynthetic machinery remains intact (Cornic, 2000). As a consequence of stomatal closure, a consistent hypothesis is that both the internal and chloroplastic CO₂ molar ratio decline, and CO₂ is progressively replaced by O₂ thus favouring the oxygenase activity of Rubisco.

Maize is particularly sensitive to water stress during its reproductive stage which leads to a strong impairment of grain filling. Under a short-term, moderate water stress induced by sorbitol, the activity of C₄ PEPC showed a 50% increase in maize leaf discs. This increase was correlated with enhanced proline levels, thus suggesting that C₄ PEPC could be involved in plant stress adaptation (Rodriguez-Penagos and Munoz-Clares, 1999). Although the C₄ PEPC of *Amaranthus edulis* has an estimated flux-control coefficient over the CO₂ assimilation rate of 0.26 for plants grown at ambient CO₂, this becomes much higher (0.68) when plants are grown at low CO₂. Therefore, the enzyme is expected to contribute significantly to the control of C flux in the photosynthetic pathway under the limited internal CO₂ conditions promoted by drought (Dever et al., 1997). Based on these data, it can be hypothesized that increasing the C₄ PEPC content by genetic engineering may positively impact on (1) osmotic adjustment, providing precursors for proline biosynthesis and (2) the capacity to fix CO₂ at lower gas conductance. Enhancing the performance of the CO₂ trapping system would ensure both higher CO₂ levels in the vicinity of Rubisco and lower rates of photorespiration, and may contribute to a better tolerance of C₄ plants to water deficit.

To test this hypothesis, maize plants were engineered by *Agrobacterium*-mediated infection or biotic techniques with a plasmid containing a full length C₄ PEPC (from sorghum) cDNA under the control of the C₄ gene promoter (leaf specific). Transgenic maize overexpressing (2.2-fold) or underexpressing (0.2-fold by a co-suppression effect) this C₄ PEPC were produced and characterized at the molecular and physiological levels. Sorghum C₄ PEPC was expressed in the mesophyll cell cytosol and found to undergo phosphorylation in the light. However, the phosphorylation status of the enzyme was significantly lower in overexpressor plant leaves subjected to non-
saturating light. This was not due to a decrease in PEPC levels and down-regulation of the phosphorylation cascade. In PEPC-deficient mutants of the C4 plant *Amaranthus edulis*, a compensatory mechanism increasing the C4 PEPC phosphorylation status has been described (Devet et al., 1997). Therefore, the plant can respond to the modification of PEPC levels by modulating the phosphorylation status of the enzyme.

Physiological studies showed that ectopic expression of C4 PEPC has a pleiotropic effect on maize plants. In particular, the engineered plants displayed a better capacity to fix CO2 in water-sufficient conditions. Under moderate drought, most of the characteristics investigated (CO2 fixation rate, CO2 compensation point, fresh and dry weight, leaf surface, and stomatal density) were improved in the overexpressors and depressed in the underexpressors. Furthermore, in addition to the above-mentioned characteristics, the overexpressor showed a significant (+30%) improvement in the intrinsic water use efficiency (WUE). Elevated values of WUE can be due to the better capacity of the plants to fix CO2 when the gas conductance is reduced by water-limiting conditions. Contributing to the limitation in gas conductance is the observed reduction in leaf stomatal density of the overexpressor. Recent results have shown that the stomatal density of developing leaves depends on light intensity and CO2 concentration sensed by mature leaves (Brownlee, 2001). Studies on *Arabidopsis* mutants altered in stomatal density response and patterning suggest that *HIC* and *SDD1* genes are involved in favouring diffusion of long-distance systemic signals and processing of signalling proteins. Along these lines, it is interesting to note that the stomatal density of transgenic maize leaves (4th leaf) varied in an opposite way with respect to C4 PEPC amount. Thus, the lower gas conductance, due to the reduction of stomatal aperture (under stress) and density, and high C4 PEPC activity, that maintain higher CO2 fixation levels, may account for the improved WUE and biomass production of the C4 PEPC overexpressor.

As mentioned above, PEPC has been suggested to be implicated in the lipid filling of castor oil seeds during maturation (Smith et al., 1992). The highest enzyme content coincided with the most active phase of storage oil accumulation. Furthermore, it has been demonstrated that malate supports very high rates of fatty acid synthesis in isolated leucoplasts from developing seeds (Smith et al., 1992). In another set of experiments, *Agrobacterium*-mediated and biolistic transformation techniques were used to alter the PEPC content of maize seeds with DNA constructs containing the C4 PEPC cDNA from sorghum in fusion with the High-Molecular-Weight Glutelin (HMWG) promoter from wheat. Biochemical analyses showed that there was no significant change in the lipid content of transformed seeds compared to wild-type seeds (although the transgenics contained up to 10-fold more PEPC than the wild-type seeds). By contrast, a weak but statistically significant positive correlation was detected with protein, starch and soluble sugar levels. Several hypotheses can account for these observations. Perhaps the PEPC is not contributing significantly to fluxes through the different metabolic pathways leading to storage seed components. Maybe the functional and regulatory properties of the C4 PEPC (e.g. $K_m$ for PEP, regulatory phosphorylation and malate sensitivity) are not well adapted to the physiological context of seeds. It is also possible that the HMWG promoter activity does not coincide with the phase of seed filling in which PEPC plays a role.

**Concluding remarks**

It is now possible to produce transgenic C3 and C4 plants that ectopically express foreign PEPC cDNA and genes. These techniques pave the way to studies devoted to engineering crop plants with PEPC in an attempt to improve photosynthetic rates, yield, biomass, and tolerance to abiotic stress. However, future advances could be hindered by the fragmented state of the current understanding of plant metabolism and the growing gap between an ability to clone, study and manipulate individual genes and proteins and an understanding of how they are integrated into and impact on the complex metabolic networks in plants (DellaPenna, 2001). Indeed, this approach is still in its infancy and suffers from several technical and conceptual limitations. From a general point of view, the control analysis theory states that control over a metabolic flux is generally shared by several enzymes of a pathway, and it is expected that ‘large increases in flux cannot be generated by activation of single enzymes, but can be by activation at several sites along the pathway’ (Fell and Thomas, 1999). This has been observed in a number of cases and, to overcome this problem, it will be necessary simultaneously to increase, together with PEPC, those enzymes which exert a control over the flux pathway, and perhaps also regulatory genes impacting on their activity. Furthermore, in the case of highly regulated enzymes, compensatory mechanisms to offset the effect of the genetic modification do occur. Finally, for the recombinant enzyme in a given physiological context, the substrate may be limiting, or the increased amount of enzyme may have deleterious effects on the subtle balance of related metabolites. It is now known that many metabolites trigger signalling processes impacting on target gene expression and metabolic controls of several enzymes. Collectively, these data emphasize the point that a correct dosage and location of the introduced enzymes must be achieved. However, how is it possible to install the Kranz anatomy with its division of labour between specialized photosynthetic cells in a C3 leaf? Assessment of the single cell-C4 system present in some aquatic plants...
like *Hydrilla verticillata* as an alternative approach is currently under investigation (Leegood, 2002; Häusler et al., 2002), yet it is thought that a certain degree of compartmentation will be required to achieve the goal (Leegood, 2002). In spite of the wealth of data currently available to guide genetic manipulation, it is still mandatory to develop knowledge about the physiological context in which the enzyme (or gene) of interest is to be inserted and the functional and regulatory mechanisms modulating its activity. Even when this knowledge becomes available, the introduction of a new gene may have pleiotropic effects on the host plant, making the result of the transgenic experiment somewhat unpredictable and implying an unavoidable touch of empiricism. This is the case, for example, of the transgenic maize overexpressing the C₄ PEPC, for which both growth and developmental processes have been altered by the single gene transformation. In addition, this work has revealed that improved performances induced by the recombinant protein might only be detected following exposure of the plant to limiting environmental conditions.

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