Plant Physiology, April 2000, Vol. 122, pp. 1187–1192, www.plantphysiol.org © 2000 American Society of Plant Physiologists

The *rug3* Locus of Pea Encodes Plastidial Phosphoglucomutase[1]

Christopher J. Harrison, Ruth M. Mould, Mark J. Leech, Susan A. Johnson, Lynda Turner, Sabine L. Schreck, Kathleen M. Baird, Peter L. Jack[2], Stephen Rawsthorne, Cliff L. Hedley, and Trevor L. Wang*

John Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom (C.J.H., M.J.L., S.A.J., L.T., S.L.S., S.R., C.L.H., T.L.W.); Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom (R.M.M.); Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom (K.M.B.); and Plant Breeding International Cambridge, Cambridge CB2 2LQ, United Kingdom (P.L.J.)

Two cDNA clones were isolated from pea (*Pisum sativum* L.) and their deduced amino acid sequences shown to have significant homology to phosphoglucomutases from eukaryotic and prokaryotic sources. The longer cDNA contained a putative transit-peptide-encoding sequence, supporting the hypothesis that the isolated clones represent the cytosolic and plastidial isoforms of phosphoglucomutase in pea. Plastid protein import assays confirmed that the putative plastidial isoform was targeted to the plastid stroma where it was proteolytically processed. Expression, co-segregation, linkage, and molecular analyses have confirmed that the *rug3* locus of pea encodes plastidial phosphoglucomutase. Mutations at this locus result in a near-starchless phenotype of the plant.

Phosphoglucomutase (PGM) is of significant importance in the partitioning of stored carbon. In starch-storing plants, multiple isoforms of PGM often exist, since the enzymes are required both in the cytoplasm and in the plastids. Within the amyloplast, plastidial PGM (PGM[P]) provides the substrate, Glc-1-P, for the committed pathway of starch synthesis. In plants that rely upon the import of Glc-6-P exclusively, there is an absolute requirement for PGM activity within the plastids, and its absence has been shown to result in starchless (or near-starchless) mutants (Caspar et al., 1985; Hanson and McHale, 1988; Harrison et al., 1998).

The seeds of an allelic series of *rug3* mutant lines are severely wrinkled at maturity and have starch contents ranging from approximately 1% (w/w) to 12% as a proportion of the dry weight depending on genotype (Harrison et al., 1998). The *rug3* plants are unique in that they are the first near-starchless mutants of a starch-storing crop that are completely viable, the mutation having little effect on the plant when grown under normal conditions. This is in contrast to near-starchless mutants of other higher plant species (Caspar et al., 1985; Hanson and McHale, 1988; Lin et al., 1988). In the *rug3* mutant pea (*Pisum sativum* L.) lines, the activity of the enzyme plastidial phosphoglucomutase is reduced to a virtually undetectable level as a result of the most severe alleles. Linkage analysis has shown that the wrinkled character of seeds with the *rug3rug3* genotype maps very close to the previously ascertained location of the PGM(P) isozyme (Weeden and Marx, 1984; Weeden et al., 1984). Together with biochemical data, this has provided evidence that the *rug3* locus encodes the PGM(P) structural gene (Harrison et al., 1998). Following the cloning of the gene, targeting experiments, restriction fragment-length polymorphism (RFLP) and expression analyses, we now demonstrate conclusively that the *rug3* locus encodes plastidial phosphoglucomutase.

MATERIALS AND METHODS

Plant Material

Seeds of each of the *rug3rug3* pea (*Pisum sativum* L.) lines and seeds near-isogenic to these lines except for alleles at the *rug3* locus were grown in 5-inch pots containing John Innes no. 1 compost to which 30% (w/v) chick grit had been added. Once established, the plants were fed weekly with low-nitrogen fertilizer. Greenhouses were maintained in a 15°C/10°C minimum day/night cycle with supplementary lighting to provide a minimum photoperiod of 16 h. For the purpose of chloroplast isolation, pea shoots (cv Feltham First) were grown as described by Mould and Gray (1998a).

Extraction of RNA

RNA was extracted from pea tissues according to Harrison (1996). Poly(A^+) mRNA was prepared from total RNA using the Poly-A-Tract mRNA isolation kit (Promega, Southampton, UK).

Preparation of cDNA Libraries and Screening

RNA was isolated from pea embryos (round-seeded BC1; Hedley et al., 1986) with fresh weights of 1 to 10 mg (a), 10 to 100 mg (b), 100 to 200 mg (c), 200 to 300 mg (d), and 300 to 400 mg (e). Poly(A^+) mRNA was prepared from an

[1] The John Innes Centre is supported by a grant-in-aid from the Biotechnology and Biological Sciences Research Council. S.L.S. has a research studentship from the John Innes Foundation and R.M.M. has a fellowship from the Royal Society.

[2] Present address: Monsanto Agriculture, Maris Lane, Cambridge CB2 2LQ, United Kingdom.

* Corresponding author; e-mail trevor.wang@bbsrc.ac.uk; fax 44–1603–456844.

equimolar mixture of total RNA from the five classes as described above. Poly(A^+) mRNA was isolated directly from 200-mg embryos of cv Novella using a mRNA purification kit (Quickprep, Amersham-Pharmacia Biotech, Little Chalfont, UK). In independent cloning experiments, two cDNA libraries were used: a ZAP XR library was constructed from 5 g of the mRNA from BC1 by Stratagene (La Jolla, CA), and a second cDNA library was constructed from the mRNA isolated from cv Novella using the ZAPII construction kit (Stratagene). Libraries were amplified and approximately 10^6 colonies from each were used in the screening procedure.

The ZAP XR library screen was carried out using a cDNA clone isolated from potato (a gift from R. Trethewey, Max-Plank-Institute, Golm, Germany) showing considerable homology to published PGM sequences. A DNA fragment comprising the entire potato cDNA clone was radiolabeled using the Ready-To-Go labeling kit (Amersham-Pharmacia Biotech). The resulting probe was hybridized to nylon membranes (HyBond; Amersham-Pharmacia Biotech), bearing approximately 5×10^5 PFUs from the cDNA library, overnight at a temperature of 50°C in hybridization buffer consisting of 5× Denhardt's mixture, 6× SSC , and 0.1% (w/v) SDS (Sambrook et al., 1989). The membranes were washed at low stringency in 2× SSC at 50°C twice for 30 min each time. The membranes were exposed to x-ray film overnight. Positive plaques were purified. In vitro excision of pBluescript plasmids containing putative PGM inserts was carried out and plasmids purified using plasmid purification kits (Qiagen, Crawley, UK). PCR amplification was carried out on the plasmids to ascertain insert sizes.

A suspension of phage (1 μL) from each of the putative PGM clones was spotted onto a 15-cm Petri dish preinoculated with top-agar containing *Escherichia coli* strain XL-1-Blue plating cells. After an overnight incubation at 37°C, each spot of phage suspension had produced a plaque approximately 5 mm in diameter. Duplicate plaque lifts were taken from this plate and probed with a radiolabeled fragment obtained from one of the putative PGM clones (PGM1) as above. One membrane was washed at low stringency (50°C, 2× SSC and 0.1% [w/v] SDS) and the other at high stringency (65°C, 0.1× SSC and 0.1% [w/v] SDS). The membranes were then exposed to x-ray film overnight.

In the ZAP II library screen, the PCR product cloned from amplification of cv Novella cDNA described above was radiolabeled and hybridized to library filters at 65°C in 5× Denhardt's and 6× SSC overnight. The filters were washed at high stringency (0.1× SSC at 65°C) and exposed to x-ray film. Positive plaques were purified and PCR amplified to ascertain insert size.

Amplification of PGM Gene Fragment

Complementary DNA was prepared from pea embryo mRNA according to the method of Sambrook et al. (1989). Degenerate primers (5′-ACIGCIWSICAYAAYCC and 5′-CKRTCICCRTCICCRTCRAAIGC) were synthesized based on previously identified (Harrison, 1996) regions of conserved amino acid sequence in known PGM genes from *E. coli*, yeast, rabbit, rat, and human. PCR amplification of pea embryo cDNA (cv Novella) using standard conditions (25-μL reaction mixture containing: 0.2 mM dNTPs, 0.3 μM each oligonucleotide primer, l0 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 units of *Taq* DNA polymerase [Advanced Biotechnologies, Epsom, UK]) and a PCR cycle with an annealing temperature of 44°C yielded a fragment of the expected size (500 bp). The fragment was cloned using the pT7Blue T vector kit (Novagen, Madison, WI) and sequenced to confirm homology with known PGM sequences.

Sequence Analysis and Data Assembly

Sequence analysis of all putative PGM clones was carried out using an automated system (PRISM 377XL, Applied Biosystems, Foster City, CA). Sequencing reactions were carried out using the PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) following the manufacturer's instructions. M13 and internal primers were used to obtain complete sequences of the clones. Sequence data assembly was carried out with the GCG package (Wisconsin Package, version 10.0, Genetics Computer Group, Madison, WI). Phylogenetic analysis of the peptide sequence was carried out using PHYLIP (PHYLogeny Inference Package, version 3.5, University of Washington, Seattle).

Co-Segregation Analysis

Segregating populations were constructed by crossing a conventional (*Rug3Rug3,rr*) vining cultivar (Harrier) with the *rug3-erug3-e* line and generating F_2 and F_4 material. Plants were phenotyped through a combination of visual inspection of their seed and iodine staining of leaf tissue. DNA from segregating populations was isolated (Dellaporta et al., 1983), digested with *Eco*RI, and fractionated by electrophoresis through 1% (w/v) agarose prior to capillary blotting (Sambrook et al., 1989). The insert from a putative pea plastidial PGM clone was radiolabeled and used as a probe.

Protein Import Analysis

Intact chloroplasts were prepared from approximately 100 g of pea shoots using Percoll step gradients (Mould and Gray, 1998a). Plasmids bearing the pea PGM clones were linearized by digestion with *Xho*I. The linearized plasmids were transcribed in vitro using T3 RNA polymerase. Radiolabeled precursor proteins were synthesized in a wheat germ translation system, including [^{35}S]Met, [^{35}S]Cys, and transcription products, essentially as described in Mould and Gray (1998b).

Import assays contained intact pea chloroplasts (600 mg of chlorophyll), 5 mM Met, 5 mM Cys, and 10 mM MgATP in a final volume of 600 mL of import buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, pH 8.0, and 0.33 M sorbitol) with 45 μL of radiolabeled products from translation in vitro. Assays were incubated

in the light (100 mmol photons m^{-2} s^{-1}) for 60 min at 25°C. After the incubation, intact chloroplasts were treated with thermolysin (0.2 mg/mL final concentration in import buffer) for 30 min on ice and then the protease reaction was stopped by the addition of EDTA to 50 mM in import buffer. Chloroplasts were re-isolated by centrifugation through a cushion of 40% (w/v) Percoll in import buffer and then washed in import buffer (Mould and Gray, 1998b). An aliquot (1/10) of the thermolysin-treated chloroplast sample was taken for analysis and the remainder was fractionated essentially as described by Schnell and Blobel (1993). Samples of thermolysin-treated chloroplasts, stromal fraction, thylakoids, and thermolysin-treated thylakoids were quantified by SDS-PAGE (Laemmli, 1970) followed by Coomassie staining and scanning densitometry of stained protein bands (Rubisco subunits and the light-harvesting chlorophyll-protein complex). Equivalent amounts of these fractions (approximately equal to 2% of the chloroplasts recovered from the Percoll gradient), and 50% of the inner and outer envelope fractions recovered were analyzed by electrophoresis on a 10% (w/v) polyacrylamide gel in the presence of SDS, followed by fluorography.

Northern Blotting

Poly(A^+) RNA was isolated from pea leaves and embryos as described above. Northern blotting was carried out according to the method of Sambrook et al. (1989).

Linkage Analysis

RFLPs were identified in parental lines of a recombinant-inbred population previously used for linkage analysis in pea (Ellis et al., 1998; Laucou et al., 1998) using putative pea plastidial PGM and cytosolic PGM cDNA clones. Corresponding Southern blots from the RI population were screened with the two cDNA clones.

Molecular Analysis of Mutant Alleles

RNA was extracted from 200- to 300-mg *rug3* embryos as described above. Complementary DNA was prepared and PCR amplified using pairs of primers based on the wild-type PGM(P) sequence. Primers were designed such that the coding region could be completely sequenced from three overlapping PCR products. PCR was carried out on cDNA from each of the mutant lines using *pfu* polymerase (Stratagene), following the recommended protocols for this enzyme with annealing temperatures of 50°C.

RESULTS

Cloning and Sequencing of PGM cDNAs from Pea

PCR products generated from consensus sequence data were used successfully to isolate one class of cDNA clones from one of the library screens. The longest of these clones was fully sequenced and found to have significant homology to known PGMs. From the low-stringency screen of a cDNA library with the potato cDNA clone, two classes of clones were identified and found to cross-hybridize at low but not at high stringency. Subsequent sequence analysis revealed that these two classes were indeed related (64% identity at the DNA sequence level over approximately 1,700 bp) and that both showed significant homology to known PGMs on the basis of their deduced peptide sequence. One class of clone, referred to herein as PGM2 (EMBL accession no. AJ250770), was identical to that obtained by both PCR and library screening, and showed an extended region at the 5′ end corresponding to approximately 60 amino acids. The shorter cDNA clone is referred to herein as PGM1 (EMBL accession no. AJ250769). A comparison spanning the region of the active site of the deduced peptide sequences from the cDNAs with published PGM peptide sequences produced the PRETTYBOX diagram shown in Figure 1. The consensus phylogenetic tree produced from 10 replicates of the PROTDIST program is shown in Figure 2, and represents a possible relationship between the PGM sequences shown in Figure 1.

Co-Segregation Analysis

Segregating populations of plants were generated by crossing the *Rug3Rug3* line Harrier with the *rug3-erug3-e* mutant line. Genomic DNA from these parental lines showed a RFLP when digested with *Eco*RI and probed with the full-length PGM2 cDNA clone. F_2 and F_4 populations were analyzed for their phenotype with regard to this polymorphism, the seed shape, and the reaction of the leaves to iodine staining. Highly wrinkled-seeded (*rug3rug3*) plants (21 plants for F_2 and nine for F_4) showing an absence of starch in their leaves were all homozygous for the RFLP allele corresponding to that from the *rug3-erug3-e* parental line. Plants (77 for F_2 and 33 for F_4) having starch in their leaves and possessing the seed phenotype of the Harrier

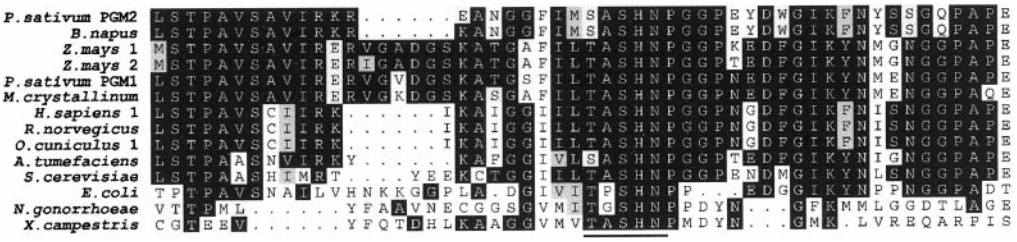

Figure 1. Comparison of PGM amino acid sequences across the active site of the enzyme (underlined). Identical residues are highlighted in black, similar residues in gray.

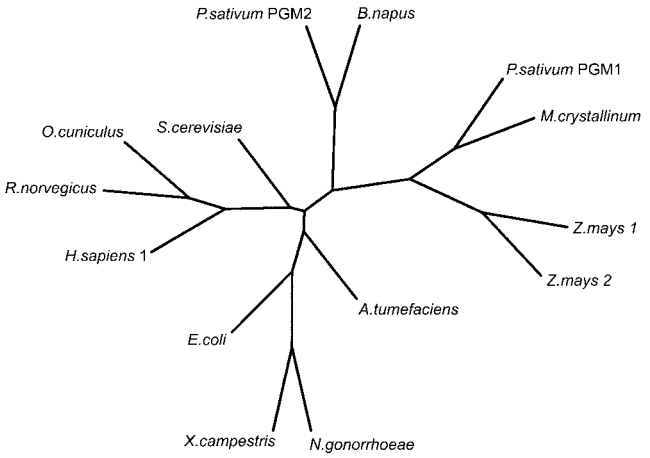

Figure 2. Tree representing a possible phylogenetic relationship between the PGM sequences shown in Figure 1. The sequence region compared was a 54-amino acid section including the known PGM active site. The *B. napus* sequence (EMBL accession no. AJ250771) was obtained by sequencing a clone from a screen of a silique cDNA library with PGM2.

parent (mildly wrinkled) showed the RFLP alleles of either the Harrier parent or of both parents (heterozygotes).

Linkage Analysis

RFLPs for the two PGM cDNAs were obtained for the crosses JI281 × JI399. The phenotypes with respect to the RFLPs were scored in the RI population from the corresponding cross, and this information was integrated into the existing genetic map of pea generated from this cross (Ellis et al., 1998; Laucou et al., 1998). Linkage group positions were deduced by multiple two-point tests. The PGM1 and PGM2 cDNAs mapped to linkage groups VII and II, respectively, at positions corresponding to those reported previously for PGM isozymes (Weeden and Marx, 1984, 1987; Weeden et al., 1984).

Expression of PGM Genes

Figure 3 shows northern-blot analyses of embryo mRNA using the PGM1 and PGM2 cDNA clones as probes. RNA transcripts were detected in all lines with the PGM1 probe, with the transcript being approximately 2.3 kb. Between lines, however, variation in transcript levels was evident.

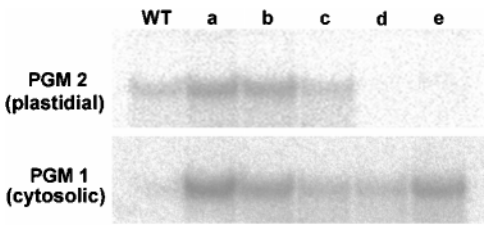

Figure 3. Northern-blot analysis of PGMs. WT, Wild type. a to e, *rug3-a* to *rug3-e*. Each lane contains 5 μg of poly(A$^+$) RNA isolated from immature pea embryos. Image produced using a phosphor imager.

When probed with DNA from the PGM2 clone, no transcript was detectable in RNA isolated from the *rug3-d* line, whereas in the *rug3-e* mutant the transcript level was reduced to 10% of the wild type based on densitometric measurements of bands after a long exposure.

In Vitro Transcription and Protein Targeting Analysis

Plasmids were transcribed in vitro and the products translated in a wheat germ system including [^{35}S]Met and [^{35}S]Cys to produce radiolabeled protein. The major translation product from PGM1 transcripts was approximately 64 kD. Two major translation products were generated from PGM2 transcripts of approximately 69 and 65 kD. When isolated intact pea chloroplasts were incubated with radiolabeled protein from the latter in the presence of ATP, a radiolabeled polypeptide of approximately 59 kD was generated (Fig. 4), which is consistent with the cleavage of the putative transit peptide. After import, incubations were treated with the protease thermolysin (which, under the conditions used does not penetrate the chloroplast envelope) and intact chloroplasts re-isolated and fractionated. The resistance of the 59-kD polypeptide to degradation by exogenously added thermolysin (Fig. 4, lane C) indicates that it is located within the chloroplast and is a product of radiolabeled protein import. Fractionation of the intact thermolysin-treated chloroplasts into stroma (Fig. 4, lane S), washed thylakoids (lane T), thermolysin-treated thylakoids (lane tT), inner envelopes (lane I), and outer envelopes (lane O) demonstrated that the radiolabeled polypeptide was present in the stromal fraction. When isolated intact pea chloroplasts were incubated with radiolabeled PGM1 protein in the presence of ATP, no radiolabeled protein was associated with the chloroplast fraction after thermolysin treatment (results not shown).

Molecular Analysis of *rug3* Mutants

The complete sequence of the coding region of the PGM2 gene from the *rug3-brug3-b* and *rug3-drug3-d* lines was obtained from overlapping clones obtained in duplicate from independent PCR reactions. The analysis of the sequence revealed one base change in the *rug3-brug3-b* line

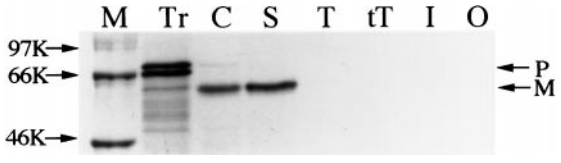

Figure 4. Import of putative PGM(P) by isolated pea chloroplasts. ^{35}S-Labeled PGM(P) was incubated with isolated intact pea chloroplasts at 25°C for 60 min. Chloroplasts were subsequently treated with thermolysin, re-isolated, lysed, and fractionated. Samples were analyzed on a SDS-10% (w/v) polyacrylamide gel followed by fluorography. Lane M, Molecular mass markers; lane Tr, translation products; lane C, chloroplasts reisolated and thermolysin treated after import incubation; lane S, stromal fraction; lane T, washed thylakoids; lane tT, thermolysin-treated thylakoids; lane I, inner envelope fraction; lane O, outer envelope fraction. P and M, Putative precursor and mature forms of PGM(P) respectively. K, Kilodaltons.

compared with the sequence of the wild type. The single base change (C to T at position 1,744) would result in a change in the amino acid sequence, converting an Arg to a Cys at amino acid position 528. The sequence of the *rug3-drug3-d* line was identical to that of the wild type.

DISCUSSION

A distinctive feature of the amino acid sequence of all known PGMs is the T/SASHN motif that is known to form part of the active site of the enzyme. This motif formed the basis for one of the primers for the PCR-based cloning of the gene, and was subsequently identified in both classes of clones obtained, which provided some evidence that the two cDNA clones were PGMs (or closely related sequences). The sequence of the two clones in general showed 72% (PGM2) and 88% (PGM1) similarity at the amino acid level to the one isoform from maize and 67% (PGM2) and 69% (PGM1), respectively, to the human protein. On the basis of DNA and deduced amino acid sequence, it was considered that the two clones were strong candidates for the two PGM isoforms known to exist in pea.

The PGM2 clones showed an extended 5′ region corresponding to approximately 60 amino acids. This was consistent with the presence of a transit peptide sequence and suggested that this cDNA corresponds to plastidial PGM in pea. Transcription of the putative PGM2 cDNA followed by translation in vitro generated two major polypeptide products (69 and 65 kD). Plastidial protein import analyses revealed the presence of a radiolabeled polypeptide of 59 kD in the stromal fraction after intact chloroplasts were incubated with radiolabeled protein from the PGM2 clone. This is consistent with the protein being translated as a larger precursor form in vivo that is targeted across the chloroplast envelope into the chloroplast stroma and proteolytically processed to generate the mature form. The 69-kD form is likely to be the true precursor form, and high levels are associated with the chloroplast fraction before thermolysin treatment (results not shown), indicating that it was associated with the outer face of the outer envelope membrane. The 65-kD form is likely to be a product of internal translation initiation. Transcription and translation of the PGM1 cDNA gave rise to a polypeptide product of approximately 59 kD, which was not imported by isolated chloroplasts. These results are wholly consistent with the hypothesis that the PGM1 and PGM2 clones represent the cytosolic and plastidial isoforms of PGM, respectively. Furthermore, the data from the northern analyses are consistent with these clones representing the cytosolic and plastidial isoforms, respectively, of PGM in pea.

Phylogenetic analysis of highly conserved regions of known PGM amino acid sequences and those deduced from the two pea cDNAs and a *Brassica napus* cDNA indicated separation of the PGMs into classes corresponding to plant, bacterial, and mammalian origin. The putative plastidial PGM sequences from pea (PGM2) and *B. napus* appear to be approximately equidistant in the tree from the plant and prokaryote branches. This is perhaps consistent with the hypothesis that plastids are derived from ancient prokaryote symbionts. A spinach PGM sequence reported by Penger et al. (1994) does not show a high degree of homology to any of the identified plant PGMs. With the sequence data now available from numerous plant species, it seems highly unlikely that this clone was a PGM. Multiple amino acid sequence comparisons between all known PGMs, including those described here, show a high degree of similarity between plant (and eukaryote in general) sequences. The spinach sequence, however, appears to be more closely related to PGMs of prokaryotic origin. A possible explanation for this is the bifunctionality of the enzymes in prokaryote systems. In bacteria, the PGM enzyme also has phosphomannomutase activity, and it is possible that in plants, a separate enzyme (of which the spinach clone is an example) performs this activity. Since the spinach clone was isolated by complementation of a yeast hexokinase mutant, there is no de facto reason why it should correspond to a PGM.

The *rug3* locus has been shown to be closely linked to the flower character *k* and therefore to the PGM(P) isozyme locus (Weeden et al., 1984; Harrison et al., 1998). In addition, the position of PGM(C) is known from isozyme mapping (Weeden and Marx, 1987). The mapping data presented here are consistent with previous data for the PGM isozymes and with the previous analysis showing linkage between the *rug3* locus and *k*. This is further evidence that the PGM1 cDNA represents the cytosolic isoform of PGM and that the PGM2 cDNA corresponds to the plastidial isoform. Furthermore, these data link the *rug3* locus (the wrinkled-seed character) with the PGM(P) isozyme and the PGM2 cDNA clone. Cosegregation analysis of the wrinkled-seed character (*rug3rug3*) and an RFLP character for the PGM2 cDNA has also established that the cDNA and the wrinkled character map very close to or on top of each other. The data from the genetic analyses are very strong evidence that the *rug3* locus includes the structural gene for plastidial PGM and that the PGM2 cDNA corresponds to the mRNA transcript for this gene.

Three *rug3* alleles give rise to seeds with very low levels of starch and undetectable plastidial PGM activity (*rug3-b*, *rug3-d*, and *rug3-e*; Harrison et al., 1998). Analysis of the expression of the plastidial PGM gene in mutant embryos carried out using the PGM2 cDNA revealed that the *rug3-d* and *rug3-e* alleles caused a large reduction in the PGM(P) transcript level. The *rug3-d* allele appears to cause complete absence of a transcript and is probably due to a mutation in the promoter region of the gene, since no mutations were found in the coding region of the mutant gene. In contrast, sequencing of the coding region of the PGM gene from *rug3-b* revealed the presence of a single base change that resulted in the substitution of Arg by Cys in the deduced amino acid sequence. Such a change in a residue that is conserved in all described PGM sequences is likely to inactivate the enzyme. The starchless phenotype of *rug3-erug3-e* line, however, cannot be explained simply, because expression analysis indicated that about 10% of the mRNA remains. It is possible that, in this line, the mutation results in an unstable transcript that is rapidly degraded. This phenomenon, called "nonsense-mediated mRNA decay" (Zhang et al., 1995),

has been observed in yeast and, recently, in Arabidopsis (Marchant and Bennett, 1998). If this were the case, then the mutation could be located within an intron of the gene at a site important in its correct splicing. This has been shown to be the case in several Arabidopsis mutants showing reduced transcript levels (Marchant and Bennett, 1998). The molecular analysis of this mutant line will now be extended to the genomic DNA sequence.

CONCLUSIONS

Two cDNA clones have been isolated from wild-type pea and have high similarity to PGMs from other species. We conclude that these clones represent the two isoforms of PGM in pea, and that they encode plastidial and cytosolic forms of the enzyme. Linkage analyses and expression data support the conclusion that the *rug3* locus of pea contains the structural gene for plastidial PGM and that the absence of a message for this protein results in the almost complete absence of starch in the plant, which leads to the wrinkled-seed phenotype.

ACKNOWLEDGMENTS

We wish to thank Richard Trethewey for the kind gift of the potato PGM cDNA clone and Martin Fulda for the *B. napus* library.

Received November 8, 1999; accepted November 30, 1999.

LITERATURE CITED

Caspar T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. Plant Physiol **79:** 11–17

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Ellis THN, Poyser SJ, Knox MR, Vershinin AV, Ambrose MJ (1998) *Ty1-copia*class retrotransposon insertion site polymorphism for linkage and diversity analysis in pea. Mol Gen Genet **260:** 9–19

Hanson KR, McHale NA (1988) A starchless mutant of *Nicotiana sylvestris* containing a modified plastid phosphoglucomutase. Plant Physiol **88:** 838–844

Harrison CJ (1996) The *rug3* locus of pea. PhD thesis. University of East Anglia, Norwich, UK

Harrison CJ, Hedley CL, Wang TL (1998) Evidence that the *rug3* locus of pea (*Pisum sativum* L.) encodes plastidial phosphoglucomutase confirms that the imported substrate for starch synthesis in pea amyloplasts is glucose-6-phosphate. Plant J **13:** 753–762

Hedley CL, Smith C, Ambrose MJ, Cook S, Wang TL (1986) An analysis of seed development in *Pisum sativum*. II. The effect of the *r* locus on the growth and development of the seed. Ann Bot **58:** 371–379

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Laucou V, Haurogne K, Ellis THN, Rameau C (1998) Genetic mapping in pea. Theor Appl Genet **97:** 905–915

Lin TP, Caspar T, Somerville CR, Preiss J (1988) A starch deficient mutant of *Arabidopsis thaliana* with low ADP glucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol **88:** 1175–1181

Marchant A, Bennett MJ (1998) The *Arabidopsis AUX1* gene: a model to study mRNA processing in plants. Plant Mol Biol **36:** 463–471

Mould RM, Gray JC (1998a) Preparation of chloroplasts for protein synthesis and protein import. *In* JE Celis, ed, Cell Biology: A Laboratory Handbook, Ed 2, Vol 2. Academic Press, New York, pp 81–86

Mould RM, Gray JC (1998b) Import of nuclear-encoded proteins by isolated chloroplasts and thylakoids. *In* JE Celis, ed, Cell Biology: A Laboratory Handbook, Ed 2, Vol 2. Academic Press, New York, pp 286–292

Penger A, Pelzer-Reith B, Schnarrenberger C (1994) cDNA sequence for the plastidic phosphoglucomutase from *Spinacia oleracea* (L.). Plant Physiol **105:** 1439–1440

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schnell DJ, Blobel G (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localisation to envelope contact sites. J Cell Biol **120:** 103–115

Weeden NF, Marx GA (1984) Chromosomal locations of twelve isozyme loci in *Pisum sativum*. J Hered **75:** 365–370

Weeden NF, Marx GA (1987) Further genetic analysis and linkage relationships of isozyme loci in the pea. J Hered **78:** 153–159

Weeden NF, Provvidenti R, Marx GA (1984) An isozyme marker for resistance to bean yellow mosaic virus in *Pisum sativum*. J Hered **75:** 411–412

Zhang S, Ruiz-Echevarria MJ, Quan Y, Peltz SW (1995) Identification and characterisation of a sequence specific motif involved in nonsense-mediated mRNA decay. Mol Cell Biol **15:** 2231–2244