Molecular and biochemical characterization of the sunflower (*Helianthus annuus* L) cytosolic and plastidial enolases in relation to seed development

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Abstract

In the present study, we describe the molecular and biochemical characterization of sunflower (*Helianthus annuus* L.) enolase (ENO, EC 4.2.1.11) proteins, which catalyze the formation of phosphoenolpyruvate, the penultimate intermediate in the glycolytic pathway. We cloned and characterized three cDNAs encoding different ENO isoforms from developing sunflower seeds. Studies using fluorescently tagged ENOs confirmed the predicted subcellular localization of ENO isoforms: *HaENO1* in the plastid while *HaENO2* and *HaENO3* were found in the cytosol. The cDNAs were used to express the corresponding 6(His)-tagged proteins in *Escherichia coli*. The proteins were purified to electrophoretic homogeneity, using immobilized metal ion affinity chromatography, and biochemically characterized. Recombinant *HaENO1* and *HaENO2*, but not *HaENO3* were shown to have enolase activity, in agreement with data obtained with the *Arabidopsis* homolog proteins. Site directed mutagenesis of several critical amino acids was used to attempt to recover enolase activity in recombinant *HaENO3*, resulting in very small increases that were not additive. A kinetic characterization of the two active isoforms showed that pH had similar effect on their velocity, that they had similar affinity for 2-phosphoglycerate, but that the k_{cat}/K_m of the plastidial enzyme was higher than that of the cytosolic isoform. Even though *HaENO2* was always the most highly expressed transcript, the levels of expression of the three *ENO* genes were remarkably distinct in all the vegetative and reproductive tissues studied. This indicates that in seeds the conversion of 2-phosphoglycerate to phosphoenolpyruvate takes place through the cytosolic and the plastidial pathways therefore both routes could contribute to the supply of carbon for lipid synthesis. The identity of the main source of carbon during the period of stored products synthesis is discussed.

Keywords: sunflower, enolase, seed, glycolytic metabolism, lipids

Abbreviations:

ENO enolase

G6P glucose-6 phosphate

OPPP oxidative pentose phosphate pathway

TP triose phosphate

2-PGA 2-phosphoglycerate

PEP phospho*enol*pyruvate

PK pyruvate kinase

Pyr pyruvate

1. Introduction

Sunflower (*Helianthus annuus* L.) heterotrophic seeds, photosynthetically inactive during their development, are unable to use light energy to produce ATP and NADPH, and therefore rely completely on the maternal supply from photosynthetic tissues for building up reserves. Photosynthetic sucrose is exported to sink tissues providing the majority of carbon in seed lipids [1, 2]. Accordingly, sucrose is the principal source of carbon provided by the mother plant to the developing embryos [3]. Sucrose is enzymatically cleavage and hexoses and hexoses-phosphates enter the glycolysis pathway generating carbon, reducing equivalents, and energy for *de novo* intraplastidial fatty acid synthesis [4, 5]. Measurement of soluble carbohydrate levels in sunflower seeds have shown high hexose consumption associated with the synthesis of storage products, such as lipids [6]. Hexoses fate is mainly the cytosolic and plastidial glycolysis, both pathways interconnected by membrane transporters [7]. This transport of metabolites is fundamental for sunflower plastids due to previously described incapacity to use light energy [8]. In sunflower seed metabolism glucose-6 phosphate (G6P) has been identified like a major connexion between both subcellular compartments. Within plastids G6P feeds the oxidative pentose phosphate pathway (OPPP), generating the NADPH required to the *de novo* fatty acid synthesis [8, 9]. Triose phosphates (TP) constitute another chiefly connexion between cytosolic and plastidial glycolysis, most of the carbon in fatty acids is derived from these glycolytic intermediates. Although TP are found in both locations the comparison between enzyme activities found in the cytosol and within plastids in developing sunflower embryos has allowed the identification of the cytosolic pathway as the predominant source of carbon for lipid biosynthesis [6].

The glycolytic pathway is a catabolic route that ends with the synthesis of pyruvate (Pyr). In *Arabidopsis* embryos, the conversion of phosphoenolpyruvate (PEP) to Pyr, catalyzed by pyruvate kinase (PK), is mainly located in the plastids [10, 11]. The preceding reaction is catalyzed by enolase (ENO) which insures the Mg^{2+} -dependent conversion of 2-phosphoglycerate (2-PGA) to PEP. Plant ENOs are encoded by a small gene family [12, 13]. Previous studies have provided evidence that *Arabidopsis* ENO isoforms are present in both the plastid and the cytosol [14, 15]. Furthermore, in *Arabidopsis*, cytosolic glycolysis is thought to provide PEP necessary to support plastidial oil production [15]. Although, recently, the participation of the plastidial glycolysis has been claimed as a potential contributor to the process [16].

Previously, we have shown that in sunflower embryos crude extracts ENO activity increases during development in conjunction with oil deposition, and that most of this ENO activity is associated to the cytosol [6]. These data could indicate that, in developing sunflower seeds, oil accumulation is mainly supported by PEP generated in the cytosol. However, further characterization of the different ENOs expressed in the embryos is required in order to better understand the participation of cytosolic and plastidial glycolysis during oil synthesis.

In this study, we have identified and characterized three sunflower ENO isoforms. We surveyed the expression of *ENO* genes and the subcellular localization of the corresponding protein products. Recombinant ENO proteins were expressed in *Escherichia coli* in order to determine their kinetic properties. Similarly, to the situation observed in *A. thaliana*, we found that one the three ENO proteins did not have catalytic activity. Site directed mutagenesis was used to recover low levels of activity for this protein. These data extend our understanding of the contribution of ENO isoforms to the conversion of 2-PGA to PEP in the cytosolic and plastidial compartments of sunflower seeds during fatty acid synthesis. Our results are discussed in relation to a model that recapitulates current knowledge on the importance of the glycolytic pathway in sunflower seed filling.

2. Materials and methods

2.1. Biological materials, culture conditions and chemicals

Sunflower (*Helianthus annuus* L.) line CAS-6, with normal fatty acids composition and oil content, was used in this work. Plants were cultivated in growth chambers at 25/15°C (day/night), with a 16-h photoperiod, and a photon flux density of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Fertilization with Bayfolan S (Bayer, Barcelona, Spain) was done using fertirrigation lines. Seeds from 10 to 30 days after flowering (DAF) from the external seed rings of the capitulum were harvested for analysis.

E. coli (XL1-Blue strain) was used as plasmid host for cloning and protein expression. All primers were synthesized by MWG Biotech AG (Ebersberg, Germany). Bacteria were grown at 37°C in LB media (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl, pH 7). When appropriate, ampicillin (100 $\mu\text{g/mL}$) was added for plasmid selection. Except when mentioned otherwise, buffers, chemicals and reagents were of analytical grade from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific (Nepean, ON, Canada).

2.2. Cloning of the cDNAs encoding three sunflower enolases

Approximately 0.4 g of developing sunflower seeds were harvested at 15 DAF. Seeds were ground in liquid N₂ with a precooled sterile mortar and pestle and mRNA was isolated using the MicroFastTrack Kit (Invitrogen, Groningen, The Netherlands). The mRNA pellet was resuspended in 33 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and the cDNA was obtained using a Ready-To-Go T-Primed First Strand Kit (Amersham Bioscience, Roosendaal, The Netherlands). Known plant ENO protein sequences from public databases were aligned using the ClustalX v.2.0 program [17] to identify highly conserved regions. PCR fragments were amplified with primers designed from these regions (Table S1). The fragments were cloned into the pGEM-TEasy[®] vector (Promega, Madison, USA) and several clones were sequenced on both strands by Secugen (Madrid, Spain). The identity of the clones was confirmed using the BLAST software [18], identifying three different clones, *Ha*ENO1, *Ha*ENO2 and *Ha*ENO3. The 5'-ends were obtained using the Smart[™]-RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and specific reverse internal oligonucleotides pairs for each cloned sequence (Table S1). The 3'-end of the cDNAs was obtained by PCR using the external oligo FA2Z (5'-AACTGGAAGAATTCGCGG-3'), complementary to the sequences incorporated during the initial cDNA synthesis, and specific internal

oligonucleotides for each cloned sequence (Table S1). The PCR fragments were cloned, sequenced and assembled to obtain DNA sequences coding for a plastidial enolase, *HaENO1* (1471 bp), and two cytosolic isoforms, *HaENO2* (1339 bp) and *HaENO3* (1426 bp). These cDNA sequences were deposited in GENBANK under accession numbers EF137905, KU764663 and KU764664, respectively.

2.3. cDNA and protein sequence analyses

Sequences homologous to the predicted protein sequences of sunflower ENOs were retrieved using the BLASTP program (www.ncbi.nlm.nih.gov). Alignment of the amino acids sequences for ENO proteins deposited at GENBANK, including the transit peptides for plastidial isoforms, was performed using the ClustalX v.2.0 program with the default settings [17]. These alignments were used to generate a phylogenetic tree based on the neighbour-joining algorithm [19], and the resulting phenogram was drawn using the MEGA 4.0 program [20]. Analysis of predicted subcellular localization was done with Target P1.1 [21]; MultiLoc2 [22]; and PredoTAR [23].

2.4. Modelling of the three-dimensional structure of sunflower ENOs

Homology modelling studies were performed using Swiss-Model Workspace (swissmodel.expasy.org) [24] and JPred4 secondary structure prediction server [25]. The sequences used as templates were the human beta enolase 1 alpha and the specific enolase-2 (respective UniProtKB accession numbers P06733 and P09104). The chosen templates were the most homologous ENO for which X-ray structure information were available (PDB entries: 2psn and 3ucd), showing 68.5% sequence identity with *HaENO1*, 73% with *HaENO2* and 57% with *HaENO3*. SWISS-MODEL was used in first approach and project (optimise) modes using default parameters. Structures were visualized using Swiss-PDBViewer (www.expasy.org/spdbv; [26]).

2.5. Constructs for recombinant sunflower ENOs expression in *E. coli* and site directed mutagenesis

Initial attempts to produce a full-length recombinant *HaENO1* were unsuccessful (see results). We therefore opted to generate a recombinant protein lacking the predicted signal peptide. Primers with internal *KpnI* and *HindIII* restriction sites, respectively named *KpnIHaENO1* and *HindIIIHaENO1*, were designed in order to amplify the truncated version of *HaENO1* named Δ *HaENO1*. The PCR product

obtained was subcloned into the *KpnI-HindIII* sites of pQE-80L (Qiagen, Hilden, Germany) to produce a fusion protein with a 6(His) tag at the N terminus. The full *HaENO2* cDNA sequence was cloned into pQE-80L as a *PstI-SphI* fragment using the *PstIHaENO2* and *SphIHaENO2* primers. In a similar way, a full *HaENO3* cDNA was amplified using *SalIHaENO3* and *SphIHaENO3* primers and the PCR product was subcloned into the *SalI-SphI* sites of pQE-80L. Ligation into the correct reading frame was confirmed by sequencing and the resulting constructs were designated pQE-ENO1, pQE-ENO2 and pQE-ENO3, respectively. The recombinant plasmids were introduced and expressed in *E. coli* XL1-Blue strain. The predicted molecular masses for recombinant proteins 6(His) Δ *HaENO1*, 6(His)*HaENO2*, 6(His)*HaENO3* were respectively 46.7, 48.9 and 52.3 kDa. The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to make point mutations in the *HaENO3* sequence within the pQE-ENO3 construct. The amino acids mutated were: P84S, E251D, D252E and G362D. Primers used to generate those mutations are listed in Table S1.

2.6. Heterologous protein expression and purification

E. coli cells harbouring recombinant plasmids, pQE-ENO1, pQE-ENO2 and pQE-ENO3 were grown under continuous shaking at 37°C in LB broth containing ampicillin. The cells were induced at an OD₆₀₀ of 0.5 with 0.6 mM IPTG, and grown for an additional 2 h at 37°C. Cells were harvested by centrifugation (10 min at 10,000 x g), and pellets were frozen at -80°C until used. Purification by IMAC was carried out at 4°C as previously described by Dorion et al. [27]. Protein concentration was determined according to Bradford [28]. Glycerol was added to the purified enzyme preparation at a final concentration of 50% (v/v) and the solution was stored at -20°C until used. Loss of activity of the enzymes stored in these conditions was less than 10% over a three months period.

2.7. ENO activity assay and kinetic analyses

ENO activity was measured by following NADH oxidation using a coupled enzyme assay on a VersaMax (Molecular Devices, Sunnyvale, CA, USA) microplate reader according to a protocol modified from Forsthoefel et al. [29]. The specific activities were expressed in international units (U) mg⁻¹ protein with 1 U corresponding to the disappearance of NADH at the rate of 1 μ mol min⁻¹ at 25°C. Briefly, the 200 μ L

reaction mixture contained 80 mM triethanolamine, pH 7.4, 0.12 mM NADH, 25 mM MgCl₂, 100 mM KCl, 1.3 mM ADP, 1 U of pyruvate kinase (EC 2.7.1.40), 2 U of lactate dehydrogenase (EC 1.1.1.27) and recombinant protein. Reactions were initiated by addition of 1.9 mM 2-phosphoglycerate. Activity was linear with time and proportional to the amount of recombinant protein in the assay. Kinetic analyses were independently repeated at least 3 times, with four to six technical replicates for each experiment. Kinetic parameters were calculated from experimental data using SigmaPlot 12.5 (SPSS Inc, Chicago, IL). Statistical analysis of the data was done using Student's *t*-test tool of SigmaPlot 12.5. A value of $P < 0.05$ was considered significant.

2.8. Gene expression analysis by real time quantitative PCR and data mining

cDNAs from developing sunflower seeds, roots, stems, green cotyledons and leaves were obtained as described above. The cDNAs were subjected to real time quantitative PCR (RT-QPCR) with specific pairs of primers (Table S1: q*HaENO1*-F and q*HaENO1*-R for *HaENO1*; q*HaENO2*-F and q*HaENO2*-R for *HaENO2*; and q*HaENO3*-F and q*HaENO3*-R for *HaENO3*) and using SYBR Green I (QuantiTect[®] SYBR[®] Green PCR Kit, Qiagen, Crawley, UK) in a MiniOpticon system to monitor the resulting fluorescence (Bio-Rad). The reaction mixture was heated to 95°C for 15 min before subjecting it to 40 PCR cycles consisting of: 94°C for 15 s; 56°C for 30 s; and 72°C for 15 s. Efficiency curves were drawn up using sequential dilutions of cDNA. The Pfaffl method [30] was applied to calculate comparative expression levels between samples and the sunflower actin gene *HaACT1* (GenBank Accession number FJ487620) was used as the reference gene using a specific pair of primers (Table S1). In order to compare the sunflower expression profiles with *Arabidopsis* enolases data, *AtENO1* (At1g74030), *AtENO2* (At2g36530) and *AtENO3* (At2g29560) expression values estimated from microarrays [31] were used.

2.9. Transient expression in *Nicotiana benthamiana* and microscopy

Construction of the Pro35Sdual:*HaENO1*:mGFP, Pro35Sdual:*HaENO2*:mCherry and Pro35Sdual:*HaENO3*:mCherry transgenes: cDNAs were amplified with the proofreading *PfuUltra* DNA polymerase (Stratagene) from a mixture of seed cDNAs using primers listed in Table S1. The PCR products were introduced by BP recombination into the pDONR207 entry vector (Invitrogen) and transferred into the destination vector pMDC83 [32] by LR recombination (Figure S2).

Leaves of *N. benthamiana* were imaged with a Zeiss LSM710 confocal microscope as described in Miart et al. [33].

3. Results

3.1. Isolation, sequence analysis of three sunflower *ENO* cDNAs and identification of their genomic sequences

Conserved regions from known enolase sequences were used to design oligonucleotide primers. Using these primers, three fragments were PCR amplified from developing sunflower seeds cDNA, which corresponded to three distinct internal regions of *ENO* mRNAs. Subsequently, the three full-length cDNA clones were obtained by RACE. These PCR fragments were cloned and sequenced, and their homology to other plant *ENO* was confirmed using the BLAST software [18]. The conceptual translation of the cDNA sequences and the alignment of the resulting *HaENO* proteins (Eudicotyledon subclass Asterids family Asteraceae) along with other *ENOs* from *Arabidopsis thaliana* (Eudicotyledon subclass Rosids family Brassicaceae), revealed a high degree of identity (Fig. 1). The various *HaENO* isoforms were named after their respective *Arabidopsis* homologs.

The full-length *ENO* cDNAs were predicted to encode proteins of 489 amino acids (*HaENO1*, predicted molecular mass of 52 kDa and pI of 6.07), 445 amino acids (*HaENO2*, predicted molecular mass of 48 kDa and pI of 5.34) and 474 amino acids (*HaENO3*, predicted molecular mass of 51.4 kDa and pI of 5.64) (Fig. S1). Among sunflower *ENOs*, identity percentages in amino acids sequences were around 50%. *HaENO1* and *HaENO2* were the most similar with 58.2% sequence identity. Interestingly, the *HaENO2* sequence contained four amino acids insertions and one deletion compared to the two other sequences (Fig. 1). The *HaENO2* sequence lacked a conserved Lys found at positions 113 and 98 respectively in *HaENO1* and *HaENO3*. The first insertion was a Gln located at position 95 of the *HaENO2* sequence. A second insertion, five amino acids long, was found between positions 102 and 107 (Glu-Trp-Gly-Trp-Cys). The subsequent insertions were a Lys residue found at position 260 and a doublet Gly-Glu at positions 320-321 in addition to a four amino acids extension at the C-terminus. The sunflower protein sequences were further subjected to analysis with three different subcellular localisation prediction tools. The consensus results (Table S2) predict that *HaENO1* is targeted to the plastid whereas *HaENO2* and *HaENO3*, which lack an identifiable targeting signal, probably localize to the cytoplasm. The *HaENO1*

sequence contained a predicted plastidial transit peptide of 60 residues at its N-terminus. For this reason, the molecular mass for the mature *HaENO1* protein was predicted to be around 52 kDa. The conserved amino acid residues involved in the interaction with the ion and the substrate, are present even in distant phylogenetic groups, but are absent in *HaENO3*. These differences are located at position 80 (P/S), 252 (D/E), and 414 (Q/H). The first position participates in the enzyme-metal interaction and the last two interact with the substrate (Figs. 1 and 4d).

Using the sequences reported here and other known cytoplasmic or plastidial ENO sequences from Viridiplantae, a phylogenetic tree was generated. The ENO sequence from the green alga *Chlamydomonas reinhardtii* was used as an outgroup to root the tree (Fig. 2). As expected, sunflower ENOs showed high homology with ENO proteins from other Asterids clade species, with identity values around 92%. For example, sequences from Asterales (e.g. *Cynara cardunculus*), Lamiales (e.g. *Sesamum indicum*), or Solanales species (e.g. *Solanum tuberosum*) showed the highest identity values with sunflower ENOs (only the latter ones are included in the phylogenetic tree to allow the inclusion of more distant clades). Phylogenetic analysis indicates that *HaENO3* and other *AtENO3* homologs belong to the same group and are genetically distant from the other ENO genes (Fig. 2). The two other sunflower genes belong to the same group. *HaENO1* is genetically close to *AtENO1* and their homologs encoding for predicted plastidial enolases [14]. In addition, *AtENO1* and *AtENO3* also contain an N-terminal extension similarly to what was observed in *HaENO1* and *HaENO3* (Fig. 1), whereas *AtENO2* is considered to be cytosolic and reported to be a bifunctional protein encoding a regulator of cold-responsive gene expression generated by alternative translation [15, 34, 35].

We took the opportunity of the recent availability of the sunflower genome ([36]; <https://www.heliagene.org/HanXRQ-SUNRISE/>) to identify the genes coding for cytosolic and plastidial glycolytic enzymes (Table S3). Among these, we were able to recover the genes corresponding to the three ENO cDNAs cloned. Only one copy of the genes for isoforms ENO1 (HanXRQChr05g0157781) and ENO3 (HanXRQ05g0135571). Notwithstanding the sunflower genomic duplication, four genes encoding for ENO2-like proteins (HanXRQChr06g0181591, which encodes ENO2; HanXRQChr16g0512701, HanXRQChr01g0028341 and HanXRQChr11g0351111) were located. Compared to their homologs in *Arabidopsis*, the structures of *HaENO1* and *HaENO3* genes are conserved with six and twelve

introns receptively. *HaENO2* presents sixteen introns, three more than its *Arabidopsis* homolog (Fig. S2.). Nevertheless, based on the sunflower transcriptome data available (<https://www.heliagene.org>), the active cytosolic isoform characterized in this study, *HaENO2*, corresponds to the highest expressed enolase in sunflower (Fig. S4).

3.2 Tertiary structure prediction of *HaENO* proteins

Eukaryotic enolases are homodimers with each monomer showing a bidomain structure [37]. As mentioned, their secondary structure is divided into two domains (Fig. 3). The N-terminal domain contains an antiparallel β -sheet of three strands and four alpha helices (residues 61-195 in *HaENO1*, 1-131 in *HaENO2* and 46-180 in *HaENO3*). The C-terminal domain (residues 196-489 in *HaENO1*, 132-445 in *HaENO2* and 181-474 in *HaENO3*) contains the active site and a mixed α/β -barrel, which displays an unusual folding pattern that, in sunflower enolases models, gives $\beta\beta\alpha\beta\beta(\beta\alpha)6\alpha$ connectivity (Figs. 3 and 4). These two domains are connected through an eight residues long loop region (L2). In addition, there are three other loop regions that are important for catalytic function and are more flexible than other regions of the protein because they shift towards the active site when binding to the substrate occurs [38]. In *HaENO1* and *HaENO2*, the L1 loop, which is located near the N-terminus, contains a Ser residue involved in the metal binding site. In *HaENO3*, the L1 Ser residue is substituted by a Pro. The L3 loop contains a Ser residue beside a His involved in the substrate-binding pocket in *HaENO1* and *HaENO2*. This residue is replaced by a Lys in *HaENO3*. The L4 loop is located between the first and second $\alpha\beta$ structures in the C-terminal domain, shows relatively low levels of conservation between isoforms (Fig. 3) and introduces changes at the ternary structure (Fig. 4, yellow circle). Apart from these changes, predicted tertiary structures for *HaENO1* and *HaENO2* look very similar, suggesting that kinetics parameters will be comparable between the two proteins (Figs. 3, 4a and 4b). Similarly, the changes occurring in *HaENO3* point to a lack of normal enolase functionality (Figs. 3 and 4c).

3.3 Determination of the subcellular localization of fluorescently tagged *HaENO* fusion proteins

Protein localization prediction programs predicted that *HaENO2* and *HaENO3* were likely localized in the cytosol while *HaENO1* was recognized as a chloroplastic protein (Table S2). These predictions agree with the subcellular localization of the

Arabidopsis ENO homologs [14, 15]. To verify their subcellular localization, the *Ha*ENO proteins were transiently expressed as fusion proteins in *N. benthamiana* leaves. The full-length *Ha*ENO1 was fused at its C-terminal to GFP and full-length *Ha*ENO2 and *Ha*ENO3 were fused at their C-terminal to mCherry (Fig. S3). The cytosolic marker RanBP1-GFP was used as control [39]. Transformed areas were examined by confocal laser microscopy. The fluorescence signal from *Ha*ENO1-GFP overlapped that of chlorophyll autofluorescence, confirming *Ha*ENO1 localization in chloroplasts (Fig. 5). Conversely, the fluorescence of *Ha*ENO2-mCherry and *Ha*ENO3-mCherry completely superposed the signal obtained from RanBP1-GFP therefore confirming the cytosolic localization of both proteins.

3.4 Site directed mutagenesis of *Ha*ENO3 and expression and purification of wild type and mutant recombinant *Ha*ENO proteins

The alignment of ENO proteins from sunflower along with other ENOs from *A. thaliana* allowed the identification of several amino acid modifications within possibly important conserved domains in the sequence of *Ha*ENO3 (Fig. 3). These amino acids substitutions were located in the Mg^{2+} binding domains (P84 and G362) and in the catalytic active side (E251 and D252) [15, 40]. To later test the effects of these substitutions of presumed important residues, we performed site-directed mutagenesis on the *Ha*ENO3 sequence to obtain the following amino acid substitutions: P84S, E251D, D252E and G362D. We thus produced four different mutagenized versions of *Ha*ENO3 in which single or multiple residues of the *Ha*ENO3 protein sequence were changed to the residues present in *Ha*ENO1 and *Ha*ENO2. This generated the *Ha*ENO3^{P84S}, *Ha*ENO3^{E251D/D252E}, *Ha*ENO3^{G362D} and *Ha*ENO3^{P84S/E251D/D252E/G362D} mutants.

In early trials, the production of wild type (full-length) 6(His)*Ha*ENO1 yielded extremely low amounts of recombinant protein (data not shown). We therefore decided to use an expression strategy in which we deleted the sequence coding for the predicted 60 amino acid N-terminal signal peptide from the construct, thus generating a truncated version which was named 6(His) Δ *Ha*ENO1, in order to avoid a potential interference of the N-terminal targeting signal with expression in *E. coli*. A similar approach was previously successful for overcoming problems encountered when expressing recombinant hexokinases carrying an N-terminal hydrophobic domain [41, 42]. Wild type and mutant recombinant *Ha*ENO proteins were all successfully overexpressed in *E.*

coli and purified by IMAC (Fig. 6). All the purified proteins were >95% homogenous as judged from Coomassie blue staining. It was interesting to note that 6(His)*Ha*ENO3 and its four mutants migrated to a lower apparent molecular weight compared to 6(His)*Ha*ENO2 and 6(His) Δ *Ha*ENO1 despite the fact that their predicted sequence was longer than the two latter proteins. The effect of a limited number of sequence substitutions between the different recombinant proteins could be responsible for perturbation of protein/SDS interactions, leading to electrophoretic ‘gel shifting’ behaviour, as documented in other cases [43]. All the purified proteins were assayed for enolase activity at pH 7.4 and under saturating substrate conditions. 6(His) Δ *Ha*ENO1 had a specific activity of 116.4 U mg⁻¹ protein, while the specific activity obtained with 6(His)*Ha*ENO2 was 53.7 U mg⁻¹ protein. In the case of 6(His)*Ha*ENO3 and its mutagenized versions no significant or extremely low specific activities were detected (Table 1). Increasing 2-PGA concentration up to 5 mM or varying assay pH in the range 6.4-8.9 did not result in a significant change of ENO activity for 6(His)*Ha*ENO3 and its mutants compared to values reported in Table 1.

3.6 Analysis of kinetic parameters and inhibitor studies for 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2

Despite the importance of ENO in carbon partitioning during lipid synthesis and deposition in developing oilseeds, few studies have focused on the characterization of the kinetic properties of these isoforms [14, 15]. We took advantage of the fact that we were able to express 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 and purify them to a high specific activity to further characterize their kinetic behaviour. Since 6(His)*Ha*ENO3 and its mutants had no significant activity, kinetic studies were not performed on these proteins. The activity of purified 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 was monitored in the pH range between 6.25 and 8.75 under substrate saturating conditions (Fig. 7). The activity of both enzymes was similarly affected by pH. They displayed an optimum pH around 7 with a sharp reduction of their activities at values above the optimum (around 50% less activity at pH 8). Substrate saturation kinetics for 2-PGA were performed at the following three different physiologically relevant pH values: 6.8, 7.4, and 8 (Fig. 8). Under all conditions, both enzymes displayed Michaelis-Menten kinetics with 2-PGA as substrate. As expected from the results of Table 1, k_{cat} values for 6(His) Δ *Ha*ENO1 were higher than for 6(His)*Ha*ENO2 (Fig 8a). Assay pH did not significantly influence k_{cat} for 6(His)*Ha*ENO2, whereas that of 6(His) Δ *Ha*ENO1 was

slightly higher under alkaline conditions. K_m values for 2-PGA (Fig 8b) were strikingly similar for both enzymes and decreased with increasing pH. The resulting catalytic efficiencies (k_{cat}/K_m) were thus higher under alkaline conditions for both enzymes, particularly in the case of 6(His) Δ *Ha*ENO1 (Fig 8c).

Both recombinant enzymes were assayed at pH 6.8, 7.4, and 8 in the absence or presence of a range of possible effectors belonging to carbon metabolism (Table 2). Fluoride, a known inhibitor of enolases from various organisms [44, 45] was also tested as NaF. As expected, both recombinant proteins were strongly inhibited by NaF (Table 2). Among the plant metabolites tested, PPi (0.2-1 mM) and G6P 5 mM showed a modest (~ 20% maximum) inhibitory effect on 6(His) Δ *Ha*ENO1 (Table 2). Sensitivity to G6P was only significant at slightly acidic conditions. This isoform was also weakly sensitive to stimulation by Tyr (tyrosine) and Pi. The effect of PPi and G6P was not observed with 6(His)*Ha*ENO2, which instead exhibited a small increase in activity in the presence of respiratory intermediates citrate and α -KG (α -ketoglutarate) under neutral to acidic pHs.

3.7 Pattern of expression of sunflower ENOs and glycolysis-related genes

Expression levels of the three *ENO* genes in different tissues, including developing seeds, leaves, roots, stems and cotyledons were examined using quantitative real time PCR (Fig. 9a). *Ha*ENO2 showed the highest level of expression in all the tissues studied. The pattern of *Ha*ENO2 expression during seed development allowed distinguishing two clearly marked stages. In the initial phase, which lasted up to 12 DAF, high levels of expression were observed and transcripts of *Ha*ENO2 accounted for over 90% of the combined expression of the three genes. In the second phase of expression, between 16 and 30 DAF, there was a strong decrease of expression, characterized by lower *Ha*ENO2 transcript levels. As seed development progressed, the temporal expression pattern of *Ha*ENO1 followed an increase that reached a maximum at 12 DAF, and declined thereafter. *Ha*ENO3 was detected in all tissues and at all time points. Except in the case of leaf tissue, *Ha*ENO3 was always the weakest expressed of the three genes, generally by a factor of one to two orders of magnitude. The sunflower *ENO* genes expression pattern was compared to that of *Arabidopsis thaliana*, recovered through data mining (Fig 9b). The general pattern observed in sunflower tissues was also found for the *Arabidopsis* orthologs. The highest level of expression was observed for *At*ENO2. Low level of *At*ENO1 was found in leaves and this gene showed a peak of

expression during seed filling. Lower levels of *AtENO3* transcripts were generally found in all tissues except leaves.

Data mining in the sunflower bioinformatics database (<https://www.heliagene.org>) allowed recovering the expression levels of glycolytic and transporters related-genes in sunflower seeds listed in Table S3. These transcriptomic data are presented in Fig. S5. This analysis allowed the identification of genes corresponding to complete glycolytic pathways within the cytosol (Fig. S5a) and the plastid (Fig. S5b). In addition, genes encoding for metabolite transporters between the two compartments were also expressed (Fig. S5c). These data also indicate the predominant expression of genes encoding for enzymes of the cytosolic glycolytic pathway compared to those encoding for the plastidial route.

4. Discussion

In developing seeds, glycolysis is the paramount metabolic pathway in which hexoses are converted to Pyr that is subsequently being used as a source of carbon for fatty acids biosynthesis within plastids. PEP, the product of ENO activity, is the immediate precursor of Pyr and therefore plays a key biochemical function during lipid deposition. ENO is a ubiquitous enzyme that is present in the plant cytosolic and plastidial compartments. The present study was undertaken to characterize the *ENO* gene family in sunflower in relation to seed development and lipid reserve accumulation.

4.1 The three sunflower ENO proteins have distinct subcellular localizations and parallel the molecular features of the *A. thaliana* ENO family

Using a PCR amplification of conserved *ENO* sequences and a RACE strategy, we cloned three full-length *ENO* cDNAs from 15 DAF sunflower seeds. These sequences encoded three distinct ENO proteins. These sequences were used in conjunction with 124 other plant *ENO* sequences representing a large variety of lower and higher plant families, to build a phylogenetic tree (Fig. 2). The results indicate that the three sunflower *ENO* cDNAs characterized in the present study represent a member of each of the three *ENO* groups present in plants, documented for example in *A. thaliana* [12, 14, 15]. The evolution of the three types of plant *ENOs* is interesting, as it is assumed that it involves lateral gene transfer between eukaryotes [12]. Using sequence data (Fig. 1) to identify *A. thaliana* genes and their sunflower orthologs, the

origin of the various sunflower *ENOs* can be inferred [12] (note that *A. thaliana* ENO gene denomination in this latter publication is different from that of the current genome annotation, which is used here). Thus, *HaENO1* is putatively derived from an ancestor of fungi and animals. *HaENO3*, which is phylogenetically close to the *C. reinhardtii* ENO (Fig. 2), is likely to have evolved from a lateral transfer of chlorophyte origin. *HaENO2* carries amino acids insertions, in particular the pentapeptide ¹⁰²Glu-Trp-Gly-Trp-Cys¹⁰⁷ which is uniquely shared with alveolate ENOs [12]. This five amino acids insert has been shown to be important for ENO structure and activity in *Plasmodium falciparum* [46]. These features indicate a very complex evolutionary history, perhaps involving lateral gene transfer and recombination events early in evolution [12, 47].

Although it was originally speculated that some higher plants could lack a gene encoding for a plastidial ENO [48], it is now well established that higher plants express genes for plastidial and cytosolic ENOs [12-14]. In sunflower, using mRNA from developing seeds, we have found two cytosolic and one plastidial ENO.

In the recently published sunflower genome, [36], only one copy of the genes for isoforms *ENO1* (HaChr05g0157781) and *ENO3* (HaChr05g0135571) have been found, notwithstanding the sunflower genomic duplication, 4 genes encoding for *ENO2*-like proteins (HaChr06g0181591, *HaENO2*; HaChr16g0512701; HaChr01g0028341 and HaChr11g0351111) were located. Nevertheless, the active cytosolic isoform characterized in this study, *HaENO2*, corresponds to the highest expressed enolase in sunflower (<https://www.heliagene.org>) (Fig S4).

The sunflower ENO isoforms have the same subcellular localization as their *Arabidopsis* counterparts. The *HaENO1* sequence was predicted to encode for a protein targeted to the chloroplast, which was confirmed experimentally using expression of a fluorescently tagged version of *HaENO1* in *N. benthamiana*. The two other proteins were confirmed as cytosolic isoforms using the same approach (Fig. 5). As mentioned before, the predicted secondary and tertiary structures point to the fact that *HaENO1* and *HaENO2* maintain general structure and active site residues found in known functional ENO enzymes. This was not the case with *HaENO3*, which not only showed structural changes, for example in Loop 4 (Figs. 3 and 4c), but also had non-conserved residues in ion and substrate binding sites (Fig. 4d).

4.2 Biochemical and kinetic properties of sunflower ENOs

The three sunflower ENOs were heterologously expressed in *E. coli* and purified to electrophoretic homogeneity (Fig. 6). The recombinant proteins were used to determine their respective kinetic properties. The first striking result was the lack of activity in 6(His)*Ha*ENO3. A similar observation was previously made with recombinant *A. thaliana* ENO3 [15]. The specific activity obtained with 6(His) Δ *Ha*ENO1 was ~200-fold higher than that obtained with *A. thaliana* recombinant ENO1 [14]. The specific activity of 6(His)*Ha*ENO2 also compared very favourably (~8 times higher) with that of recombinant *A. thaliana* recombinant ENO2 [15]. Native plastidial and cytosolic ENO isoforms isolated from *Ricinus communis* developing endosperm had much higher specific activities (≥ 200 U. mg⁻¹ protein) [44]. The lack of activity of ENO3 was previously investigated in the case of *A. thaliana* [15]. This protein shares many of the amino acid substitutions noted for *Ha*ENO3, in particular the substitutions at positions 251-252 (ED in ENO3 proteins instead of DE in ENO1 and ENO2 proteins, Fig. 1). It was previously shown that a mutated form of *A. thaliana* ENO3 carrying the E251D/D252E substitutions (i.e. the same residues as in *A. thaliana* ENO1 and ENO2) was able to complement an *eno*⁻ *E. coli* strain as shown by its ability to grow on glucose as sole carbon source [15]. However, in that study, the modified ENO3 protein was not purified and its specific activity therefore not determined. In the present study, we generated four mutant forms of *Ha*ENO3, namely: *Ha*ENO3^{P84S}, *Ha*ENO3^{E251D/D252E}, *Ha*ENO3^{G362D} and *Ha*ENO3^{P84S/E251D/D252E/G362D}. In all cases, amino acids in the WT *Ha*ENO3 sequence were modified to recover the amino acids present in the *Ha*ENO1 and *Ha*ENO2 sequences. For three mutant proteins (*Ha*ENO3^{P84S}, *Ha*ENO3^{E251D/D252E}, *Ha*ENO3^{G362D}) we were able to obtain extremely low, but significant specific activities. These values were nevertheless four orders of magnitude below those obtained with 6(His)*Ha*ENO1 and 6(His)*Ha*ENO2. A logical explanation for these data is that evolution of the *Ha*ENO3 sequence has accumulated a large number of mutations that compromise the overall *Ha*ENO3 structure and/or activity. It is interesting to note that effects of the engineered modifications were not cumulative, as the protein carrying all the amino acids changes (the *Ha*ENO3^{P84S/E251D/D252E/G362D} mutant) did not exhibit any significant ENO activity. It remains to be seen whether the *A. thaliana* ENO3 mutant carrying the E251D/D252E substitutions [15] has higher specific activity than our *Ha*ENO3^{E251D/D252E}. It is nevertheless possible that equally low levels of activity would still enable growth of an *eno*⁻ *E. coli* mutant. It is intriguing to note that even though most plants seem to contain two expressed cytosolic ENO

isoforms, the ENO of chlorophytic origin is devoid of catalytic activity in each of the two organisms in which it has been characterized. Further characterization of the products of the *ENO3* orthologs in other plants will be necessary to determine if this situation is of general occurrence in the plant kingdom.

Because of its lack of activity, 6(His)*Ha*ENO3 was not further characterized for kinetic properties. The activity of 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 was examined as a function of pH (Fig. 7). The two proteins had almost identical profiles with an optimum pH near neutrality and a relatively large decrease in activity between pH 7 and 8 (~50%). Thus, activity of the sunflower plastidial ENO is expected to be the highest in the dark whereas the onset of photosynthesis, which would induce an alkalization of the stroma, would cause a significant decrease in enzyme activity. These profiles for the reaction in the direction of PEP formation appear relatively similar to those obtained for the two purified *R. communis* ENOs [44], those from *Echinochloa* species [49] and for the enzyme purified from *Synechococcus* PCC 6301 [50]. However, a much more alkaline optimum pH (~ pH 8) was obtained for recombinant *A. thaliana* ENO1 [14]. Saturation kinetics using 2-PGA as substrate were determined for 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 at three pH values (6.8, 7.4 and 8). These values represent a range of physiological pHs that can be encountered in the cytosolic and plastidial compartments [51, 52]. Under all tested pH conditions, the two enzymes displayed Michaelis-Menten kinetics and nearly identical K_m values at all considered (Fig 8b). K_m values were in the range of 0.12 to 0.25 mM and the affinity of both enzymes increased with higher pH values, similarly to the observation made with recombinant *A. thaliana* ENO1 [14]. Sunflower recombinant ENOs K_m values were similar to that of cyanobacterial ENO [50], but 3-4 fold higher than those of purified native plant enzymes [44, 49]. The k_{cat} of the recombinant plastidial isoform (~0.06-0.09 sec⁻¹) was higher than that of the cytosolic enzyme (~0.04 sec⁻¹) and only modestly affected by assay pH (Fig. 8a). However, these values are much lower than k_{cat} determined for a number of ENOs from various non-plant origins [46, 53, 54]. The resulting k_{cat}/K_m ratios (Fig 8c) were higher for the plastidial isoform and increased with increasing pH values. These results could indicate that the plastidial enzyme is more efficient for PEP formation. However, this also greatly depends on the availability of 2-PGA to the isoforms in the cytosolic and plastidial compartments.

6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 were also screened for possible effectors under different pH conditions (Table 2). The known sensitivity of ENOs to fluoride [44,

50, 55-57] was also observed for the two active recombinant sunflower proteins. Although citrate, α -KG, Tyr and Pi were found to have significant positive effects on one or both isoforms, no strong inhibition or activation pattern could be observed for any of these metabolites. However, PPi and G6P, two compounds relevant to the plastid metabolism had greater effects than the other metabolites. The two ENO isoforms were differently sensitive to PPi. The plastidial, and not the cytosolic isoform, was inhibited by up to 20% at 1 mM PPi. This effect was previously only observed for native and recombinant *Leishmania* ENOs [58] and for cytosolic and plastidial *R. communis* ENOs [44]. These results are interesting because PPi is putatively involved as a regulator of cytosolic and plastid metabolisms [59]. G6P also had inhibitory effect on 6(His) Δ HaENO1 (at pH 6.8), but not on 6(His)HaENO2. To our knowledge, this finding constitutes the first report of the sensitivity of a plant ENO to G6P. This could be meaningful because G6P (i) is relatively abundant in the plastid [60] and (ii) is utilized by the oxidative pentose phosphate pathway to provide reducing equivalents needed for plastid lipid synthesis [8, 9]. Thus, import of G6P in the stroma would inhibit the downstream plastidial ENO.

4.3 Expression of sunflower ENOs and their function during sunflower seed filling.

The plastidial PK, is the main enzyme responsible for the conversion of PEP to Pyr in *Arabidopsis* embryos [10]. Indeed, plastidial pyruvate kinase mutants have a reduced rate of lipid synthesis [10, 11]. Due to the difficulties to distinguish between cytosolic and plastidial metabolic fluxes for the lower part of glycolysis [9, 61], the contributions of cytosolic and plastidial PEP pool to the synthesis of plastidial Pyr and subsequently of fatty acids remains an open question. Besides this contribution to seed metabolism, PEP is also used for the synthesis of essential aromatic amino acids and phenylpropanoids in plants [62, 63]. Expression levels of the three sunflower *ENO* genes cloned in this study were quantified in different tissues (Fig. 9a). Compared to *HaENO1* and *HaENO3*, *HaENO2* showed the highest level of expression in all organs and developmental stages examined. During seed development, expression level of *HaENO2* peaks at the initial stages, 12 days after flowering (DAF), and account for most of the combined expression of the three genes. The temporal expression pattern of *HaENO2* and *HaENO1* followed a declining trend similar to other genes involved in carbohydrate and lipid metabolism described in sunflower seed and in other four different oilseeds [42, 64-66]. The expression levels of glycolysis and transporters

related-genes in sunflower seeds (Table S3, Fig.5) were obtained. The analysis of these data, in conjunction with the data presented in the present study have allowed us to propose a model of glycolysis in sunflower seeds in connection to lipid accumulation (Fig. 10). Transcriptomic data have allowed us to confirm the highly likely existence of two complete glycolytic pathways within the cytosol and the plastids, as well as the presence of multiple transporters connecting both subcellular compartments. Higher levels of expression were generally found for the genes encoding enzymes involved in the cytosolic glycolysis route (Figs. 9, S5). It is therefore likely that there is an active glycolytic pathway in both compartments. Thus, the transcriptome data together with the presence of an active plastidial enolase isoform, characterized in this work, supports the possible participation of the plastidial pathway providing carbon during *de novo* fatty acid synthesis [16]. Of course, gene expression levels does not always reflect events occurring at the level of metabolic flux or enzyme activity as previously shown in the case of sunflower seed PGI [64]. Nevertheless, it is tempting to speculate from the transcriptomic data and previous work [6, 42, 64, 66] that the cytosolic glycolytic pathway, in conjunction with the phosphoenolpyruvate/Pi translocator of the plastid inner membrane, provides the majority of the PEP used by plastidial PK for the synthesis of Pyr. In this model, the presence of a plastidial ENO would provide an alternative or a minor supplement to the main cytosolic source of carbon for lipid synthesis. This highlights the metabolic complexity and flexibility of the process and open new opportunities for exploring alternative pathways toward engineering oilseeds.

Acknowledgements

This work was supported by the “Ministerio de Economía y Competitividad” and FEDER (AGL2014-53537-R). JR is supported by a Discovery Grant (RGPIN 227271) from the National Science and Engineering Research Council of Canada. The authors would also like to thank the Hauts-de-France Region and the European Regional Development Fund (ERDF) 2014/2020 for the funding of this work.

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Figure Legends

Figure 1. Alignment of the deduced amino acid sequences of *H. annuus* enolases, *HaENO1*, *HaENO2* and *HaENO3*, with the closely related sequences from *A. thaliana*, *AtENO1* (gi|15221107|), *AtENO2* (gi|15227987|) and *AtENO3* (gi|15227561|). Identical residues are highlighted as black boxes and highly conserved residues as grey boxes. Residues involved in the metal binding site indicated with black triangles and those residues participating in the substrate-binding pocket are indicated with white diamonds. Asterisks indicate identical residues, colon conservative changes, and dot weakly conservative changes between the sequences.

Figure 2. Phylogenetic tree of the enolases from different plant, bryophyte and chlorophyte species. Sequence from *Chlamydomonas reinhardtii* was used as an out group to root the tree. The GenInfo identifiers (gi) follow the species name: *A. lyrata*, *Arabidopsis lyrata*; *A. thaliana*, *Arabidopsis thaliana*; *A. trichopoda*, *Amborella trichopoda*; *B. distachyon*, *Brachypodium distachyon*; *C. arietinum*, *Cicer arietinum*; *C. clementina*, *Citrus clementina*; *C. reinhardtii*, *Chlamydomonas reinhardtii*; *C. rubella*, *Capsella rubella*; *C. sativus*, *Cucumis sativus*; *C. sinensis*, *Citrus sinensis*; *D. discoideum*, *Dictyostelium discoideum*; *E. salsugineum*, *Eutrema salsugineum*; *F. vesca*, *Fragaria vesca*; *G. max*, *Glycine max*; *M. truncatula*, *Medicago truncatula*; *O. brachyantha*, *Oryza brachyantha*; *O. sativa*, *Oryza sativa*; *P. patens*, *Physcomitrella patens*; *P. persica*, *Prunus persica*; *P. trichocarpa*, *Populus trichocarpa*; *P. vulgaris*, *Phaseolus vulgaris*; *R. communis*, *Ricinus communis*; *S. bicolor*, *Sorghum bicolor*; *S. itálica*, *Setaria itálica*; *S. lycopersicum*, *Solanum lycopersicum*; *S. moellendorffii*, *Selaginella moellendorffii*; *S. tuberosum*, *Solanum tuberosum*; *T. cacao*, *Theobroma cacao*; *V. vinífera*, *Vitis vinífera*; and *Z. mays*, *Zea mays*.

Figure 3. Comparison between the deduced amino acid sequences (*HaENO1*, *HaENO2* and *HaENO3*) and predicted secondary structures (*HaENO1*, *HaENO2* and *HaENO3* respectively; *HaENO* indicates when no structural differences were found between the three predictions for the sunflower enolase proteins. Asterisks designates identical residues, a colon shows conservative changes, and a dot indicates weakly conservative changes between the sequences. Structures elements: α -helixes and β -sheets, in N- and C-terminal domains are preceded by “N” or “C”, respectively. Loop regions are boxed,

numbered and labeled with “L”. H: α -helix; S: β -sheet. Residues involved in the metal and substrate binding sites [67] are highlighted in black and grey, respectively.

Figure 4. Proposed structural models for sunflower enolase monomers *Ha*ENO1 (a), *Ha*ENO2 (b) and *Ha*ENO3 (c), and comparison of the residues involved in the metal binding site and substrate binding pocket (d). a, b and c, ribbon diagrams. Residues in α -helices are red, in β -sheets gray, in the metal binding site green, and in the substrate-binding pocket orange. Loop region L4 shown inside a yellow circle. Residues in red in panel d are those not conserved in *Ha*ENO3 respect to *Ha*ENO1 and *Ha*ENO2 residues.

Figure 5. Subcellular localization of *Ha*ENOs. Localization of *Ha*ENO2 and *Ha*ENO3 proteins in *N. benthamiana* leaves and protein co-localization with the cytosolic marker protein RanBP1-GFP by confocal microscopy. Co-expression of RanBP1 tagged to GFP with respective *Ha*ENO2 and 3 tagged to mCherry allowed identification of co-localization sites for *Ha*ENO2 and *Ha*ENO3 in the cytosol (a-h). Chloroplast localization of *Ha*ENO1 protein in *N. benthamiana* leaves. Green fluorescent signals of *Ha*ENO1-GFP were detected in the chloroplasts co-localized with chlorophyll autofluorescence pseudo-colored in red (i-j). Bar = 35 μ m.

Figure 6. SDS–PAGE analysis of purified wild type and mutant recombinant *Ha*ENO proteins after IMAC purification. Lanes M: molecular mass standards, lane 1: 6(His)*Ha*ENO2; lane 2, 6(His)*Ha* Δ ENO1; lane 3, 6(His)*Ha*ENO3; lane 4: 6(His)*Ha*ENO3^{P84S}; lane 5, 6(His)*Ha*ENO3^{E251D/D252E}; lane 6, 6(His)*Ha*ENO3^{G362D}; lane 7, 6(His)*Ha*ENO3^{P84S/E251D/D252E/G362D}. Approximately 0.7 μ g recombinant protein was loaded on each lane.

Figure 7. Effect of assay pH on the activity of 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2. Data are expressed as percent of maximal activity and are representative values from four independent experiments. Open symbols: 6(His) Δ *Ha*ENO1, closed symbols: 6(His)*Ha*ENO2.

Figure 8. Analysis of 6(His) Δ *Ha*ENO1 and 6(His)*Ha*ENO2 kinetic properties at different pHs. k_{cat} (a), K_m (b) and k_{cat}/K_m (c) were determined at three different pHs.

Data are mean values and standard deviations from three independent experiments. Open symbols: 6(His) Δ HaENO1, closed symbols: 6(His)HaENO2.

Figure 9. Expression levels of plastidial and cytosolic-localized enolases in developing seeds and vegetative tissues in *H. annuus* (a) and *A. thaliana* (b). *HaENO1*, *HaENO2* and *HaENO3* expression determined by RT-QPCR; *AtENO1*, *AtENO2* and *AtENO3* expression estimated from microarrays [31]. Sunflower seeds stages counting days after flowering (DAF); Arabidopsis stages: 3, mid globular to early heart embryos; 4, early to late heart embryos; 5, late heart to mid torpedo embryos; 6, mid to late torpedo embryos; 7, late torpedo to early walking-stick embryos; 8, walking-stick to early curled cotyledons embryos; 9, curled cotyledons to early green cotyledons embryos; 10, green cotyledons embryos. Sunflower values represent mean values of three independent samples.

Figure 10. Schematic representation of glycolysis and plastidial transporters in sunflower seeds in relation to lipid accumulation and available transcriptome data. In red, enzymes that were previously purified and enzymatically characterized. Grey arrows indicate reactions catalyzed by enzymes and transporters identified in sunflower seed transcriptome. Broken blue line represents the possible G6P inhibition effect on plastidial ENO. Wider arrows represent the putative origin of carbon from triose-phosphate to pyruvate. Enzymes and transporters: HXK, hexokinase; FK, fructokinase; PGI, phosphoglucose isomerase; PFK, ATP - dependent phosphofructokinases; PFP, pyrophosphate - dependent phosphofructokinases; FBA, fructose-bisphosphate aldolase; TPI, triose-phosphate isomerase; NADP-GAPC, non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; GLT, glucose transporter; GPT, glucose phosphate/Pi translocator; NTT, nucleotide transporter; TPT, triose phosphate/Pi translocator; PPT, phosphoenolpyruvate/Pi translocator; BASS2, Sodium/pyruvate cotransporter. Genes encoding enzymes and transporters are listed in table S3. Transcriptome data obtained from www.heliogene.org [36].

Table 1. Specific activities of purified recombinant enolases. Enolase activity was assayed under substrate saturating conditions and at pH 7.4.

Protein	$V_{\max}$ (U mg ⁻¹ protein)
6(His) Δ <i>Ha</i> ENO1	116.4 $\pm$ 4.3
6(His) <i>Ha</i> ENO2	53.7 $\pm$ 3.9
6(His) <i>Ha</i> ENO3	<0.001
6(His) <i>Ha</i> ENO3 ^{P84S}	0.002 $\pm$ 0.001
6(His) <i>Ha</i> ENO3 ^{E251D/D252E}	0.003 $\pm$ 0.001
6(His) <i>Ha</i> ENO3 ^{G362D}	0.005 $\pm$ 0.003
6(His) <i>Ha</i> ENO3 ^{P84S/E251D/D252E/G362D}	<0.001

Table 2. Assay of effectors on enolase activity at various pH. Enolase activity was measured at various pHs and under saturating 2-PGA concentration in absence or presence of the indicated concentration of effectors. SE was always < 2.5%. Data in bold show differences of > 10% compared to control and are significant at $p < 0.001$ (Student's t test)

Effectors	Concentration (mM)	6(His) Δ <i>Ha</i> ENO1			6(His) <i>Ha</i> ENO2		
		(% activity)			(% activity)		
		pH6.8	pH7.4	pH8.0	pH6.8	pH7.4	pH8.0
None	-	100	100	100	100	100	100
G6P	5.0	79	99	106	91	103	99
G1P	5.0	109	103	103	104	101	97
F6P	5.0	103	100	98	97	101	96
3-PGA	2.0	103	99	104	107	106	99
Citrate	5.0	112	104	110	112	114	104
α -KG	5.0	107	102	101	113	107	98
TYR	0.2	111	102	103	109	105	91
PHE	1.0	106	102	96	106	108	107
Pi	5.0	112	99	98	98	99	91
PPi	0.2	96	95	98	102	105	102
	0.5	92	88	88	108	107	98
	1.0	82	80	81	107	105	99
NaF	0.1	91	95	93	92	99	92
	1.0	32	33	39	39	39	38
	10.0	1	1	1	8	5	4

Fig. 1

HaEN01 MALAPPTTTTSLNPLLSKSPKLSPPFVNLPVTSRRSPTKFTVRNSVSVAQSPSVSAAKSSSTVK_S 65
AtEN01 MALT--TKPHHLQRSFLS--PSRVSGERYLESAP--SCLRFR-RSGVQC-----VVAKECRVKG 53
HaEN02 -----MATIKA 6
AtEN02 -----MATITV 6
HaEN03 -----MSVQDYLDKHMLSRKIEDAVNAAVRAKTADPVLFI SNHMRKAVP SAITK 49
AtEN03 -----MSVQEYLDKHMLSRKIEDAVNAAVRAKTSDPVLFIANHLKKAVSSVITK 49

▼

HaEN01 VKARQIILDSRGNPTVEVDLVTDD--LYRSAVPSGASTGIYEAELELRDGDKSIVYGKGVLNAVKN 128
AtEN01 VKARQIILDSRGNPTVEVDLITDD--LYRSAVPSGASTGIYEAELELRDGDKSIVYGKGVLQA IKN 116
HaEN02 VEARQIFDSRGNPTVEVDITLSDGT LARA AVPSGASTGIYEAELELRDGG-SGYLGKGVSKAVANV 70
AtEN02 VKARQIFDSRGNPTVEVDIHTSNGIKVTA AVPSGASTGIYEAELELRDGG-SDYLGKGVSKAVGNV 70
HaEN03 VKARQIILDSRGIPTEVDLYTNKG-MFRASAPSGAPSGMYGAELERDGD KATYI LGNGVSKAVKN 113
AtEN03 VKARQIILDSRGIPTEVDLHTNKG-VFRASAPSGDSSGTIYEAELELRDGD KGMYLGNVSKAVKN 113

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HaEN01 NEVLGPKLIGVDVRNCAEFDGIML-EIDGTPN----KSKLGANAILGVLSACRAGAGAKSIPL 187
AtEN01 NELVAPKLIGVDVRNCADVDAIML-ELDGTNP----KSKLGANAILGVLSVC RAGAGAKGVPL 175
HaEN02 NTII GPALVGKDPTDQTGIDNFVMQQLDGTONEWGWCQKQLGANAILAVSLAV KAGASVLKTPL 135
AtEN02 NNIIGPALIGKDPTQTAIDNFMVHELDGTONEWGWCQKQLGANAILAVSLAVCKAGAAVVSGIPL 135
HaEN03 NEKISEAIVGMDPILOQNIDQAMT-DLDKTEK-----KGLGANAILAVSVAACRAGAAEKEVPL 172
AtEN03 NEKISEALIGMDPKLOGQIDQAMT-DLDKTEK-----KSELGANAILAVSTAACKAGAAEKEVPL 172

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HaEN01 YKHIQETISGTELMPVPFAFNVI NGGSHAGNNLAMOEFMILPVGATSF AEALRMGS EYHYTLTKGI 252
AtEN01 YKHIQETS GTELMPVPFAFNVI NGGSHAGNSLAMOEFMILPVGATSFSEAFQMGS EYHYTLTKGI 240
HaEN02 YKHIANLAGNKNLVLPVPFAFNVI NGGSHAGNKLAMOEFMILPVGATSF KEAMKMGEVYHNLSKV 200
AtEN02 YKHIANLAGNPKIIVLPVPFAFNVI NGGSHAGNKLAMOEFMILPVGAASF KEAMKMGEVYHNLSKV 200
HaEN03 YKHIADLSGGGNHVLPVPAFTLISGGKHAGNNLALREIMILPVGAQT FEEAMQMGSETYHHLKAV 237
AtEN03 CKHLSDLSCRANMVLVPVPAFTVLSGGKHASNTFAIQEIMILPVGASRF EALQWGS ETYHHLKAV 237

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HaEN01 IKAKYQGDAQNVGDEGGFAPNVQDNREGVLVLLIDA TEKAGYTGTKIKIGMDVAASEFLTK-DGKYD 316
AtEN01 IKTKYQGDAQNVGDEGGFAPNVQDNREGVLVLLIDA TEKAGYTGTKIKIGMDVAASEFFMK-DGRYD 304
HaEN02 IKKKYQDATNVGDEGGFAPNIQENKEGLELLKTAIAKAGYTDKVVIGMDVAASEFYAEKD KTYD 265
AtEN02 IKKKYQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYSE-DKTYD 264
HaEN03 IA EKYGHGCNVVEDGGLAPDITSFREGLDLMVEATGRTGYANKLKIAIDVAATDFCIG--SKYD 300
AtEN03 ISEKNGGLGCNVGEDGGLAPDISSLKEGLELVKEAINRTGYNDKIKIAIDIAATNFCLG--TKYD 300

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HaEN01 LNFKKQPNDGAHVL SAPSLGDIYREFVRDFPIVSIEDPFDDQDWTSWTSLQSSV--DIOLVGDDL 379
AtEN01 LNFKKQPNDGAHVL SAESLADIYREFIKDFPIVSIEDPFDDQDWSWASLQSSV--DIOLVGDDL 367
HaEN02 LNFKKEENN DGKQKISGEQLKDLYKSFVSEYP IVSIEDPFDDQDWEHYGKMTAECGEQVOIVGDDL 330
AtEN02 LNFKKEENN S QKISGDALKDLYKSFVSEYP IVSIEDPFDDQDWEHYAKMTTECGTEVOIVGDDL 329
HaEN03 LD FKSPKKS GNFKSGEEMIEYKELCDYPIVSIEDPFDDKDEWQVKFSGLR--ICQVVGDDL 363
AtEN03 LD IKSPKKS GNFKSAEDMIDMYKEICNDYPIVSIEDPFDDKDEWHTKYFSSLG--ICQVVGDDL 363

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HaEN01 LVTNPKRIAEG IQKKACNALLLVNQIGTVTESIQ AALDSKAAGVGMVSHRSGETEDNFIADLS 444
AtEN01 LVTNPKRIAEAIKKQSCNALLLVNQIGTVTESIQ AALDSKAAGVGMVSHRSGETEDNFIADLS 432
HaEN02 LVTNPKTRVKAIDEKTCNALLLVNQIGSVTESIEAVRMSKRAGVGMVSHRSGETEDTFIADLS 395
AtEN02 LVTNPKRVAKIAEKSCNALLLVNQIGSVTESIEAVKMSKKAAGVGMVSHRSGETEDTFIADLA 394
HaEN03 LMSNPKRIERAIEQNACDALLLVNQIGTVTEAIEVVKMAKDAKAGVVISORSGETEDSFLADLS 428
AtEN03 LMSNSKRVERAIOESSCNALLLVNQIGTVTEAIEVVKMARDAGVGVTSHRGETEDSFIADLS 428

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HaEN01 VGLASGQIKTGAPCSRERLAKYNOLLRIEELG-NVRVAGEAFRSP---- 489
AtEN01 VGLASGQIKTGAPCSRERLSKYNOLLRIEELG-NVRVAGEAFRSP---- 477
HaEN02 VGLATGQIKTGAPCSRERLAKYNOLLRIEELGSEAVYAGANFRKPVEPY 445
AtEN02 VGLSTGQIKTGAPCSRERLAKYNOLLRIEELGSEAVYAGVNRFPKPEPY 444
HaEN03 VGLATGQIKAGAPCGERLAKYNOLLRIEELIGE QASYVGENWRHS---- 474
AtEN03 VGLATGVIKAGAPCGERTMKYNOLLRIEELGDAQVYAGEDWKLSL--- 475

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Fig. 2

Fig. 3

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Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

**Molecular and biochemical characterization of the sunflower (*Helianthus annuus*
L) cytosolic and plastidial enolases in relation to seed developmen**

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SUPPORTING INFORMATION

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Table S1. PCR primers used in this work. Restriction sites are underlined.

Primer	Sequence (5' → 3')
<i>Ha</i> ENO3-F80	GCCAGATCCGATTTACTCC
<i>Ha</i> ENO3-F714	CTGCATCACGCTAATAGTGG
<i>Ha</i> ENO2-R1120	CGATGTGTAAACATCTAGTAG
<i>Ha</i> ENO2-R341	ACACCCATCTTCATGGCTTC
<i>Ha</i> ENO2-R88	ACCAACCCCATTCATTTTGA
<i>Ha</i> ENO1-F79	CCGCCACTACTCACCACACT
<i>Ha</i> ENO1-F711	CTTGTTATGCCAGTTCCAGC
5UTR <i>Ha</i> ENO2-F	AGATCTTAACCTAATGGCGACC
5UTR <i>Ha</i> ENO2-R	CAGCTCGGGCAATGTACC
3UTR <i>Ha</i> ENO2-R	GTGTAACATCTAGTAGGGTTCC
5UTR <i>Ha</i> ENO3-F	ATAACAATGTCAGTACAAGACTATTTGG
5UTR <i>Ha</i> ENO3-R	CCGAAGGAACTGCTTTCCGC
3UTR <i>Ha</i> ENO3-R	TAAAGGATCAGGAGTGCCTCC
5UTR <i>Ha</i> ENO1-F	GCGGACTTTCCCATACATCC
5UTR <i>Ha</i> ENO1-R	AGTGTGGTGAGTAGTGGCGG
q <i>Ha</i> ENO1-F	TGAAGCATTTCAGATCTCCATGAGA
q <i>Ha</i> ENO1-R	GCCTAGTAAGGTTTACAACCTCTAA
q <i>Ha</i> ENO2-F	GGAGCTTGGATCAGAAGCG
q <i>Ha</i> ENO2-R	ATCCTTGACTCTTAATCATCTCGA
q <i>Ha</i> ENO3-F	AGGTGCTCCTTGCCGAGGA
q <i>Ha</i> ENO3-R	TCAGGAGTGCCTCCAGTTCT
<i>Ha</i> ACT1-F	GCTAACAGGGAAAAGATGACT
<i>Ha</i> ACT1-R	ACTGGCATAAAGAGAAAGCACG
<i>Sall</i> <i>Ha</i> ENO3	AAAAGT <u>CGACT</u> TCAGGAGTGCCTCCAGTTC
<i>Sph</i> I <i>Ha</i> ENO3	TTAAGCATGCATGTCAGTACAAGACTATTTGG
<i>Pst</i> I <i>Ha</i> ENO2	CTGACTGCAGCTAGTAGGGTTCCACAGGCTTGC
<i>Sph</i> I <i>Ha</i> ENO2	GACT <u>GCATGC</u> ATGGCGACCATCAAGGCAGTCG
<i>Kpn</i> I <i>Ha</i> ENO1	ATC <u>GGGTACC</u> AGGCAGATTATTGACAGCAGAGG
<i>Hind</i> III <i>Ha</i> ENO1	CGATA <u>AGCTT</u> TCATGGAGATCTGAATGCTTCACC
GW-ENO2-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACGACCATCAA GGCAGTCG
GW-ENO2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTAGGGTTCCACA GGCTTGCGG
GW-ENO3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAGTACAA GACTATTTGG
GW-ENO3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGAGTGCCTCCAG TTCTCGCC
GW-ENO1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCTTAGCA CCTCCTACC
GW-ENO1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGAGATCTGAAT GCTTCACC
P84S-F	CTGCTCCAAGTGGTGCTTCTTCTGGAATGTACGGG
P84S-R	CCCGTACATTCCAGAAGAAGCACCACTTGGAGCAG
E251D-D252E-F	GGATGTAATGTTGTTGATGAAGGCGGCCTTGCTCCTG
E251D-D252E-R	CAGGAGCAAGGCCGCCTTCATCAACAACATTACATCC
G362D-F	GAATTTGCCAGGTTGTTGGAGATGATTTGTTGATGTCGAATC
G362D-R	GATTCGACATCAACAAATCATCTCCAACAACCTGGCAAATTC

Table S2. Prediction of subcellular targeting or localization for the three *Ha*ENO isoforms. The deduced primary sequences were analyzed using the following prediction tools: Target P1.1, MPLoc2, and Predotar. For each prediction tool and protein, the Table reports the likelihood (score) for targeting to or subcellular localization in a particular compartment. For each protein and prediction tool, the highest score (in bold) indicates the most likely subcellular localization or targeting.

Prediction tool	Subcellular localization or targeting	Protein		
		<i>Ha</i> ENO1	<i>Ha</i> ENO2	<i>Ha</i> ENO3
TargetP1.1	cTP	0.976	0.144	0.092
	mTP	0.030	0.143	0.174
	SP	0.003	0.086	0.055
	NSS	0.038	0.780	0.748
MultiLoc2	Chloroplast	0.90	0.16	0.11
	Cytoplasm	0.06	0.81	0.79
	Mitochondria	0.04	0.02	0.08
	SP	0.00	0.00	0.00
	Nuclear	0.00	0.00	0.01
PredoTAR	Plastid	0.97	0.01	0.01
	Mitochondria	0.03	0.02	0.01
	ER	0.00	0.00	0.00
	not organellar	0.03	0.98	0.98

Abbreviations: cTP, chloroplastic targeting; mTP, mitochondrial targeting; SP, secretory pathway; NSS, no signal sequence; SP: secretory pathway; ER: endoplasmic reticulum

Table S3. Identification of sunflower genes related to carbohydrate metabolism in seeds.

At gene ID	Protein	Location	Ha gene ID
At4G29130	H XK1	Cytosol	HanXRQChr10g0313171 HanXRQChr14g0438061
At2G19860	H XK2	Cytosol	HanXRQChr12g0355131 HanXRQChr04g0095871
At1G47840	H XK3	Plastid	HanXRQChr06g0184771
At2G31390	FRK1	Cytosol	HanXRQChr01g0023821 HanXRQChr11g0343711
At1G06030	FRK2	Cytosol	HanXRQChr01g0023821 HanXRQChr11g0343711
At3G59480	FRK3	Cytosol	HanXRQChr01g0023821 HanXRQChr11g0343711
At1G06020	FRK4	Cytosol	HanXRQChr01g0023821 HanXRQChr11g0343711
At4G10260	FRK5	Cytosol	HanXRQChr09g0266931
At1G66430	FRK6	Plastid	HanXRQChr14g0440331
At5G51830	FRK7	Cytosol	HanXRQChr04g0105761
At5G42740	PGI	Cytosol	HanXRQChr09g0268611
At4G24620	PGI	Plastid	HanXRQChr09g0250891
At4G26270	PFK3	Cytosol	HanXRQChr06g0172111
At4G29220	PFK1	Cytosol	HanXRQChr04g0111111
At4G32840	PFK6	Cytosol	HanXRQChr07g0190021
At5G56630	PFK7	Cytosol	HanXRQChr03g0078011

At5G61580	PFK4	Plastid	HanXRQChr08g0223231
At2G22480	PFK5	Plastid	HanXRQChr16g0518061
At1G12000	Beta PFP	Cytosol	HanXRQChr01g0006531 HanXRQChr17g0558271 HanXRQChr10g0310151
At1G20950	PFP	Cytosol	HanXRQChr16g0512491 HanXRQChr08g0216771
At1G76550	Alpha PFP	Cytosol	HanXRQChr16g0512491 HanXRQChr14g0427931
At4G04040	PFP	Cytosol	HanXRQChr16g0516901
At3G52930	FBA	Cytosol	HanXRQChr01g0028911 HanXRQChr14g0432581 HanXRQChr13g0402651 HanXRQChr17g0549641
At4G26530	FBA	Cytosol	HanXRQChr15g0488011
At1G18270	FBA	Cytosol	HanXRQChr17g0559181
At4G38970	FBA	Plastid	HanXRQChr14g0441641 HanXRQChr16g0531101 HanXRQChr13g0414711 HanXRQChr15g0482861
At2G01140	FBA	Plastid	HanXRQChr17g0567101 HanXRQChr16g0529531
At3G55440	TPI	Cytosol	HanXRQChr08g0215681 HanXRQChr14g0428971 HanXRQChr06g0184891
At2G21170	TPI	Plastid	HanXRQChr17g0556391 HanXRQChr13g0389071
At2G24270	non-phosphorylating NADP-GAPC	Cytosol	HanXRQChr03g0064801 HanXRQChr03g0084911
At1G12900	GapA-2	Plastid	HanXRQChr17g0559241
At1G42970	GapB	Plastid	HanXRQChr05g0144801 HanXRQChr15g0492501
At3G04120	GapC1	Cytosol	HanXRQChr08g0207371 HanXRQChr17g0540201 HanXRQChr15g0475141

			HanXRQChr17g0549561 HanXRQChr10g0296431 HanXRQChr03g0079671
At1G13440	GapC2	Cytosol	HanXRQChr12g0372271 HanXRQChr16g0497871 HanXRQChr09g0272941 HanXRQChr15g0487051
At1G79530	GapCp-1	Plastid	HanXRQChr11g0335661 HanXRQChr11g0321921 HanXRQChr11g0335651 HanXRQChr15g0490921
At1G79550	PGK	Cytosol	HanXRQChr11g0335611 HanXRQChr05g0160531
At1G56190	PGK	Plastid	HanXRQChr13g0408831
At1G09780	i-PGM	Cytosol	HanXRQChr10g0286701 HanXRQChr15g0464621 HanXRQChr01g0000311 HanXRQChr11g0329261
At4G09520	i-PGM	Cytosol	HanXRQChr07g0200151 HanXRQChr17g0559871 HanXRQChr03g0070911
At1G22170	d-PGM	Plastid	HanXRQChr05g0162211 HanXRQChr04g0112961
At2G29560	ENO	Cytosol	HanXRQChr05g0135571
At2G36530	ENO	Cytosol	HanXRQChr06g0181591 HanXRQChr01g0028341 HanXRQChr16g0512701 HanXRQChr11g0351111
At1G74030	ENO	Plastid	HanXRQChr05g0157781
At2G36580	PK	Cytosol	HanXRQChr11g0350981 HanXRQChr01g0028101 HanXRQChr08g0218131
At3G52990	PK	Cytosol	HanXRQChr15g0491551 HanXRQChr05g0147521
At5G63680	PK	Cytosol	HanXRQChr15g0478461 HanXRQChr17g0543441 HanXRQChr10g0296281

At4G26390	PK	Cytosol	HanXRQChr14g0437131 HanXRQChr01g0021491 HanXRQChr17g0555661 HanXRQChr15g0494911
At5G56350	PK	Cytosol	HanXRQChr07g0186531 HanXRQChr01g0021521
At3G49160	PK	Plastid	HanXRQChr14g0462941
At1G32440	PKbeta subunit	Plastid	HanXRQChr03g0085681 HanXRQChr03g0086931
At3G22960	PKalpha subunit	Plastid	HanXRQChr16g0514671 HanXRQChr15g0468861
At5G52920	PKbeta subunit	Plastid	HanXRQChr13g0393011 HanXRQChr14g0462681 HanXRQChr07g0207121
At5G46110	TPT	Plastid	HanXRQChr17g0552681 HanXRQChr11g0342621 HanXRQChr11g0342581 HanXRQChr11g0342591
At5G33320	PPT1	Plastid	HanXRQChr05g0155751 HanXRQChr05g0149571 HanXRQChr02g0036001
At3G01550	PPT2	Plastid	HanXRQChr07g0198931 HanXRQChr07g0198891
At5G54800	GPT1	Plastid	HanXRQChr15g0471391 HanXRQChr03g0077511 HanXRQChr08g0234061 HanXRQChr05g0159151
At5G16150	GLT1	Plastid	HanXRQChr14g0438481 HanXRQChr07g0194541
At2G26900	BASS2	Plastid	HanXRQChr09g0238611 HanXRQChr01g0027191
At1G80300	NTT1	Plastid	HanXRQChr02g0032561 HanXRQChr04g0096661 HanXRQChr03g0081331

Figure S1. Sunflower ENO protein sequences.

HaENO1

1	MALAPPTTTT	SLSNPLLSSK	PSLSSPFVNL	PTVSRRSPTK	FTVRNSVSVA
51	QSPSVSAAKS	STVKSVKARQ	IIDSRGNPTV	EVDLVTDGLY	RSVPSGAST
101	GIYEALRLD	GDKSVYGGKG	VLNAVKNINE	VLGPKLIGVD	VRNQAEFDGI
151	MLEIDGTPNK	SKLGANAILG	VSLSACRAGA	GAKSIPLYKH	IQEISGTKEL
201	VMPVPAFNVI	NGGSHAGNNL	AMQEFMILPV	GATSFAEALR	MGSEVYHTLK
251	GIIKAKYGQD	ACNVGDEGGF	APNVQDNREG	LVLLIDAIEK	AGYTGKIKIG
301	MDVAASEFLT	KDGKYDLNFK	KQPNDDAHVL	SAPSLGDLYR	EFVRDFPIVS
351	IEDPFDQDDW	TSWTSLQSSV	DIQLVGDDLL	VTNPKRIAEG	IQKKACNALL
401	LKVNQIGTVT	ESIQAAALDSK	AAGWGVMSH	RSGETEDNFI	ADLSVGLASG
451	QIKTGAPCRS	ERLAKYNQLL	RIEEELGNVR	YAGEAFRSP	

HaENO2

1	MATIKAVKAR	QIFDSRGNPT	VEVDITLSDG	TLARAAVPSG	ASTGIYEALE
51	LRDGGSDYLG	KGVS KAVANV	NTIIGPALVG	KDPTDQTGID	NFMVQQLDGT
101	QNEWGWCKQK	LGANAILAVS	LAVCKAGASV	LKTPLYKHIA	NLAGNKNLVL
151	PVPAFNVI	GSHAGNKLAM	QEFMILPIGA	SSFKEAMKMG	VEVYHNLKSV
201	IKKKYGQDAT	NVGDEGGFAP	NIQENKEGLE	LLKTAIAKAG	YTDKVVIGMD
251	VAASEFYAEK	DKTYDLNFKE	ENNDGKQKIS	GEQLKDLYKS	FVSEYPIVSI
301	EDPFDQDDWE	HYGKMTAECG	EQVQIVGDDL	LVTNPTRVKK	AIDEKTCNAL
351	LLKVNQIGSV	TESIEAVRMS	KRAGWGVMS	HRSGETEDTF	IADLSVGLAT
401	GQIKTGAPCR	SERLAKYNQL	LRIEEELGSE	AVYAGANFRK	PVEPY

HaENO3

1	MSVQDYLDKH	MLSRKIEDAV	NAAVRAKTAD	PVLFI SNHMR	KAVPSAITKV
51	KARQILDSRG	IPTVEVDLYT	NKGMFRASAP	SGAPSGMYGA	IELRDGD KAT
101	YLGNGVSKAV	KNINEKISEA	IVGMDPILQN	QIDQAMIDLD	KTEKKGELGA
151	NAILAVSVAA	CRAGAAEKEV	PLYKHIADLS	GGGNHVLPVP	AFTLISGGKH
201	AGNNLALREI	MILPVGAQTF	EEAMQMGSET	YHHLKAVIAE	KYGP HGCNVV
251	EDGGLAPDIT	SFREGLDLVM	EAIGRTGYAN	KLKIAIDVAA	TDFCIGSKYD
301	LDFKSPK KSG	QNFKSGEEMI	EIYKELCKDY	PIVSIEDPFD	KEDWEQVKFF
351	SGLRICQVVG	DGLLMSNP KR	IERAIQENAC	DALLLKVNQL	GTVTEAIEVV
401	KMAKDAKWGV	VISQRSGETD	DSFLADLSVG	LATGQIKAGA	PCRGERLAKY
451	NQLIRIEEEI	GEQASYVGEN	WRHS		

Figure S2. Genomic structure of the genes encoding EN1, ENO2 and ENO3. Genes are represented in the 5'-3' orientation. Boxes represent exons and lines represent introns, white boxes denote untranslated regions and black boxes indicate the coding sequences.

Figure S3. Sunflower ENO constructions used for transient expression in *Nicotiana benthamiana* and microscopy.

pMDC83-Pro35Sdual:*HaENO1*:mGFP

pMDC83-Pro35Sdual:*HaENO2*:mCherry

pMDC83-Pro35Sdual:*HaENO3*:mCherry

Figure S4. Expression levels of cytosolic and plastidial enolase isoforms found in sunflower genome. ENO1 (HanXRQChr05g0157781), ENO3 (HanXRQ05g0135571), and ENO2-like proteins (HanXRQChr06g0181591, which encodes ENO2; HanXRQChr16g0512701, HanXRQChr01g0028341, and HanXRQChr11g035111, ENO2-like 1, 2, and 3 respectively). Transcriptome data obtained from www.heliagene.org. Genes encoding enolase isoforms are listed in table S3.

Figure S5. Expression levels of cytosolic (a) and plastidial (b) glycolytic enzymes and plastidial transporters (c) in sunflower seeds. Transcriptome data obtained from www.heliogene.org. HXK, hexokinase; FK, fructokinase; PGI, phosphoglucose isomerase; PFK, ATP - dependent phosphofructokinases; PFP, pyrophosphate - dependent phosphofructokinases; FBA, fructose-bisphosphate aldolase; TPI, triose-phosphate isomerase; NADP-GAPC, non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; GLT, glucose transporter; GPT, glucose phosphate/Pi translocator; NTT, nucleotide transporter; TPT, triose phosphate/Pi translocator; PPT, phosphoenolpyruvate/Pi translocator; BASS2, Sodium/pyruvate cotransporter. Genes encoding enzymes and transporters are listed in table S3.