

1
2
3
4 Title: Molecular cloning and biochemical characterization of three phosphoglycerate kinase
5
6 isoforms from developing sunflower (*Helianthus annuus* L.) seeds.
7
8
9

10
11 Authors: Troncoso-Ponce MA^a, Rivoal J^b, Venegas-Calación M^a, Dorion S^b, Sánchez R^a, Cejudo
12
13 FJ^c, Garcés R^a, and Martínez-Force E^{a*}.
14
15

16 Authors' affiliation: ^aInstituto de la Grasa, CSIC, Avda. Padre Garcia Tejero 4, 41012 Seville,
17
18 Spain. ^bInstitut de Recherche en Biologie Végétale, Université de Montréal, 4101 Rue
19
20 Sherbrooke est, Montréal, Que., Canada H1X 2B2. ^cInstituto de Bioquímica Vegetal y
21
22 Fotosíntesis, Universidad de Sevilla y CSIC, Avda. Américo Vespucio 49, 41092 Seville, Spain.
23
24
25
26
27
28
29
30

31 *Correspondence to: Martínez-Force E.
32

33 Email: emforce@cica.es
34

35
36 Tel: 34-954611550
37

38
39 Fax: 34-954616790
40
41
42

43 Keywords: Phosphoglycerate kinase, Sunflower, *Helianthus annuus*, Seed, Oil, Fatty acids.
44

45 Abbreviations: TAG, triacylglycerides; PGK, Phosphoglycerate kinase; 3-PGA, 3-
46
47 phosphoglycerate; 1,3-bPGA, 1,3-diphosphoglycerate Ni-NTA, nickel-nitrilotriacetic acid; LB,
48
49 Luria Bertani; IPTG, Isopropyl β -D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl
50
51 sulfate polyacrylamide gel electrophoresis.
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 **Abstract**
5

6
7 Three cDNAs encoding different phosphoglycerate kinase (PGK, EC 2.7.2.3) isoforms,
8
9 two cytosolic (*HacPGK1* and *HacPGK2*) and one plastidic (*HapPGK*), were cloned and
10
11 characterized from developing sunflower (*Helianthus annuus* L.) seeds. The expression profiles
12
13 of these genes showed differences in heterotrophic tissues, such as developing seeds and roots,
14
15 where *HacPGK1* was predominant, while *HapPGK* was highly expressed in photosynthetic
16
17 tissues. The cDNAs were expressed in *Escherichia coli*, and the corresponding proteins purified
18
19 to electrophoretic homogeneity, using immobilized metal ion affinity chromatography, and
20
21 biochemically characterized. Despite the high level of identity between sequences, the *HacPGK1*
22
23 isoform showed strong differences in terms of specific activity, temperature stability and pH
24
25 sensitivity in comparison to *HacPGK2* and *HapPGK*. A polyclonal immune serum was raised
26
27 against the purified *HacPGK1* isoform, which showed cross-immunoreactivity with the other
28
29 PGK isoforms. This serum allowed the localization of high expression levels of PGK isozymes
30
31 in embryo tissues.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. Introduction

Phosphoglycerate kinase (PGK, EC 2.7.2.3) is an essential soluble enzyme for the basic metabolism in all living organisms. It catalyses the reversible transfer of phosphate from the C1-position of 1,3-diphosphoglycerate (1,3-DPG) to ADP to form 3-phosphoglycerate (3-PG) and ATP, in the presence of Mg^{2+} . PGK, as a typical kinase, is a monomeric enzyme with two structural domains of about equal size joined by a flexible hinge (Watson et al., 1982). The C-terminal domain binds the nucleotide, Mg-ADP or Mg-ATP, while the N-terminal domain binds 3-PG or 1,3-DPG. During catalysis the domains move toward each other to bring the substrates into the proper vicinity for the transfer of the phosphoryl group (Zerrad et al., 2011). In higher plants there are two classes of phosphoglycerate kinase, one is localized in the cytosol (cPGK), and the other in plastids (pPGK) (Anderson and Advani, 1970). The cytosolic enzyme functions primarily in glycolysis for substrate-level ATP generation, one of the two glycolytic steps that produce ATP (Plaxton, 1996), and is also involved in gluconeogenesis. The plastidial isoform is involved in the photosynthetic carbon reduction cycle and in chloroplast glycolysis. In plants, both isozymes generally show a high degree of conservation (Longstaff et al., 1989). The most significant difference in their sequences is the presence of a chloroplast transit peptide at the N-terminus of the chloroplastic isoform. In tobacco leaves, the genes encoding these isozymes are differentially expressed in a developmental and tissue-specific manner and both genes show light-modulated expression *in vivo* (Bringloe et al., 1996). In addition, both isozymes appear to be involved in the regulation of carbon partitioning between starch and sucrose (Shah and Bradbeer, 1994). Additionally, a sub-population of cytosolic PGK has been localized in the nucleus in pea leaves and shoots, due to the presence of a functional nuclear localization signal near its N-terminus (Brice et al., 2004). Interestingly, it has been hypothesized that PGK has

1
2
3
4 more than one role (Jeffery, 1999) and it has been regarded as a “moonlighting” protein. Several
5
6 lines of evidence support this view, as PGK participates in DNA replication and repair in both
7
8 the mammalian (Jindal and Viswanatha 1990; Kumble and Viswanatha 1991; Viswanatha et al.,
9
10 1992) and the plant nucleus (Al-Rashdi and Bryant 1994; Burton et al., 1997, Bryant and
11
12 Anderson 1999, Bryant et al., 2000), and is involved in *in vitro* mRNA synthesis of the Sendai
13
14 virus (Ogino et al., 1999; Popanda et al., 1998).
15
16
17
18

19 During the synthesis of reserve lipids in heterotrophic oilseeds, sucrose is converted into
20
21 hexose-phosphates and metabolized by the glycolytic pathway to produce carbon skeletons,
22
23 energy and reducing power necessary for lipid biosynthesis (Schwender and Ohlrogge, 2002;
24
25 Hill et al., 2003). In developing sunflower (*Helianthus annuus* L.) embryos, one of the glycolytic
26
27 activities that increases in conjunction with the oil content during the period of active storage
28
29 lipid synthesis is PGK (Troncoso-Ponce et al., 2009). Moreover, the study of the glycolytic
30
31 enzymatic activities in developing seeds from standard and low oil content sunflower lines, has
32
33 pointed to phosphoglycerate kinase and enolase as two of the activities probably implicated in
34
35 the differences in fat content between these lines (Troncoso-Pone et al., 2010a). In addition,
36
37 labelling and modelling studies have shown that the major source of carbon for the synthesis of
38
39 fatty acids in sunflower plastids originates from the metabolism of triose phosphates to
40
41 phosphoenolpyruvate (Alonso et al., 2007). PGK activity is involved in this sequence of
42
43 reactions, which can take place in the cytosolic and plastidial compartments (Plaxton and
44
45 Podestá, 2006; Alonso et al., 2007). It is also one of the highest glycolytic activities measured in
46
47 rapeseed (*Brassica napus*) embryo cultures (Junker et al., 2007) and has been identified in the
48
49 group of the most abundant proteins in castor bean (*Ricinus communis*) developing seeds
50
51 (Houston et al., 2009), both important crops characterized by high rate of fatty acid synthesis in
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 their seeds . In transgenic canola seeds with increased oil content, expression of a subset of genes
5
6 involved in fatty acid biosynthesis and glycolysis is up-regulated in developing seeds, including
7
8 PGK (Tan et al., 2011). All these observations suggest that this activity may participate in the
9
10 generation of energy and carbon for the fatty acid synthesis.
11
12
13

14 In order to better understand the carbon metabolism in sunflower developing seeds, we
15
16 have focused on the characterization of cytosolic and plastidial PGK isoforms. In this study, we
17
18 have identified three cDNAs from developing sunflower seeds, which are predicted to encode
19
20 two cytosolic and one plastidic PGK. We describe their phylogenetic relationship with other
21
22 homologous genes, and perform model predictions for the corresponding protein structures. The
23
24 biochemical characterization of the recombinant proteins provides evidence of differences in
25
26 kinetic properties between PGK isoforms. A survey of the expression pattern of these genes and
27
28 the immunolocalisation of PGK isoforms in developing embryos supports the view that
29
30 sunflower PGKs are differentially regulated.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2. Results and discussion

2.1. Isolation and sequence analysis of three sunflower PGK cDNAs

Conserved regions from known phosphoglycerate kinase sequences were used to design degenerated oligonucleotide primers dPGK-F and dPGK-R (Table 1). Using these primers, a 575 bp fragment was PCR amplified from developing sunflower seeds cDNA, which corresponded to an internal region of PGK mRNAs. Subsequently, three full-length cDNA clones were obtained by RACE using the primers shown in the Materials and Methods. These PCR fragments were cloned and sequenced, and their homology to other plant PGKs was confirmed using the Blast software (Altschul et al., 1990). The cDNA sequences were identified as two cytosolic PGK isoforms, *HacPGK1* (1206 bp) and *HacPGK2* (1203 bp), and a plastidial one *HapPGK* (1446 bp).

The full-length PGK cDNAs were predicted to encode proteins of 401 amino acids (*HacPGK1*, predicted molecular mass of 42.3 kDa and pI of 6.06), 400 amino acids (*HacPGK2*, predicted molecular mass of 42.4 kDa and pI of 7.61) and 481 amino acids (*HapPGK*, predicted molecular mass of 50.1 kDa and pI of 7.18). The *HapPGK* sequence contained a predicted plastid transit peptide of 76 residues at its N-terminus, not present in the two cytoplasmic isoforms, which would produce a 405 amino acids mature polypeptide of 42.2 kDa and pI of 5.16. The alignment of *HaPGK* proteins from sunflower (Eudicotyledon subclass Asterids family Asteraceae) along with other PGKs from *Arabidopsis thaliana* (Eudicotyledon subclass Rosids family Brassicaceae), cytosolic and chloroplastic forms, and *Bacillus stearothermophilus* (Bacteria phylum Firmicutes family Bacillaceae), showed a high degree of identity (Fig. 1).

Using the sequences reported here and other known PGK sequences, either cytoplasmic or plastidial isoforms, from Viridiplantae, a phylogenetic tree was generated using the PGK

1
2
3
4 sequence from the bacteria firmicute *Geobacillus stearothermophilus* as an outgroup to root the
5
6 tree (Fig. 2). This comparison indicated that *HacPGK1* and *HacPGK2* grouped with the other
7
8 plant cPGKs and were more closely related to PGKs of Solanaceae, like *Nicotiana tabacum*, than
9
10 to other known PGKs, although PGKs have been highly conserved throughout evolution.
11
12 Moreover, *HapPGK* grouped with other dicot plant PGK plastidial isoforms. The species
13
14 distribution was essentially consistent with conventional species trees, with Monocots (Poaceae
15
16 species) and Dicots species differentiating from chlorophytas, mosses and bacteria.
17
18
19
20
21
22

23 24 2.2. Tertiary structure prediction of *HaPGK* proteins

25
26 Although crystal structure data for plant PGKs are not yet available, the sequence from
27
28 *HaPGK* proteins showed enough identity, around 60%, with the *Bacillus stearothermophilus*
29
30 PGK protein, the structure of which is well-known (PDB 1PHP). The use of protein modelling
31
32 software programs based on the homology with proteins of a known crystalline structure (Swiss-
33
34 Model, Schwede et al., 2003; 3D-JIGSAW, Bates et al., 2001) allowed us to obtain structural
35
36 models for sunflower PGK proteins. Since the models for *HacPGK1* and *HacPGK2* were very
37
38 similar, only the models for *HacPGK1* and *HapPGK* are shown in Fig. 3. The predicted
39
40 structures show that both proteins are typical kinases (Watson et al., 1982) with two structural
41
42 domains joined by a waist region (Fig. 3), each domain binds one of the two substrates, 3-
43
44 phosphoglycerate (3-PG) and ADP. For the phospho-transfer reaction to take place the substrates
45
46 must be brought closer by a hinge-bending domain closure. The polypeptide chains are distinctly
47
48 folded into two domains of unequal size (Fig. 3): the large and small domains separated by a
49
50 deep cleft containing the residues involved in the enzyme active site. In the secondary structure,
51
52 15 α -helices and 17 β -strands are predicted for *HacPGK1* and 14 α -helices and 19 β -strands for
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 *Hap*PGK (Fig. 3 A and C). The 3-phosphoglycerate will fit into the cleft between the two PGK
5 domains, interacting with the residues D25, N27, R41, H64 and R122 in the case of *Hac*PGK1
6 and with residues D99, N101, R115, H138 and R196 in the case of *Hap*PGK (white arrow heads
7 in Fig. 1 and residues in green in Fig. 3B and D) forming the substrate binding site. Similarly, it
8 is predicted that ADP will interact with residues G225, G297, N321, P323, G325, V326, F327,
9 E328, G358, D359 and S360 in the case of *Hac*PGK1 and with residues G299, G371, N395,
10 P397, G399, V400, F401, E402, G432, D433 and S434 in the case of *Hap*PGK (black arrow
11 heads in Fig. 1 and residues in pink in Fig. 3B and D) those residues forming the ADP binding
12 site in pink and the catalytic site in blue). PGK hinge regions, allowing flexibility of relative
13 domain positions (open and closed state), are formed by residues V187, S188, N189, P190, I376,
14 S377 and T378 in the case of *Hac*PGK1 and with residues V261, S262, N263, P264, I450, S451
15 and T452 in the case of *Hap*PGK (grey arrow heads in Fig. 1). These models identify residues
16 likely to be important in the catalytic function of the enzyme that are potential targets for further
17 studies involving site-directed mutagenesis and kinetic characterisation of the resultant enzymes.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 2.3. Expression and purification of recombinant *Hac*PGK

42
43 The proteins encoded by the three PGK cDNAs were expressed in *E. coli* with a (6His)
44 tag at the N-terminus and purified by NTA chromatography (Fig. 4). The purified proteins
45 (lanes 2, 4 and 6) were > 95% homogenous as judged from Coomassie staining and image
46 analysis and showed apparent MWs around 45 kDa close to the predicted MWs of the
47 recombinant proteins encoded by the expression plasmid. (6His)*Hac*PGK1 was purified to a
48 specific activity of 254.4 nkat mg⁻¹ protein at pH 8.2 (Table 2). This value is comparable to that
49 reported for recombinant *Plasmodium falciparum* PGK (Pal et al., 2004), but 4-fold lower than
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 affinity-purified native barley leaf cPGK (MacMorrow and Bradbeer, 1990). While the specific
5
6 activities obtained with (6His)*Hac*PGK2 and (6His)*Hap*PGK were over 5000 nkat mg⁻¹ protein
7
8 (Table 2). Highlighting, especially in the case of the two cytosolic isoform, the fact of how very
9
10 similar proteins are able to show such striking kinetic differences, although the possibility of a
11
12 misfolding in the case of (6His)*Hac*PGK1 cannot be ruled out.
13
14
15
16
17
18

19 *2.4. Thermal stability of recombinant PGKs*

20
21 To investigate the temperature stability of these enzymes, the purified recombinant PGKs
22
23 were subjected to 3 min treatments at various temperatures ranging from 20 to 96°C (Fig. 5).
24
25 Treatments were conducted in the presence of glycerol, a co-solvent that inhibits heat-induced
26
27 aggregation for yeast PGK (Strucksberg et al., 2007). Near maximum activity was retained by all
28
29 the enzyme preparations incubated from 22 to 35°C. (6His)*Hac*PGK1 was clearly the most
30
31 temperature sensitive of the three enzymes tested, as it underwent a rapid thermal denaturation at
32
33 temperatures between 40 and 65°C. Activity was completely lost with treatments over 65°C. The
34
35 two other recombinant enzymes showed similar thermal stability, retaining about 80% activity at
36
37 temperatures up to 65°C, then rapidly decreasing between 65 and 80°C (Fig. 5).
38
39
40
41
42
43
44

45 *2.5. Kinetic characteristics of recombinant PGKs*

46
47 The activity of purified recombinant PGKs was monitored in the pH range between 5.75
48
49 and 9.25 (Fig. 6). All the enzymes displayed a broad optimum pH between 7.5 and 8.75, similar
50
51 to that reported for barley leaf and spinach leaf PGKs (Köpke-Secundo et al., 1990; MacMorrow
52
53 and Bradbeer, 1990). Their activity remained relatively high at alkaline pHs with more than 75%
54
55 activity retained at pH 9.2 in all cases. At pH values below 7, a sharp reduction of
56
57
58
59
60
61
62
63
64
65

1
2
3
4 (6His)*Hac*PGK1 activity was observed, in contrast to the two other enzymes. Interestingly,
5
6 (6His)*Hac*PGK2 activity was not significantly affected by pH. The sensitivity of
7
8 (6His)*Hac*PGK1 to pH below 7 would render the enzyme responsive to acidic conditions in the
9
10 cytoplasm. Cytoplasmic acidosis has been shown to occur in conditions of hypoxia (Roberts et
11
12 al., 1984; Drew, 1997; Gout et al., 2001), which is common in seed maternal and embryonic
13
14 tissues (Rolletschek et al., 2002; Borisjuk et al., 2004; Borisjuk and Rolletschek, 2009).
15
16
17
18

19 Substrate saturation kinetics for 3-PGA and ATP substrates were studied at three
20
21 physiologically relevant pH values (7.0 , 7.6 and 8.2) (Table 2). All enzymes displayed
22
23 Michaelis-Menten kinetics with 3-PGA and ATP at all pH values. ATP saturation kinetics
24
25 showed that both V_{max} and K_m values were stable across all pHs tested for the three enzymes.
26
27 Consistent with the specific activity data, V_{max} values were always much higher for
28
29 (6His)*Hac*PGK2 and (6His)*Hap*PGK. We did not observe biphasic kinetics or inhibition at high
30
31 ATP concentrations as previously reported for the spinach leave enzymes (Köpke-Secundo et al.,
32
33 1990). The affinity of (6His)*Hac*PGK1 for 3-PGA was comparable to values previously reported
34
35 for *Trypanosoma brucei* PGKs (Zomer et al., 1998) but was 2.4 to 3 times lower than the values
36
37 obtained with purified spinach leave PGKs (Köpke-Secundo et al., 1990) and also with the two
38
39 other recombinant *H. annuus* enzymes characterized here. The K_m values obtained using ATP
40
41 as varying substrate were comparable to previously reported values (Köpke-Secundo et al., 1990;
42
43 Zomer et al., 1998). Interestingly, (6His)*Hac*PGK1 displayed the higher affinity for this substrate
44
45 compared to the two other recombinant *H. annuus* PGKs. Under the conditions tested here, the
46
47 calculated catalytic efficiencies (V_{max}/K_m) of the recombinant enzymes were not strongly
48
49 affected by pH.
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 The effects of glycerol-2-P and glycerol-3-P were tested on all three PGK enzymes
5
6 because these two metabolites are known inhibitors of animal PGKs (Szilagyι and Vas, 1998).
7
8 However, the activity of three enzymes was unaffected by these compounds, even in the
9
10 presence of relatively high concentrations (Table 3). Likewise, UDP (10 mM) did not
11
12 significantly affect the activity of the recombinant enzymes. The inclusion of ADP and AMP in
13
14 the assay mixture resulted in a significant decrease in activity for all PGKs (Table 3). The effect
15
16 was more pronounced with ADP, with concentrations as low as 0.1 mM resulting in up to 40%
17
18 inhibition. These data indicate that sunflower seed PGKs could be sensitive to variations in the
19
20 ATP/ADP ratio or the adenylate energy charge ($[ATP] + 0.5 [ADP]$)/ ($[ATP] + [ADP] +$
21
22 $[AMP]$). Such behaviour was described before for pig muscle PGK (Molnar and Vas, 1993;
23
24 Kóvari et al., 2002) and yeast PGK (Larsson-Raznikiewicz and Arvidsson, 1971). This feature
25
26 could be physiologically relevant for the regulation of the seed enzyme in the gluconeogenic
27
28 direction because high ADP status is commonly observed under hypoxic conditions known to
29
30 prevail in seed tissues (Shelp et al., 1995; Borisjuk and Rolletschek, 2009).
31
32
33
34
35
36
37
38
39
40

41 2.6. Expression of *HacPGK1*, *HacPGK2* and *HapPGK* genes

42
43 Expression levels of the three PGK genes in different tissues have been studied (Fig. 7).
44
45 *HacPGK1* showed the highest level of expression during seed development, especially at the
46
47 initial stages, 12 days after flowering (DAF), and accounted for most (approx. 89%) of the
48
49 combined expression of the three genes. As seed development progressed, the level of expression
50
51 for this gene decreased. The temporal expression pattern of *HacPGK1* followed a declining trend
52
53 similar to other genes involved in carbohydrate and lipid metabolism described in sunflower seed
54
55 and recently in four different oilseeds (Troncoso-Ponce et al., 2010b, 2011a and 2011b).
56
57
58
59
60
61
62
63
64
65

1
2
3
4 Transcripts of the other two genes, *HacPGK2* and *HapPGK*, remained low in all the stages
5 studied though, as *HacPGK1* decreased the proportional participation of *HacPGK2* and *HapPGK*
6 in the combined pool of transcripts increased.
7
8
9

10
11 The comparison of *HacPGK1*, *HacPGK2* and *HapPGK* expression levels in different
12 tissues (Fig. 7A) shows that *Hacpgk1* gene is strongly expressed in the initial stages of the seed's
13 development. Meanwhile, highest expression of *HapPGK* in photosynthetically active tissues,
14 cotyledons and leaves, might reflect the role of this activity in the Calvin cycle. It is relevant to
15 compare these data to the expression data of *Arabidopsis thaliana* PGK genes (Fig. 7B) taken
16 from published microarrays analyses (Schmid et al., 2005), since both plants are model systems
17 for the accumulation of seed lipid reserves. *A. thaliana* contains one cytosolic PGK (At1g79550)
18 and two PGKs predicted to be targeted to the plastid (At1g56190 and At3g1270). The expression
19 patterns of *H. annuus* and *A. thaliana* cPGKs are broadly similar during seed development, with
20 higher levels at early stages. However, in *A. thaliana*, the difference between the early and the
21 later stages of cPGK expression was not as great. Expression of genes encoding pPGKs was
22 lower than that of cPGK in *A. thaliana*, as was observed in *H. annuus*. Interestingly, in both
23 systems, high levels of PGK expression coincided with the onset of lipid deposition in the
24 developing seed. This process occurs around 10-12 DAF in sunflower (Troncoso-Ponce et al.,
25 2009, 2010b) and around the torpedo stage (approximately 4-5 DAF) in *A. thaliana* (Becerra et
26 al., 2006). Expression of *A. thaliana* genes encoding cPGKs and pPGKs in various tissues (Fig.
27 7B) was also relatively similar to observations made in sunflower, with cPGK being more
28 predominant in roots and cPGKs more abundantly expressed in leaves.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57

58 2.7. PGK protein and activity profile during seed development.

59
60
61
62
63
64
65

1
2
3
4 A polyclonal antibody was generated against (6His)*HacPGK1*, which efficiently
5 recognized all PGK isoforms as shown by Western blot analysis of the recombinant proteins
6 (Fig. 8A). This immune serum was used to analyse PGK protein profiles in extracts of
7 developing seeds (Fig. 8B). A single band was observed in Western blot analysis of developing
8 seed extracts. The intensity of the band appeared to decline slightly between 15 and 20 DAF, to
9 rise at later developmental stages. Total PGK activity was measured in extracts of developing
10 seed harvested between 10 and 26 DAF (Fig. 8C). PGK activity was low at early stages of seed
11 development (10-14 DAF) and increased steadily to reach a plateau at 18 DAF, at which point
12 PGK activity remained relatively constant up to 26 DAF. This activity profile paralleled the
13 accumulation of fatty acids in seeds (Troncoso-Ponce et al., 2009). The immunoblot profiles
14 were compared to total PGK activity profiles in developing seeds (Fig. 8C). It was apparent from
15 this comparison that the weakest signal observed on immunoblot corresponded to a high level of
16 PGK activity suggesting that the regulation of total PGK activity was complex, and could
17 involve, for example, a differential contribution of the more active isoforms, *HacPGK2* and
18 *HapPGK* throughout the developmental process.
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42

43 *2.8. Immunocytochemical localisation of PGK isoforms in developing seeds*

44
45 Localization of cells with the highest PGK isoforms content has been achieved.
46 Immunohistochemistry was used to study the spatial distribution of PGKs in developing 15DAF
47 sunflower seed tissues (Fig. 9). The polyclonal antiserum generated against purified sunflower
48 (6His)*HacPGK1* could recognize the three PGK isoforms studied, allowing the visualization of
49 those cells with maximum presence of these enzymes. The spatial distribution of PGK proteins
50 overlaps with the localization of the cytosolic phosphoglucose isomerase (cPGI), a glycolytic
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 enzyme previously studied in developing sunflower seeds (Troncoso-Ponce et al., 2010b). The
5
6 fact that both enzymes are found in all cell types is not surprising. However, stronger signals
7
8 were observed in the procambial ring and especially in the case of PGK at the most external cell
9
10 layers of the proximal end of the seed.
11
12
13
14

15 16 **3. Concluding remarks** 17

18
19 In spite of the central role play by the glycolysis pathway during fatty acid synthesis in
20
21 oilseed and in other plant tissues (Bourgis et al., 2011; Troncoso-Ponce et al., 2011b), little
22
23 information is available about the characteristics of the individual enzymes. Although the study
24
25 of *E. coli* plant recombinant expressed enzymes may not have identical kinetics of a plant
26
27 expressed proteins, we have tackled the characterization of different isoforms of PGK. Our data
28
29 show that at least two cytosolic and one plastidial PGK are differently expressed during
30
31 sunflower seed development and within different plant tissues. The three genes encode proteins
32
33 that have distinct physical properties and analyses of the recombinant proteins in *E. coli* suggest
34
35 that these isozymes have different kinetic characteristics. Thus the changes in total PGK activity
36
37 measured during the development of sunflower seeds represent the complex pattern of variation
38
39 in both the expression and kinetic properties of these isozymes.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4. Experimental

4.1. Plant culture conditions, biological materials and chemicals

Sunflower (*Helianthus annuus* L.) line CAS-6, with normal fatty acid 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 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Fertilization with Bayfolan S (Bayer) was done using fertirrigation lines. Seeds from 10 to 26 DAF from the external seed rings of the capitulum were harvested for analysis. *Escherichia 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) or Fisher Scientific (Nepean, ON, Canada).

4.2. Cloning of the cDNAs encoding three sunflower PGKs

Approximately 0.4 g of developing sunflower seeds was 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). PGK protein sequences from public databases were aligned using the ClustalX v.2.0 program (Thompson et al. 1997) to identify highly conserved regions. PCR fragments were amplified with two degenerate primers designed from these regions: dPGKF and dPGKR (Table

1
2
3
4 1). The fragments were cloned into the pGEM-T-Easy® vector (Promega) and several clones
5
6 were sequenced on both strands by GATC GmbH (Konstanz, Germany). The identity of the
7
8 clones was confirmed using the BLAST software (Altschul et al. 1990), identifying three
9
10 different clones, *HacPGK1*, *HacPGK2* and *HapPGK*. The 5'- ends were obtained using the
11
12 Smart™- RACE cDNA amplification kit (Clontech) and specific reverse internal
13
14 oligonucleotides pairs for each cloned sequence: *HacPGK1*-R1 and *HacPGK1*-R2, *HacPGK2*-
15
16 R1 and *HacPGK2*-R2, and *HapPGK*-R1 and *HapPGK*-R2 (Table 1). The 3'-end of the cDNAs
17
18 was obtained by PCR using the external oligo FA2Z (Table 1), complementary to the sequences
19
20 incorporated during the initial cDNA synthesis, and specific internal oligos for each cloned
21
22 sequence: *HacPGK1*-F, *HacPGK2*-F and *HapPGK*-F (Table 1). The PCR fragments were
23
24 cloned, sequenced and assembled to obtain DNA sequences coding for two PGK cytosolic
25
26 isoforms, *HacPGK1* (1206 bp) and *HacPGK2* (1203 bp), and a plastidial one *HapPGK* (1446
27
28 bp). These cDNA sequences were deposited in GenBank under accession numbers DQ835564,
29
30 HM490307 and HM490308, respectively.
31
32
33
34
35
36
37
38
39
40

41 4.3. cDNA and protein sequence analyses

42
43 Sequences homologous to the predicted sequences of sunflower PGKs were retrieved
44
45 using the BLASTP program (www.ncbi.nlm.nih.gov). Alignment of the amino acid sequences,
46
47 including the transit peptides, for PGK proteins deposited at GENBANK was performed using
48
49 the ClustalX v.2.0 program with the default settings (Thompson et al. 1997). These entire
50
51 alignments were used to generate a phylogenetic tree based on the neighbor-joining algorithm
52
53 (Saitou and Nei 1987), and the resulting 'phenogram' was drawn using the MEGA 4.0 program
54
55 (Tamura et al. 2007).
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7 4.4. *Modelling of the three-dimensional structure of sunflower PGKs.*
8

9 Homology modelling studies were performed using Swiss Model server
10 (<http://swissmodel.expasy.org/>) (Schwede et al. 2003) and JPred prediction server (Cuff and
11 Barton 2000). The sequence used as a template was a bacterial PGK from *Bacillus*
12 *stearothermophilus*, accession number P18912 (gi129919). The chosen template was the most
13 homologous PGK for which X-ray structure information is available (PDB Entry: 1php; Davies
14 et al. 1994), showing 60.6% sequence identity with 392 residues of *HacPGK1*. SWISS-MODEL
15 was used in first approach and project (optimise) modes using default parameters. Structures
16 were visualized using Swiss-PDBViewer (Guex and Peitsch 1997).
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 4.5. *Enzyme extraction from plant tissues and protein assays*
32

33 Typically, 5 peeled sunflower seeds were ground in 1 mL of 50 mM Tris-HCl (pH 8.0), 5
34 mM DTT using an ice-cooled glass homogenizer. The resulting homogenate was centrifuged for
35 10 min at 10,000 × g. The supernatant was used immediately for enzymatic assays. Protein
36 concentration was determined according to Bradford (1976), using the Bio-Rad protein assay
37 reagent (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine gamma globulin as
38 standard.
39
40
41
42
43
44
45
46
47
48
49

50 4.6. *Constructs for recombinant sunflower PGKs expression in E. coli*
51

52 Primers with internal *SphI* and *PstI* restriction sites were designed, *SphHacPGK1-F* and
53 *PstHacPGK1-R* respectively, to amplify the entire coding sequence of *HacPGK1* by PCR (Table
54 1). The PCR product obtained was subcloned into the *SphI-PstI* sites of pQE-80L (Qiagen,
55 Hilden, Germany) to produce a fusion protein with a hexahistidine tag at the N terminus. In a
56
57
58
59
60
61
62
63
64
65

1
2
3
4 similar way, full *HacPGK2* cDNA was cloned into pQE-80L as a *SphI-SalI* fragment using
5
6 *SphHacPGK2-F* and *SalHacPGK2-R* pair of primers (Table 1) and *HapPGK* cDNA was cloned
7
8 into pQE-80L as a *SphI-PstI* fragment missing the signal peptide using *SphHapPGK-F* and
9
10 *PstHapPGK-R* primers (Table 1). Ligation into the correct reading frame was confirmed by
11
12 sequencing and the resulting constructs were designated pQEcPGK1, pQEcPGK2 and
13
14 pQEpPGK, respectively. The recombinant plasmids were introduced and expressed in *E. coli*
15
16 strain XL1-Blue. The predicted molecular mass for each recombinant protein 6(His)*HacPGK1*,
17
18 6(His)*HacPGK2* and 6(His)*HapPGK* was 43.1, 43.2 and 43.0 kDa, respectively.
19
20
21
22
23
24
25

26 4.7. Heterologous protein expression and purification

27
28 *E. coli* cells harbouring recombinant plasmids, pQEcPGK1, pQEcPGK2 and pQEpPGK
29
30 were grown under continuous shaking at 37°C in LB broth containing ampicillin. The cells were
31
32 induced at OD₆₀₀ 0.5 with 0.6 mM IPTG, and grown for an additional 2 h at 37°C. Cells were
33
34 harvested by centrifugation (10 min at 10,000 xg), and pellets were frozen at -80°C until used.
35
36 Purification steps were carried out at 4°C as previously described (Dorion et al., 2006). Protein
37
38 concentration was determined according to Bradford (1976) as described above. Glycerol was
39
40 added to the purified enzyme preparation at a final concentration of 50% (v/v) and the solution
41
42 was stored at -20°C until used.
43
44
45
46
47
48
49

50 4.8. PGK activity assay and kinetic analyses

51
52 PGK activity assays were conducted according to a protocol modified from Journet et al.
53
54 (1986). The PGK reaction was coupled to the glyceraldehyde-3-phosphate dehydrogenase
55
56 (GAPDH, EC 1.2.1.12) reaction and assayed at 25 °C by monitoring NADH oxidation at 340 nm
57
58
59
60
61
62
63
64
65

1
2
3
4 using a VersaMax (Molecular Devices, Sunnyvale, CA, USA) microplate reader. The 200 μ L
5
6 standard reaction mixture contained 100 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.2 mM NADH, 2
7
8 mM ATP, 2 mM DTT, 5 mM 3-phosphoglycerate and 0.5 U/mL GAPDH. Reaction rates were
9
10 linear with time and proportional to the amount of enzyme added to the assay within a range
11
12 spanning one order of magnitude. To study the effect of glycerol-3-phosphate, glycerol-2-
13
14 phosphate UDP, ADP and AMP on PGK activity, these metabolites were added to the standard
15
16 assay mix described above. For all analyses, presented data are means \pm SD of determinations
17
18 carried out with three independent enzyme preparations and quadruplicate assays unless
19
20 otherwise mentioned. Statistical analysis of the kinetic data was performed using Student's t test
21
22 (SigmaPlot 8.0, SPSS, Chicago, IL, USA), with $P < 0.05$ considered significant.
23
24
25
26
27

28
29 The temperature stability of the purified sunflower recombinant PGKs was tested by
30
31 incubating 20 μ l samples of pure enzyme for 3 min at various temperatures using a water bath.
32
33 After heat treatment, the samples were cooled on ice for 3 min. Residual PGK activity was then
34
35 immediately assayed as described above. The effect of pH on sunflower recombinant PGKs
36
37 activity was studied using a three-component buffer to maintain a constant ionic strength
38
39 throughout the pH range (Ellis and Morrison, 1982). The Tris-HCl buffer used in the standard
40
41 reaction mixture was therefore replaced with a mixture of 0.05 M acetic acid, 0.05 M 2-(N-
42
43 morpholino)-ethanesulfonic acid (MES) and 0.1M Tris-HCl adjusted at different pH values with
44
45 1 M NaOH or HCl. The pH value assay was measured directly in the reaction mixture using a
46
47 microelectrode immediately after completion of the spectrophotometric assay. Assays were
48
49 initiated by addition of enzyme preparation and corrected for background activity by omitting 3-
50
51 phosphoglycerate from the reaction mixture. Apparent K_m and V_{max} values were calculated
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 from the Michaelis-Menten equation using a non-linear least-squares regression program
5
6 (SigmaPlot 8.0, SPSS, Chicago, IL, USA).
7
8
9

10 11 4.9. *Quantitative real time PCR* 12

13
14 cDNAs from developing seeds, roots, stems, green cotyledons and leaves were obtained
15
16 as previously described. The cDNAs were subjected to quantitative real time PCR (QRT-PCR)
17
18 with specific pairs of primers (Table 1; *QHacPGK1-F* and *QHacPGK1-R* for *HacPGK1*;
19
20 *QHacPGK2-F* and *QHacPGK2-R* for *HacPGK2*; and *QHapPGK-F* and *QHapPGK-R* for
21
22 *HapPGK*) and using SYBR Green I (QuantiTect® SYBR® Green PCR Kit, Qiagen, Crawley,
23
24 UK) in a MiniOpticon system to monitor the resulting fluorescence (Bio-Rad). The reaction
25
26 mixture was heated to 50°C for 2min and then to 95 °C for 15 min before subjecting it to 40 PCR
27
28 cycles consisting of: 94°C for 15 s; 60.5°C for 30 s; and 72°C for 15 s. Calibration curves were
29
30 drawn up using sequential dilutions of cDNA. The Livak method (Livak and Schmittgen, 2001)
31
32 was applied to calculate comparative expression levels between samples and the sunflower actin
33
34 gene *HaACT1* (GenBank accession number FJ487620) was used as the reference gene using a
35
36 specific pair of primer (Table 1; *QHaActin-F4* and *QHaActin-R4*).
37
38
39
40
41
42
43
44

45 46 4.10. *HacPGK1 immune serum* 47

48 To generate antibodies against purified sunflower recombinant *HacPGK1*, approximately
49
50 1 mg of protein was subjected to preparative SDS-PAGE, followed by electroelution (Dorion et
51
52 al. 2006). After collection of the preimmune serum, a polyclonal antiserum was raised in a New
53
54 Zealand SPF rabbit at the Centre of Animal Production and Experimentation, University of
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 Seville (Spain), following standard methods. The crude immune serum was used for
5
6 immunoblots and immunocytochemical analysis.
7
8
9

10 11 *4.11. SDS-PAGE and immunoblot analysis* 12

13
14 Proteins, previously separated by SDS-PAGE on a gel containing 16.5% (w/v)
15
16 polyacrylamide (Schagger and Vonjagow 1987), were transferred to a nitrocellulose membrane
17
18 (Sigma-Aldrich, 0.45 μm pore size) using an XCell II mini-Cell system (Novex) and Tris-
19
20 Glycine buffer (12 mM Tris base, 96 mM glycine, 20% methanol). The transfer was carried out
21
22 at 25 V for 2 h. The membrane was stained with a Ponceau solution (Sigma-Aldrich) for 15 min
23
24 to verify the quality of the transfer and then washed with TBST (0.8% NaCl, 20 mM Tris-HCl
25
26 pH 7.6, 0.1% Tween 20). For immunodetection, blots were incubated with anti-*HacPGK*
27
28 immune serum (1/5,000 dilution). Polypeptides were detected using an anti-rabbit IgG-
29
30 Peroxidase (1/10,000 dilution) from Sigma-Aldrich. A Pierce ECL Western Blotting Substrate
31
32 kit was used for the detection of antigen-antibody complexes, according to the manufacturer's
33
34 instructions. Alternatively, an anti-rabbit IgG coupled to alkaline phosphatase (Promega,
35
36 Nepean, ON, Canada) was used as described in Dorion et al. (2005).
37
38
39
40
41
42
43
44

45 46 *4.12. Immunocytochemical analysis* 47

48
49 Freshly cut organs from 15 DAF developing seeds were immediately fixed by incubation
50
51 in FAE (3.7% formaldehyde: 5% acetic acid: 50% ethanol, by vol.) with occasional vacuum,
52
53 dehydrated in a graded series of aqueous ethanol solutions and embedded in Paraplast Plus
54
55 (Sigma Chemical Co.) as described in González et al. (1998). 10 μm sections were cut with a
56
57 Leica RM 2025 microtome and placed on poly-L-lysine coated microscope slides. After
58
59
60
61
62
63
64
65

1
2
3
4 deparaffinizing in xylol and rehydrating in decreasing concentrations of ethanol, sections were
5
6 blocked for 3 h in TBS buffer containing 1% (w/v) BSA. Anti-*Hac*PGK1 immune serum or pre-
7
8 immune serum, (diluted 1:5,000 in TBS) was added to the samples and incubated overnight at
9
10 4°C. Unbound primary antibodies were removed by 3 x 10 min washes in TBS. Tissue sections
11
12 were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 2 h at 37°C.
13
14 The reaction of alkaline phosphatase was developed with nitroblue tetrazolium and 5-bromo-4-
15
16 chloro-3-indolyl-phosphate.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

This work was supported by Ministerio de Ciencia e Innovación and FEDER, project AGL2011-23187 and by Advanta Seeds Company. This work was supported in part by a discovery grant from the Natural Sciences and Engineering Research Council of Canada to J. Rivoal.

1
2
3
4 **References**
5

- 6
7 Alonso, A.P., Goffman, F.D., Ohlrogge, J.B., Shachar-Hill, Y., 2007. Carbon conversion
8 efficiency and central metabolic fluxes in developing sunflower (*Helianthus annuus* L.)
9 embryos. *Plant J.* 52, 296-308.
10
11
12
13
14 Al-Rashdi, J., Bryant, J.A., 1994. Purification of a DNA-binding protein from a multi-protein
15 complex associated with DNA polymerase- α in pea (*Pisum sativum*). *J. Exp. Bot.* 45,
16 1867-1871.
17
18
19
20
21 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
22 search tool. *J. Mol. Biol.* 215, 403-410.
23
24
25
26 Anderson, L.E., Advani, V.R., 1970. Chloroplast and cytoplasmic enzymes: three distinct
27 isoenzymes associated with the reductive pentose phosphate cycle. *Plant Physiol.* 45,
28 583-585.
29
30
31
32
33 Anderson, L.E., Carol, A.A., 2005. Enzyme co-localization in the pea leaf cytosol: 3-P-glycerate
34 kinase, glyceraldehyde-3-P dehydrogenase, triose-P isomerase and aldolase. *Plant Sci.*
35 169, 620-628.
36
37
38
39
40
41 Bates, P.A., Kelley, L.A., MacCallum, R.M., Sternberg, M.J.E., 2001. Enhancement of protein
42 modeling by human intervention in applying the automatic programs 3D-JIGSAW and
43 3D-PSSM. *Proteins* 45, 39-46.
44
45
46
47
48 Becerra, C., Puigdomenech, P., Vicient, C.M., 2006. Computational and experimental analysis
49 identifies *Arabidopsis* genes specifically expressed during early seed development.
50 *BMC Genomics* 7, 38.
51
52
53
54
55
56 Borisjuk, L. and Rolletschek, H., 2009. The oxygen status of the developing seed. *New Phytol.*
57 182, 17-30.
58
59
60
61
62
63
64
65

- 1
2
3
4 Borisjuk, L., Rolletschek, H., Radchuk, R., Weschke, W., Wobus, U., Weber, H., 2004. Seed
5
6 development and differentiation: a role for metabolic regulation. *Plant Biol.* 6, 375-386.
7
8
- 9 Bourgis, F., Kilaru, A., Cao, X., Ngando-Ebongue, G.F., Drira, N., Ohlrogge, J.B., Arondel, V.,
10
11 2011. Comparative transcriptome and metabolite analysis of oil palm and date palm
12
13 mesocarp that differ dramatically in carbon partitioning. *Proc. Natl. Acad. Sci. U S A.*
14
15 26, 12527-12532.
16
17
- 18
19 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities
20
21 of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
22
23
- 24 Brice, D.C., Bryant, J.A., Dambrauskas, G., Drury, S.C. and Littlechild, J.A., 2004. Cloning and
25
26 expression of cytosolic phosphoglycerate kinase from pea (*Pisum sativum* L.). *J. Exp.*
27
28 *Bot.* 55, 955-956.
29
30
- 31 Bringloe, D.H., Rao, S.K., Dyer, T.A., Raines, C.A., Bradbeer, J.W., 1996. Differential gene
32
33 expression of chloroplast and cytosolic phosphoglycerate kinase in tobacco. *Plant Mol.*
34
35 *Biol.* 30, 637-640.
36
37
- 38 Bryant, J.A., Anderson, L.E., 1999. What's a nice enzyme like you doing in a place like this? A
39
40 possible link between glycolysis and DNA replication, in: Bryant, J.A., Burrell, M.M.,
41
42 Kruger, N.J. (Eds.), *Plant carbohydrate biochemistry*. Bios Scientific Publishers,
43
44 Oxford, pp. 295-304.
45
46
- 47
48 Bryant, J.A., Brice, D.C., Fitchett, P.N., Anderson, L.E., 2000. A novel DNA-binding protein
49
50 associated with DNA polymerase- α in pea stimulates polymerase activity on frequently
51
52 primed templates. *J. Exp. Bot.* 51, 1945-1947.
53
54
- 55 Burton, S.K., Van't Hof, J., Bryant, J.A., 1997. Novel DNA-binding characteristics of a protein
56
57 associated with DNA polymerase- α in pea. *Plant J.* 12, 357-365.
58
59
60
61
62
63
64
65

- 1
2
3
4 Cuff, J.A., Barton, G.J., 2000. Application of enhanced multiple sequence alignment profiles to
5
6 improve protein secondary structure prediction. *Proteins: Struct. Funct. Genet.* 40, 502-
7
8 511.
9
- 10
11 Davies, G.J., Gamblin, S.J., Littlechild, J.A., Dauter, Z., Wilson, K.S., Watson, H.C., 1994.
12
13 Structure of the ADP complex of the 3-phosphoglycerate kinase from *Bacillus*
14
15 *stearothermophilus* at 1.65 Å. *Acta Crystallogr. D Biol. Crystallogr.* 50, 202-209.
16
17
18
- 19 Dorion, S., Matton, D.P., Rivoal, J., 2006. Characterization of a cytosolic nucleoside diphosphate
20
21 kinase associated with cell division and growth in potato. *Planta* 224, 108–124.
22
23
- 24 Dorion, S., Parveen, J.J., Matton, D.P., Rivoal, J., 2005. Cloning and characterization of a
25
26 cytosolic triosephosphate isomerase developmentally regulated in potato leaves. *Plant*
27
28 *Sci.* 168, 183-194.
29
30
- 31 Drew, M.C., 1997. Oxygen deficiency and root metabolism: Injury and acclimation under
32
33 hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 223-250.
34
35
- 36 Ellis, K.J., Morrison, J.F., 1982. Buffers of constant ionic strength for studying pH-dependent
37
38 processes. *Methods Enzymol.* 87, 405-426.
39
40
- 41 González, M.C., Osuna, L., Echevarria, C., Vidal, J., Cejudo, F.J., 1998. Expression and
42
43 localization of phosphoenolpyruvate carboxylase in developing and germinating wheat
44
45 grains. *Plant Physiol.* 116, 1249-1258.
46
47
- 48 Gout, E., Boisson, A.M., Aubert, S., Bligny, R., 2001. Origin of the cytoplasmic pH changes
49
50 during anaerobic stress in higher plant cells. Carbon-13 and phosphorus-31 nuclear
51
52 magnetic resonance studies. *Plant Physiol.* 125, 912–925.
53
54
- 55 Guex, R.E.F., Peitsch, M.C., 1997. Swiss Model and Swiss Pdb Viewer: an environment for
56
57 comparative protein modelling. *Electrophoresis* 18, 2714-2723.
58
59
60
61
62
63
64
65

- 1
2
3
4 Hajduch M., Hearne L.B., Miernyk J.A., Casteel J.E., Joshi T., Agrawal G.K., Song Z., Zhou M.,
5
6 Xu D., Thelen J.J., 2010. Systems analysis of seed filling in Arabidopsis: using general
7
8 linear modeling to assess concordance of transcript and protein expression. Plant
9
10
11
12
13
14 Hill, L.M., Morley-Smith, E.R., Rawsthorne, S., 2003. Metabolism of sugars in the endosperm of
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- Hajduch M., Hearne L.B., Miernyk J.A., Casteel J.E., Joshi T., Agrawal G.K., Song Z., Zhou M.,
Xu D., Thelen J.J., 2010. Systems analysis of seed filling in Arabidopsis: using general
linear modeling to assess concordance of transcript and protein expression. Plant
Physiol. 152, 2078-2087.
- Hill, L.M., Morley-Smith, E.R., Rawsthorne, S., 2003. Metabolism of sugars in the endosperm of
developing seeds of oilseed rape. Plant Physiol. 131, 228-236.
- Houston, N.L., Hajduch, M., Thelen, J.J., 2009. Quantitative Proteomics of seed filling in castor:
comparision with soybean and rapeseed reveals differences between photosynthetic and
non-photosynthetic seed metabolism. Plant Physiol. 151, 857-868.
- Jeffery, C.J., 1999. Moonlighting proteins. Trends Biochem. Sci. 24, 8-11.
- Jindal, H.K., Vishwanatha, J.K., 1990. Functional identity of a primer recognition protein as
phosphoglycerate kinase. J. Biol. Chem. 265, 6540-6543.
- Journet, E.P., Bligny, R., Douce, R., 1986. Biochemical changes during sucrose deprivation in
higher plant cells. J. Biol. Chem. 261, 3193-3199.
- Junker, B.H., Lonien, J., Heady, L.E., Rogers, A., Schwender, J., 2007. Parallel determination of
enzyme activities an *in vitro* fluxes in *Brassica napus* embryos grown on organic or
inorganic nitrogen source. Phytochemistry 68, 2232-2242.
- Köpke-Secundo, E., Molnar, I., Schnarrenberger, C., 1990. Isolation and characterization of the
cytosolic and chloroplastic 3-phosphoglycerate kinase from spinach leaves. Plant
Physiol. 93, 40-47.
- Kovári, Z., Flachner, B., Náray-Szabó, G., Vas, M., 2002. Crystallographic and thiol-reactivity
studies on the complex of pig muscle phosphoglycerate kinase with ATP and analogues:

- 1
2
3
4 correlation between nucleotide binding mode and helix flexibility. *Biochemistry* 41,
5
6 8796-8806.
7
8
- 9 Kumble, K.D, Vishwanatha, J.K., 1991. Immunoelectron microscopic analysis of the
10
11 intracellular distribution of primer recognition proteins, annexin 2 and phosphoglycerate
12
13 kinase, in normal and transformed cells. *J. Cell Sci.* 99, 751–758.
14
15
- 16 Larsson-Raznikiewicz, M., Arvidsson, L., 1971. Inhibition of phosphoglycerate kinase by
17
18 products and product homologues. *Eur. J. Biochem.* 22, 506-512.
19
20
- 21 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
22
23 quantitative quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402-408.
24
25
- 26 Longstaff, M., Raines, C.A., McMorrow, E.M., Bradbeer, J.W., Dyer, T.A., 1989. Wheat
27
28 phosphoglycerate kinase: evidence for recombination between the genes for the
29
30 chloroplastic and cytosolic enzymes. *Nucleic Acids Res.* 17, 6569-6580.
31
32
- 33 McMorrow, E.M., Bradbeer, J.W., 1990. Separation, purification, and comparative properties of
34
35 chloroplast and cytoplasmic phosphoglycerate kinase from barley leaves. *Plant Physiol.*
36
37 93, 374-383.
38
39
- 40 Molnar, M., Vas, M., 1993. Mg^{2+} affects the binding of ADP but not ATP to 3-phosphoglycerate
41
42 kinase - correlation between equilibrium dialysis binding and enzyme kinetic data.
43
44 *Biochem. J.* 293, 595-599.
45
46
- 47
48 Ogino, T., Iwama, M., Kinouchi, J., Shibagaki, Y., Tsukamoto, T., Mizumoto, K., 1999.
49
50 Involvement of a cellular glycolytic enzyme, phosphoglycerate kinase, in Sendai virus
51
52 transcription. *J. Biol. Chem.* 274, 35999-36008.
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4 Pal, B., Pybus, B., Muccio, D.D., Chattopadhyay, D., 2004. Biochemical characterization and
5
6 crystallization of recombinant 3-phosphoglycerate kinase of *Plasmodium falciparum*.
7
8 Biochim. Biophys. Acta 1699, 277-280.
9
- 10
11 Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant
12
13 Physiol. Plant Mol. Biol. 47, 185-214.
14
- 15
16 Plaxton, W.C., Podestá, F.E., 2006. The functional organization and control of plant respiration.
17
18 Crit. Rev. Plant Sci. 25, 159-198.
19
- 20
21 Popanda, O., Fox, G., Thielmann, H.W., 1998. Modulation of DNA polymerases alpha, delta and
22
23 epsilon by lactate dehydrogenase and 3-phosphoglycerate kinase. Biochim. Biophys.
24
25 Acta 1397, 102–117.
26
- 27
28 Rivoal, J., Dunford, R., Plaxton, W.C., Turpin, D.H., 1996. Purification and properties of four
29
30 phosphoenolpyruvate carboxylase isoforms from the green alga *Selenastrum minutum*:
31
32 evidence that association of the 102-kDa catalytic subunit with unrelated polypeptides
33
34 may modify the physical and kinetic properties of the enzyme. Arch. Biochem. Biophys.
35
36 332: 47-57.
37
- 38
39
40 Roberts, J.K., Callis, J., Wemmer, D., Walbot, V., Jardetzky, O., 1984. Mechanisms of
41
42 cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under
43
44 hypoxia. Proc. Natl. Acad. Sci. USA. 81, 3379-3383.
45
- 46
47
48 Rolletschek, H., Borisjuk, L., Koschorreck, M., Wobus, U., Weber, H., 2002. Legume embryos
49
50 develop in a hypoxic environment. J. Exp. Bot. 53, 1099-1107.
51
- 52
53 Saitou, N., Nei, M., 1987. The neighbour-joining method -a new method for reconstructing
54
55 phylogenetic trees. Mol. Biol. Evol. 4, 406-425.
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4 Schagger, H., Vonjagow, G., 1987. Tricine sodium dodecyl-sulfate polyacrylamide-gel
5
6 electrophoresis for the separation of proteins in the range from 1 kDa to 100 kDa. Anal.
7
8 Biochem. 166, 368-379.
9
- 10
11 Schwede, T., Kopp, J., Guex, N., Peitsch, M.C., 2003. SWISS-MODEL: an automated protein
12
13 homology-modeling server. Nucleic Acids Res. 31, 3381-3385.
14
15
- 16 Schwender, J., Ohlrogge, J.B., 2002. Probing in vivo metabolism by stable isotope labeling of
17
18 storage lipids and proteins in developing *Brassica napus* embryos. Plant Physiol. 130,
19
20 347-361.
21
22
- 23
24 Shah, N., Bradbeer, J.W., 1994. The occurrence of chloroplastic and cytosolic isoenzymes of
25
26 phosphoglycerate kinase in a range of plant species. Planta 193, 232-237.
27
28
- 29 Shelp, B.J., Walton, C.S., Snedden, W.A., Tuin, L.G., Oresnik, I.J., and Layzell, D.B., 1995.
30
31 Gaba shunt in developing soybean seeds is associated with hypoxia. Physiol. Plant. 94,
32
33 219-228.
34
35
- 36 Strucksberg, K.H., Rosenkranz T., Fitter J., 2007. Reversible and irreversible unfolding of multi-
37
38 domain proteins. Biochim. Biophys. Acta 1774, 1591-1603.
39
40
- 41 Szilagyi, A.N., Vas, M., 1998. Anion activation of 3-phosphoglycerate kinase requires domain
42
43 closure. Biochemistry 37, 8551-8563.
44
45
- 46 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
47
48 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.
49
50
- 51 Tan, H.L., Yang, X.H., Zhang, F.X., Zheng, X., Qu, C.M., Mu, J.Y., Fu, F.Y., Li, J.A., Guan,
52
53 R.Z., Zhang, H.S., Wang, G.D., Zuo, J.R., 2011. Enhanced seed oil production in canola
54
55 by conditional expression of *Brassica napus* LEAFY COTYLEDON1 and LEC1-LIKE
56
57 in developing seeds. Plant Physiol. 156, 1577-1588.
58
59
60
61
62
63
64
65

- 1
2
3
4 Thomas, T.M., Scopes, R.K., 1998. The effects of temperature on the kinetics and stability of
5
6 mesophilic and thermophilic 3-phosphoglycerate kinases. *Biochem. J.* 330, 1087-1095.
7
8
9 Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The
10
11 CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment
12
13 aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.
14
15
16 Troncoso-Ponce, M.A., Garcés, R., Martínez-Force, E., 2010a. Glycolytic enzymatic activities in
17
18 developing seeds involved in the differences between standard and low oil content
19
20 sunflowers (*Helianthus annuus* L.). *Plant Physiol. Biochem.* 48, 961-965.
21
22
23 Troncoso-Ponce, M.A., Rivoal, J., Cejudo, F.J., Dorion, S., Garcés, R., Martínez-Force, E.,
24
25 2010b. Cloning, biochemical characterisation, tissue localization and possible post-
26
27 translational regulatory mechanism of the cytosolic phosphoglucose isomerase from
28
29 developing sunflower seeds. *Planta* 232, 845-859.
30
31
32 Troncoso-Ponce, M.A., Kilaru, A., Cao, X., Durrett, T.P., Fan, J., Jensen, J.K., Thrower, N.A.,
33
34 Pauly, M., Wilkerson, C., Ohlrogge, J.B., 2011a. Comparative deep transcriptional
35
36 profiling of four developing oilseeds. *Plant J.* 68, 1014-1027.
37
38
39 Troncoso-Ponce, M.A., Rivoal, J., Dorion, S., Moisan, M.-C., Garcés, R., Martínez-Force, E.,
40
41 2011b. Cloning, biochemical characterization and expression of a sunflower
42
43 (*Helianthus annuus* L.) hexokinase associated with seed storage compounds
44
45 accumulation. *J. Plant Physiol.* 168, 299-308.
46
47
48 Troncoso-Ponce, M.A., Kruger, N.J., Ratcliffe, R.G., Garcés, R., Martínez-Force, E., 2009.
49
50
51 Characterization of glycolytic initial metabolites and enzyme activities in developing
52
53 sunflower (*Helianthus annuus* L.) seeds. *Phytochemistry* 70, 1117-1122.
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 Vishwanatha, J.K., Jindal, H.K., Davis, R.G., 1992. The role of primer recognition proteins in
5
6 DNA replication: association with nuclear matrix in HeLa cells. *J. Cell Sci.* 101, 25-34.
7
8
9 Watson H.C., Walker N.P.C., Shaw P.J., Bryant T.N., Wendell P.L., Fothergill L.A., Perkins
10
11 R.E., Conroy S.C., Dobson M.J., Tuite M.F., Kingsman A.J., Kingsman S.M., 1982.
12
13 Sequence and structure of yeast phosphoglycerate kinase. *EMBO J.* 1, 1635-1640.
14
15
16 Zerrad, L., Merli, A., Schroder, G.F., Varga, A., Graczer, E., Pernot, P., Round, A., Vas, M.,
17
18 Bowler, M.W., 2011. A spring-loaded release mechanism regulates domain movement
19
20 and catalysis in phosphoglycerate kinase. *J. Biol. Chem.* 286, 14040-14048.
21
22
23 Zomer, A.W.M., Allert, S., Chevalier, N., Callens, M., Opperdoes, F.R., Michels, P.A.M., 1998.
24
25 Purification and characterisation of the phosphoglycerate kinase isoenzymes of
26
27 *Trypanosoma brucei* expressed in *Escherichia coli*. *Biochim. Biophys. Acta* 1386, 179-
28
29 188.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1

Sequence of PCR primers used in this work.

Primer name	Sequence ^a
<i>dPGK-F</i>	5' -GCTKVRMTYCCWGADGGBGG-3'
<i>dPGK-R</i>	5' -GGYTGGATGGGATTWGA-3'
FA2Z	5' -AACTGGAAGAATTCGCGG-3'
<i>HacPGK1-F</i>	5' -GCATCCGTGCTGCTGTTC-3'
<i>HacPGK1-R1</i>	5' -GGTCGACATCCTTGTTC-3'
<i>HacPGK1-R2</i>	5' -CCGACCTGTACGTCAACGAT-3'
<i>HacPGK2-F</i>	5' -GCATACGCGCCGCGTCC-3'
<i>HacPGK2-R1</i>	5' -GTCAACATCCTTGTGCTC-3'
<i>HacPGK2-R2</i>	5' -CAGACTTATACGTCAATGAT-3'
<i>HapPGK-F</i>	5' -ATGGTGCTAAAGTTATTC-3'
<i>HapPGK-R1</i>	5' -GTGTGACATTTTGCTTTT-3'
<i>HapPGK-R2</i>	5' -CAGATCTTTACGTCAATGAT-3'
<i>SphHacPGK1-F</i>	5' -GACTGCATGCATGGCGACTCTTAATCTCAGACACTGG- 3'
<i>PstHacPGK1-R</i>	5' -CTGACTGCAGTTATATCTTAGGCAATAGAGTTGTGG- 3'
<i>SphHacPGK2-F</i>	5' -CCCGCATGCATGGCGACAAAGAAGAGTGTAAG-3'
<i>SalHacPGK2-R</i>	5' -TTTGTCGACTCATGCCTCATTCAAGGCA-3'
<i>SphHapPGK-F</i>	5' -CCCGCATGCGCGAAGAAGAGTGTTGGTGAC-3'
<i>PstHapPGK-R</i>	5' -TTTCTGCAGTTACGCAGAGACAGTTGCCA-3'
<i>QHacPGK1-F</i>	5' -CGGCGGTTGAGAAAGTTGGAC-3'
<i>QHacPGK1-R</i>	5' -ACCCTTAACCAACAACCTTCAG-3' (3'-UTR)
<i>QHacPGK2-F</i>	5' -CAGCTGTGGAAAAGGTTGGGT-3'
<i>QHacPGK2-R</i>	5' -TTCTAGTCTCAACTAAAACCG-3' (3'-UTR)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

QHapPGK-F 5' -AAGGTTGGAGTGGCGGATGTG-3'
QHapPGK-R 5' -ATCACGATTTAGAACTACGTC-3' (3'-UTR)
QHaActin-F4 5' -GCTAACAGGGAAAAGATGACT-3'
QHaActin-R4 5' -ACTGGCATAAAGAGAAAGCACG-3'

^a Restriction sites are indicated in bold.

Table 2. Apparent kinetic parameters for (6His)*Hac*PGK1, (6His)*Hac*PGK2 and (6His)*Hap*PGK recombinant protein at different pH values. V_{\max} , maximum velocity (nkat mg⁻¹ of protein); K_m , Michaelis-Menten (μ M). Data are mean values and standard deviations from three independent experiments.

Substrate	Kinetic parameters	pH 7.0	pH 7.6	pH 8.2
(6His)<i>Hac</i>PGK1				
3-PGA	V_{\max}	243.7 ± 3.8	244.5 ± 5.2	254.4 ± 3.3
	K_m	1948.6 ± 96.4	1787.9 ± 121.1	1635.1 ± 176.7
	V_{\max}/K_m	0.12	0.13	0.15
ATP	V_{\max}	177.0 ± 1.5	179.2 ± 5.1	168.8 ± 4.6
	K_m	243.3 ± 5.7	227.6 ± 17.4	215.7 ± 15.9
	V_{\max}/K_m	0.72	0.78	0.78
(6His)<i>Hac</i>PGK2				
3-PGA	V_{\max}	5583.4 ± 58.1	6056.4 ± 61.5	5829.0 ± 123.7
	K_m	601.7 ± 27.9	711.2 ± 30.6	683.3 ± 62.1
	V_{\max}/K_m	9.27	8.51	8.53
ATP	V_{\max}	5203.5 ± 135.2	5150.2 ± 156.5	5470.2 ± 210.0
	K_m	357.1 ± 21.8	367.5 ± 26.0	404.7 ± 35.2
	V_{\max}/K_m	14.6	14.0	13.5
(6His)<i>Hap</i>PGK				
3-PGA	V_{\max}	5199.7 ± 58.2	5621.3 ± 88.7	5131.2 ± 56.2
	K_m	707.6 ± 33.5	719.6 ± 47.8	589.6 ± 29.1
	V_{\max}/K_m	7.35	7.81	8.70
ATP	V_{\max}	4665.6 ± 56.3	5682.8 ± 132.2	4485.4 ± 132.3
	K_m	300.1 ± 9.0	406.7 ± 21.4	356.9 ± 24.8
	V_{\max}/K_m	15.5	13.9	12.5

Table 3. Effect of various metabolites on (6His)*Hac*PGK1, (6His)*Hac*PGK2 and (6His)*Hap*PGK recombinant protein activities. Values in bold indicate that PGK activity was significantly different from that of the control (no effector).

Effectors	Concentration (mM)	PGK activity (%)		
		(6His) <i>Hac</i> PGK1	(6His) <i>Hac</i> PGK2	(6His) <i>Hap</i> PGK
None		100.0 ± 2.8	100.0 ± 4.3	100.0 ± 2.3
Glycerol-3P	10	92.0 ± 11.1	95.9 ± 1.2	97.2 ± 0.4
Glycerol-2P	10	97.3 ± 3.2	100.5 ± 1.2	101.0 ± 2.6
UDP	10	94.3 ± 4.4	97.9 ± 1.0	104.5 ± 1.7
ADP	0.1	63.1 ± 2.0	86.7 ± 2.9	85.6 ± 1.7
	0.25	62.4 ± 2.7	74.2 ± 2.3	69.7 ± 4.4
	0.5	62.4 ± 2.7	50.8 ± 3.4	48.1 ± 3.6
	1	32.3 ± 4.7	31.4 ± 2.2	32.5 ± 2.6
AMP	0.625	104.3 ± 6.4	95.2 ± 1.5	98.9 ± 1.5
	1.25	99.8 ± 4.8	88.4 ± 1.1	94.4 ± 1.7
	2.5	96.5 ± 3.4	77.1 ± 1.8	86.1 ± 1.4
	5	78.1 ± 6.8	62.6 ± 2.3	72.4 ± 1.8
	10	40.5 ± 5.8	44.4 ± 2.6	50.9 ± 3.4

1
2
3
4 **Figure legends**
5

6
7 **Fig. 1.** Alignment of the deduced amino acid sequences of sunflower plastidial, *HapPGK*
8 (accession number ADV16381), and cytoplasmic, *HacPGK1* (ABI18157) and *HacPGK2*
9 (ADV16380), PGKs with the closely related sequences from *Arabidopsis thaliana* (*AtpPGK1*
10 NP176015 , *AtpPGK2* NP187884 and *AtcPGK* NP178073) and the more phylogenetically distant
11 sequence from *Geobacillus stearothermophilus* (*GsPGK*, P18912). Asterisks mark identical
12 residues, colon marks conservative changes, and dot marks weakly conservative changes
13 between the sequences. Residues involved in the substrate binding site are indicated with white
14 triangles, those involved in hinge regions with grey triangles and the ones forming the ADP
15 binding site with black triangles.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 **Fig. 2.** Phylogenetic comparison of PGK proteins from plants, green algae and bacteria rooted in
32 the bacteria *Geobacillus stearothermophilus* PGK protein sequence, used as template for
33 *HaPGKs* three-dimensional structure modelling. GenBank accession numbers follow the species
34 names.
35
36
37
38
39
40
41
42

43 **Fig. 3.** Proposed structural models for *HacPGK1* and *HapPGK* phosphoglycerate kinases. (A
44 and C) Ribbon diagrams for *HacPGK1* and *HapPGK* respectively. (B and D) Views of the
45 molecular surfaces for *HacPGK1* and *HapPGK* respectively, showing the residues involved in
46 the substrate binding site in green, those forming the ADP binding site in pink and the catalytic
47 site in blue.
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 **Fig. 4.** SDS-PAGE showing the (6His)*HacPGK1*, (6His)*HacPGK2* and (6His)*HapPGK*
5 purification steps. MW, molecular mass standards; lanes 1, 3 and 5 *E. coli* crude extracts after
6 induction of (6His)*HacPGK1*, (6His)*HacPGK2* and (6His)*HapPGK* respectively; and lanes 2, 4
7 and 6 purified (6His)*HacPGK1*, (6His)*HacPGK2* and (6His)*HapPGK* proteins respectively. The
8 running positions of molecular mass standards are indicated on the left.

9
10
11
12
13
14
15
16
17
18
19 **Fig. 5.** Thermal stability of (6His)*HacPGK1*, (6His)*HacPGK2* and (6His)*HapPGK* activities.
20 Data are mean values and standard deviations from three independent experiments.

21
22
23
24
25
26 **Fig. 6.** Effect of pH variation on (6His)*HacPGK1*, (6His)*HacPGK2* and (6His)*HapPGK*
27 activities. Data are mean values and standard deviations from three independent experiments.

28
29
30
31
32
33 **Fig. 7.** PGK expression levels from developing seeds and vegetative tissues from *Helianthus*
34 *annuus* (A) and *Arabidopsis thaliana* (B). A, *HacPGK1*, *HacPGK2* and *HapPGK* expression
35 determined by real time PCR from sunflower line CAS-6; B, *AtcPGK* (At1g79550), *AtpPGK1*
36 (At1g56190) and *AtpPGK2* (At3g1270) expression estimated from microarrays of Schmid et al.
37 (2005). Sunflower seeds stages counting days after flowering (DAF); Arabidopsis stages: 3, mid
38 globular to early heart embryos; 4, early to late heart embryos; 5, late heart to mid torpedo
39 embryos; 6, mid to late torpedo embryos; 7, late torpedo to early walking-stick embryos; 8,
40 walking-stick to early curled cotyledons embryos; 9, curled cotyledons to early green cotyledons
41 embryos; 10, green cotyledons embryos. Values in panel A represent mean values of three
42 independent samples.

1
2
3
4 **Fig. 8.** PGK protein and activity levels in developing sunflower seeds. (A) Recognition of all
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

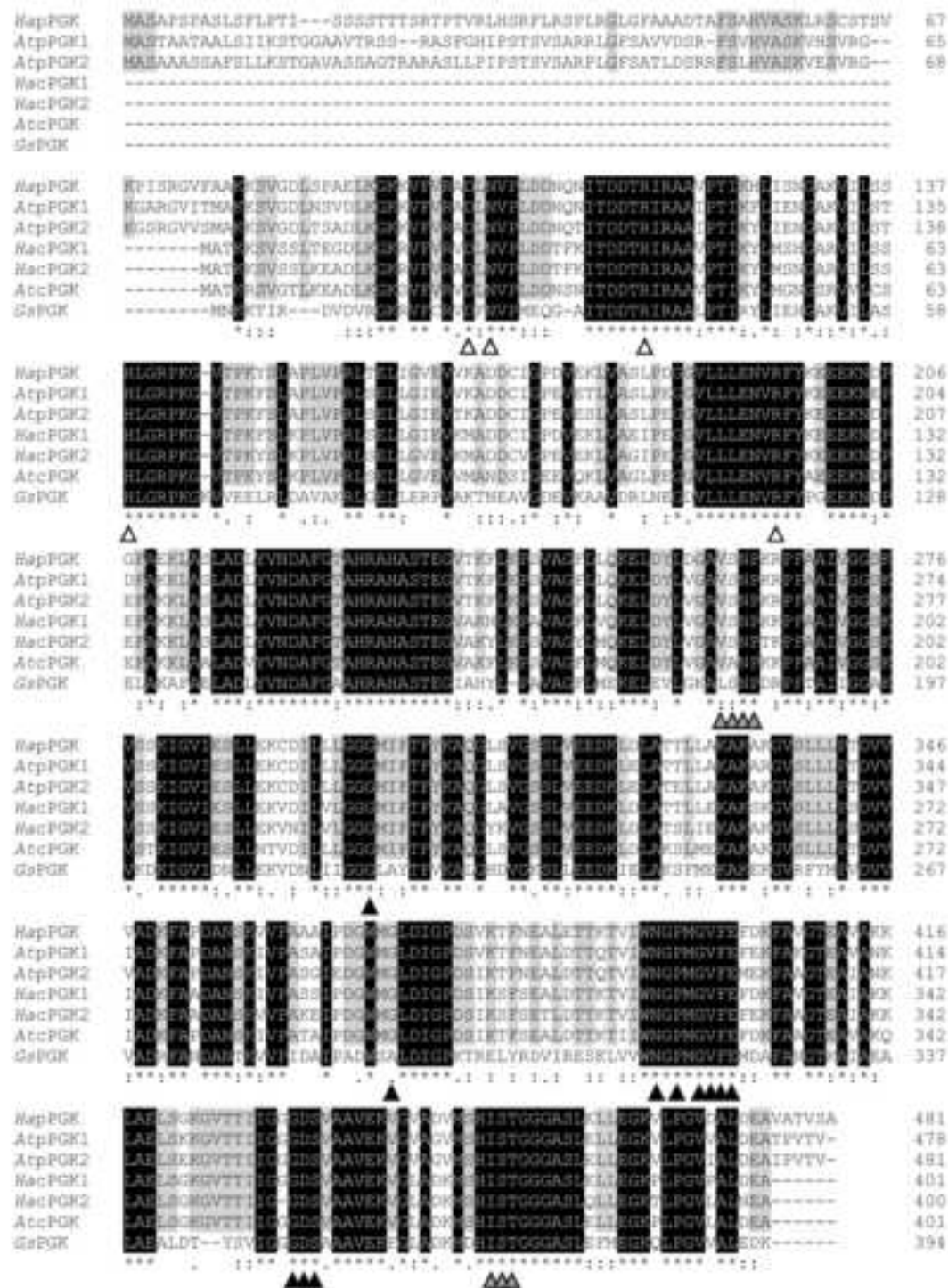
sunflower PGKs by an immune serum raised against cPGK1. Western blot analysis of 25 ng each of the following purified recombinant proteins: lane 1: (6His)*Hac*PGK1, lane 2: (6His)*Hac*PGK2 and lane 3: (6His)*Hap*PGK. (B) Immunoblot analysis of *Ha*PGKs in developing sunflower seed extracts. Developing seeds were collected at 15, 20, 25 and 30 days after flowering (number of DAF indicated above each lane). Fifteen μ g of proteins from each extract were subjected to immunoblot analysis using the immune serum as in panel (A). Purified (6His)*Hac*PGK1 protein (lane P) was used as a control. (C) Total PGK activity levels measured in developing sunflower kernels from line CAS-6. PGK activity values are the means \pm SD from three independent samples.

31 **Fig. 9.** PGKs localisation in sunflower seed tissues. Longitudinal section of the proximal end
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

incubated with antibodies raised against purified sunflower 6(His)*Hac*PGK1 (A) or with pre-immune serum (B). Bar, 500 μ m; PR, procambial ring.

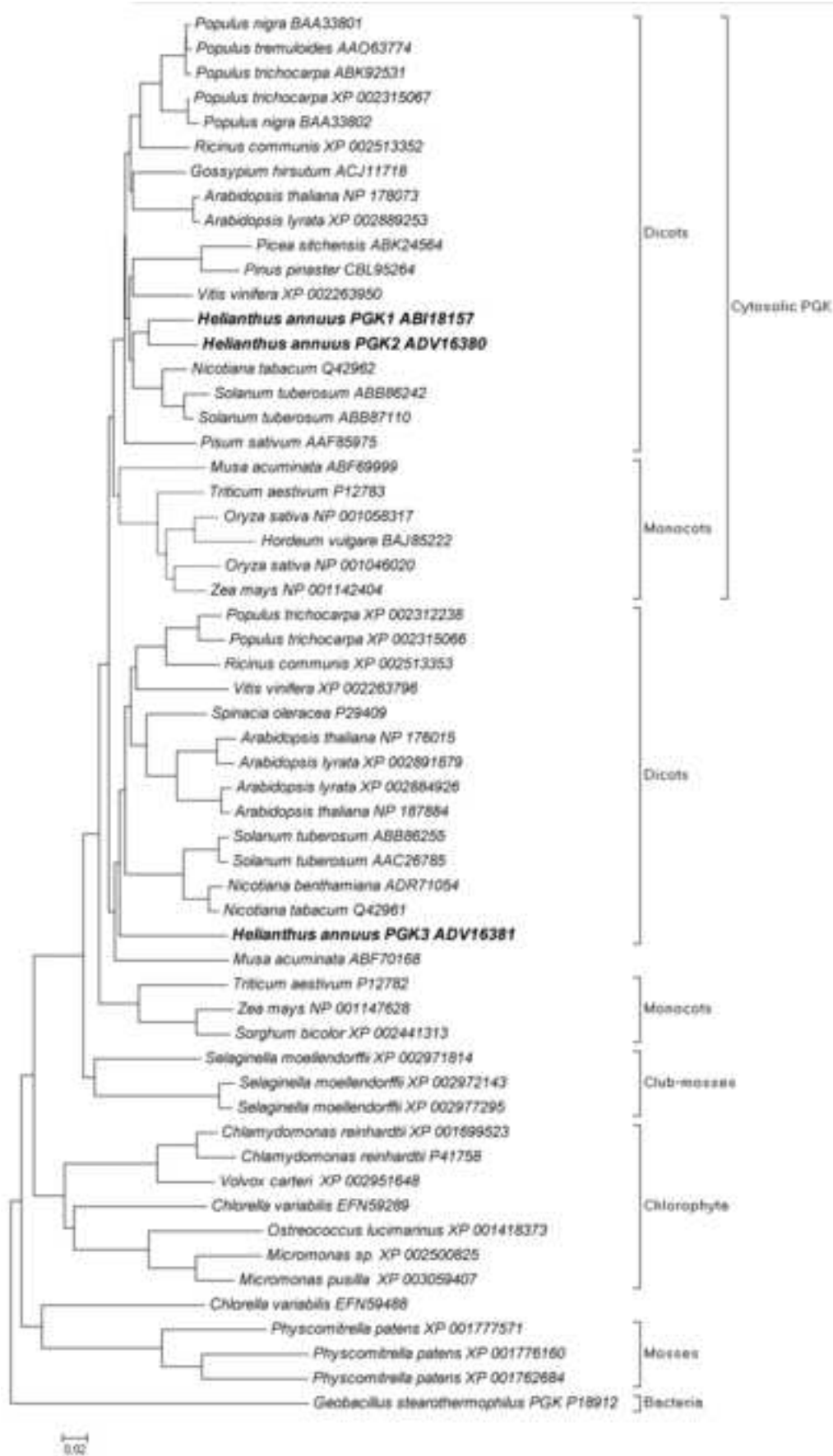
Figure(s)

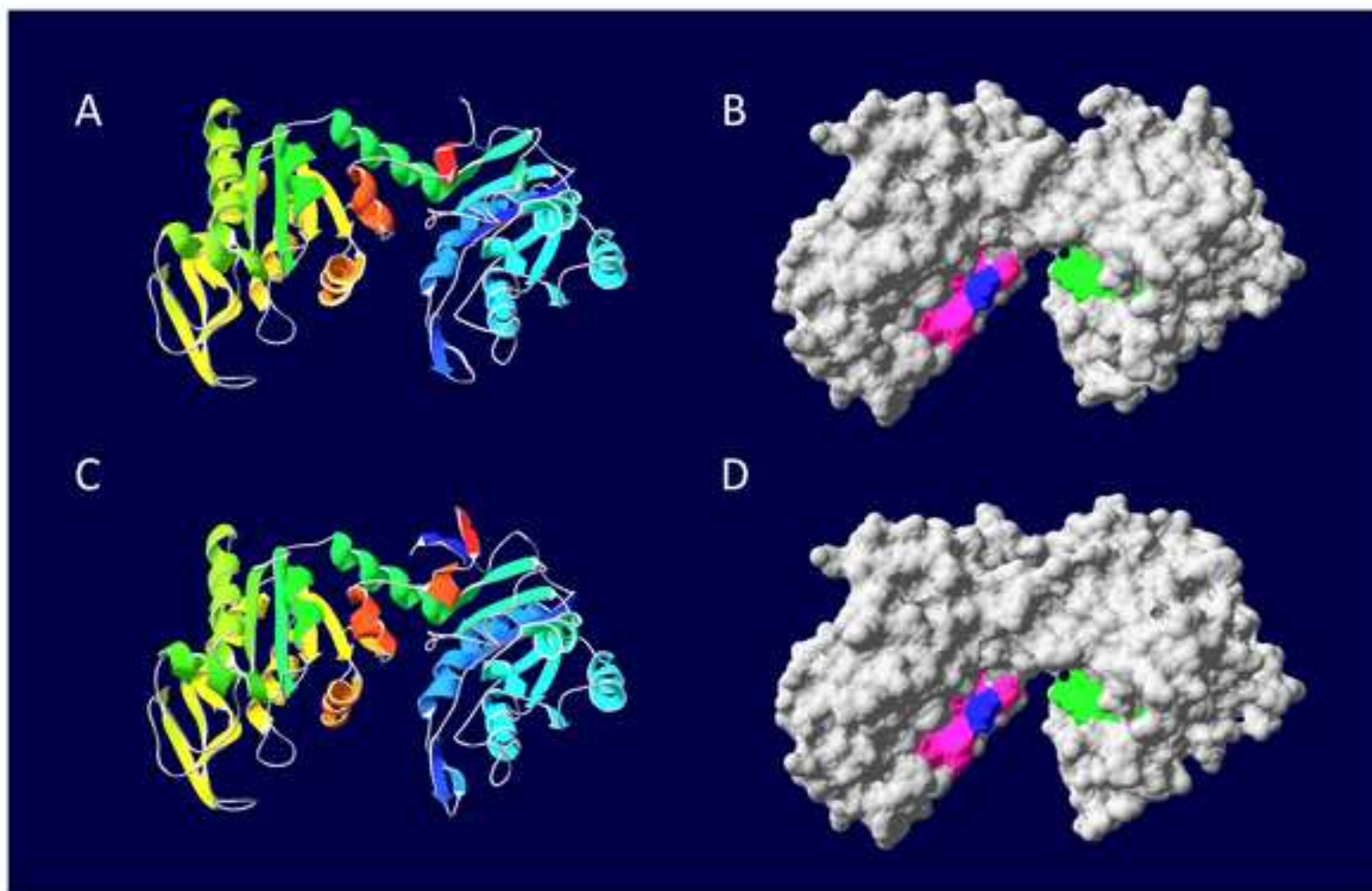
[Click here to download high resolution image](#)



Figure(s)

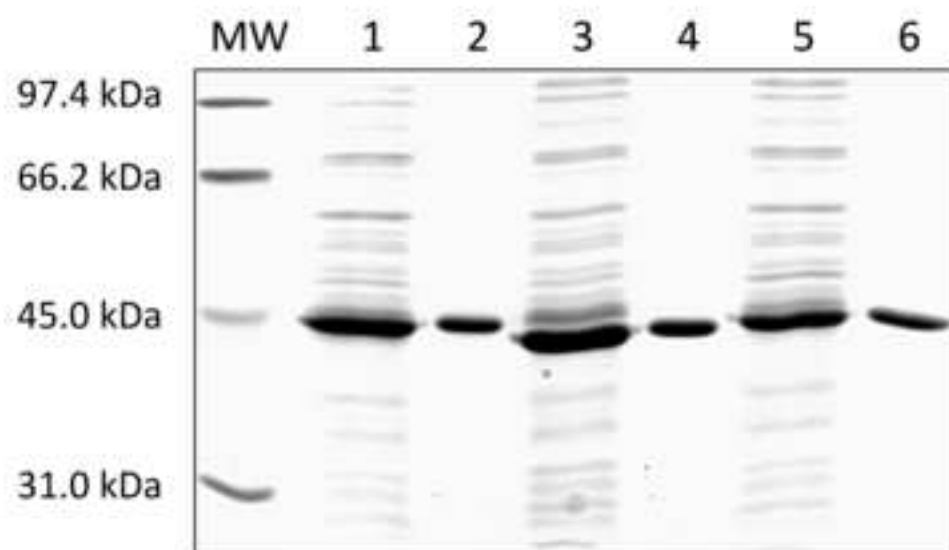
[Click here to download high resolution image](#)



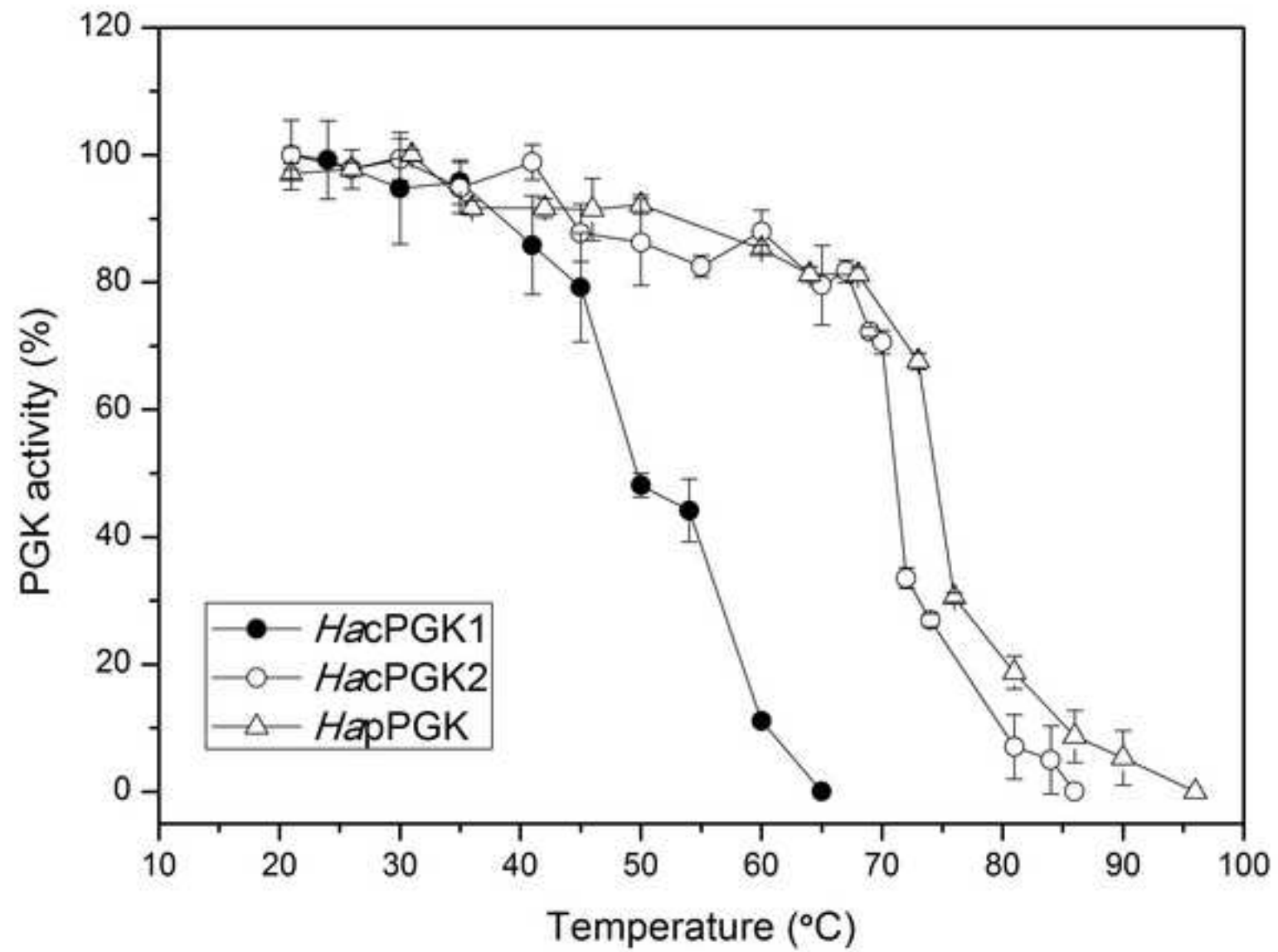


Figure(s)

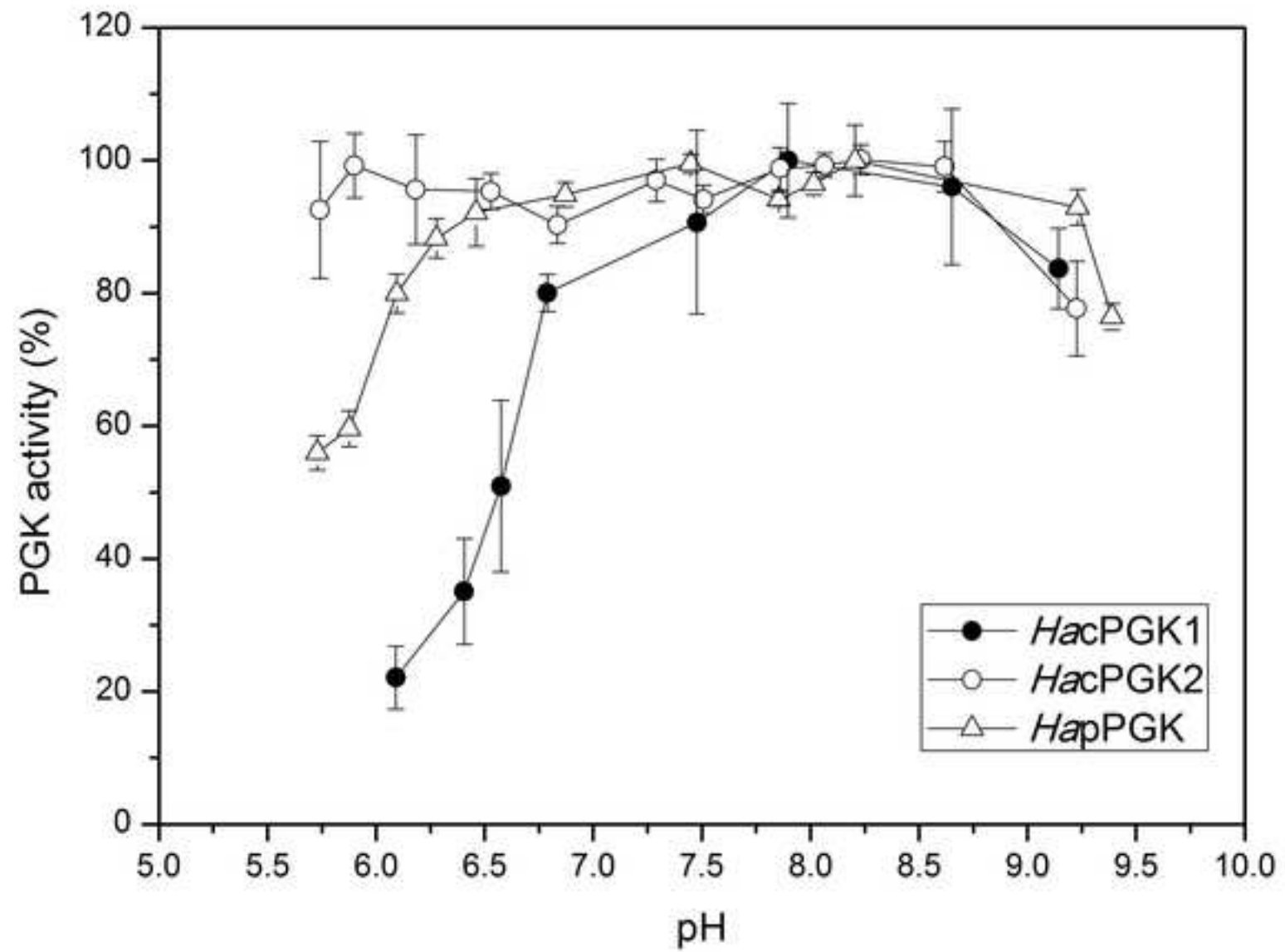
[Click here to download high resolution image](#)



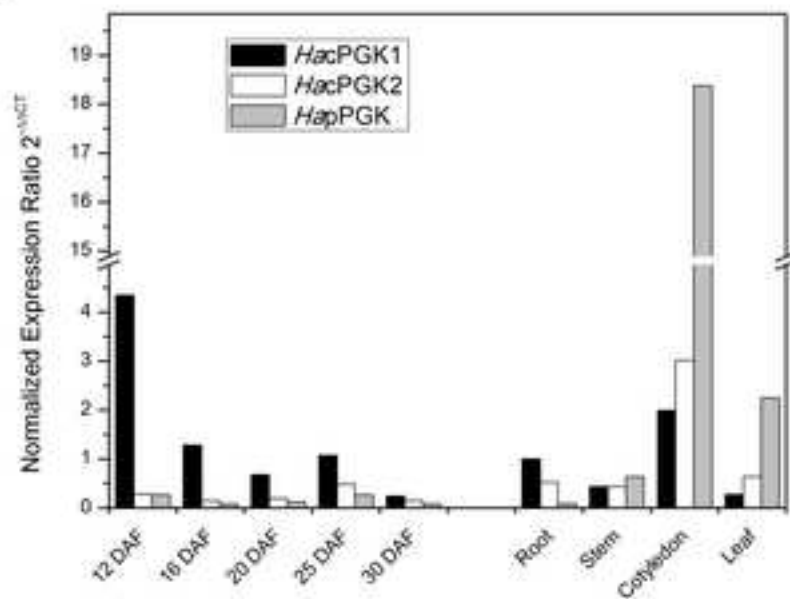
Figure(s)
[Click here to download high resolution image](#)



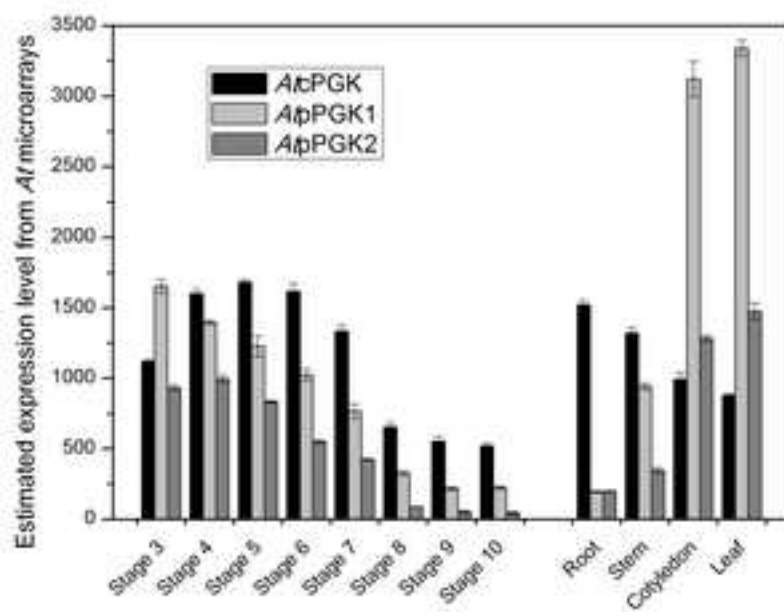
Figure(s)
[Click here to download high resolution image](#)

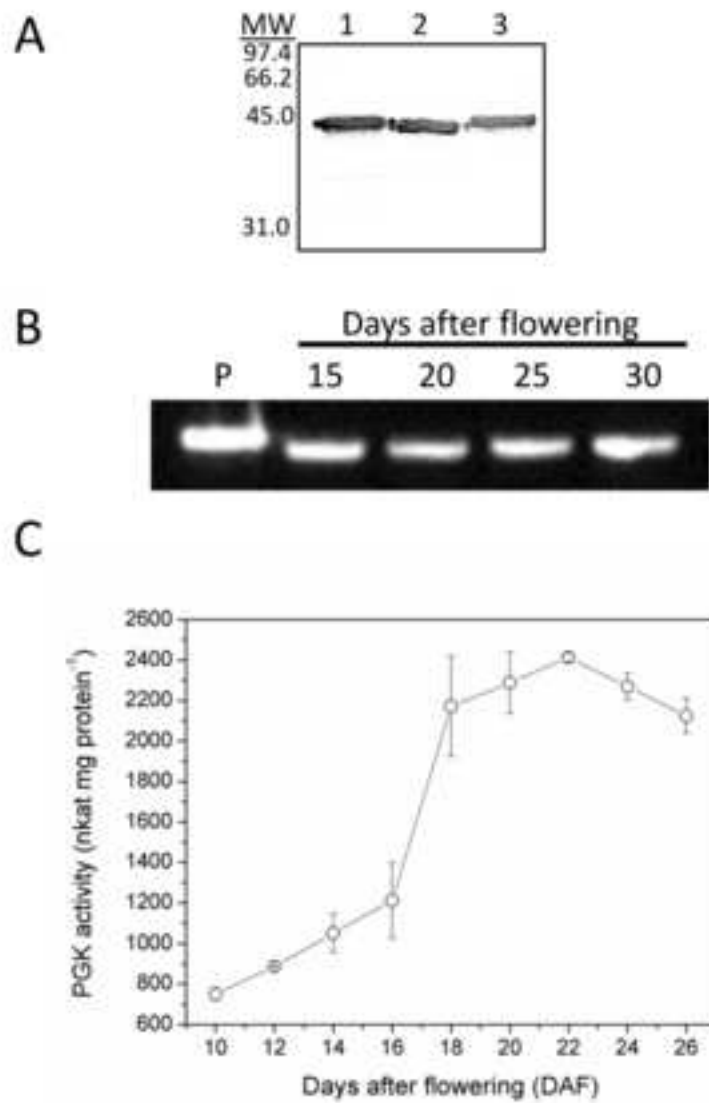


A



B





Figure(s)

[Click here to download high resolution image](#)

