Cloning and characterization of a glucose 6-phosphate/phosphate translocator from *Oryza sativa*[※]

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Abstract: Plastids of nongreen tissues import carbon as a source of biosynthetic pathways and energy, and glucose 6-phosphate is the preferred hexose phosphate taken up by nongreen plastids. A cDNA clone encoding glucose 6-phosphate/phosphate translocator (*GPT*) was isolated from a cDNA library of immature seeds of rice and named as *OsGPT*. The cDNA has one uninterrupted open reading frame encoding a 42 kDa polypeptide possessing transit peptide consisting of 70 amino acid residues. The *OsGPT* gene maps on chromosome 8 of rice and is linked to the quantitative trait locus for 1000-grain weight. The expression of *OsGPT* is mainly restricted to heterotrophic tissues. These results suggest that glucose 6-phosphate imported via *GPT* can be used for starch biosynthesis in rice nongreen plastids.

Key words: Glucose 6-phosphate/phosphate translocator, Starch synthesis, Rice (*Oryza sativa L.*)
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INTRODUCTION

Nongreen plastids of heterotrophic tissues are carbohydrate-importing organelles and, in the case of amyloplasts of storage tissues, the site of starch synthesis. Investigation of whole tissues from a variety of starch synthesizing crop plants indicated that hexose units were imported into plastids as precursors for starch. Starch synthesis in plastids isolated from pea cotyledons (Hill and Smith, 1991), pea roots (Borchert *et al.*, 1993), and cauliflower buds (Neuhaus and Maas, 1996) is dependent on Glucose 6-phosphate (Glc6P). However, in amyloplasts from wheat endosperm (Tetlow *et al.*, 1994) and potato tubers (Naeem *et al.*, 1997) Glucose 1-phosphate (Glc1P), but not Glc6P, is the precursor of starch synthesis. Maize endosperm amyloplasts can synthesize starch from both Glc1P and Glc6P (Neuhaus *et al.*, 1993). Mutants of *Arabidopsis* with a defect in the plastidic phosphoglucomutase are unable to synthesize starch (Kofler *et al.*, 2000), the rug3 locus of pea encodes plastidial phosphoglucomutase (Harrison *et al.*, 2000) and transgenic potato plants with reduced activity of plastidic phosphoglucomutase are also defective in starch accumulation (Tauberger *et al.*, 2000). These reports indicate that Glc6P is the preferred substrate taken up by plastids and the conversion of Glc6P to Glc1P inside the plastids, catalyzed by phosphoglucomutase, is a prerequisite to starch formation. The cDNA of *GPT* has been cloned from maize, potato and pea. *GPT*-specific transcripts are barely detectable in photosynthetic tissues but abundant in heterotrophic tissues (maize roots and reproductive organs, potato tubes). It has been demonstrated that *GPT* protein mediates a 1:1 exchange of Glc6P mainly with inorganic phosphate and triose phosphates, suggesting that these tissues may utilize Glc6P for starch synthesis (Kammerer *et al.*, 1998). However, little is known so far about the precursors for starch biosynthesis in amyloplasts of rice endosperm. In this paper, we report the cloning of a cDNA from rice endosperm mainly expressed in immature seeds and roots encoding *GPT*.

MATERIALS AND METHODS

1. Plant material and growth conditions

Rice (*Oryza sativa* L. cv. Zhe 733, indica)

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was grown in greenhouse conditions at 22 ℃/28 ℃ (night/day) and 80% relative humidity. Roots and seedlings from 7d-old seedlings, stems and mature leaves from 5 days after flowering (DAF) plants, immature seeds from 3-15 DAF were sampled.

2. cDNA library construction

A cDNA library was constructed using a SMART™ cDNA Library Construction Kit (CLONTECH) in the λ-TriplEx2 vector following the manufacturer's instructions. Total RNA was isolated from frozen material using Trizol (GIBCOBRL) following the kit protocols; poly (A) RNA was purified from total RNA using an mRNA PolyATtract Kit (mRNA isolation system III, Promega). Double-chain cDNA was synthesized from poly (A) RNA extracted from 5 – 10 DAF immature seeds. cDNA was prepared for directional insertion between the two S*fi*I sites of λ-TriplEx2 vector. Recombinant phages were packaged in vitro using Maxplax™ Lambda Packaging Extract (Epicentre Technolodies) and then amplified.

3. PCR amplification of cDNA and sequencing

Alignment of the predicted amino acid sequences *Arabidopsis thaliana* (AB005232), *Pisum sativum* (AF020814), *Solanum tuberosum* (AF020816), *Zea mays* (AF020813) identified several conserved domains. Two domains were used to design sense (Gu: 5'-ATT GGT GG[T/A] TGT G[C/G][T/A] CT[A/T] GC-3') and the antisense (Gl: 5'-GC[A/G] GC[T/A] CC [C/G/A] AG[T/A/G] GCA TTG-3') degenerate primers. PCR was carried out with the template of the rice cDNA library and using primers at 0.65 μmol/L, 200 μmol/L dNTPs, and 1 unit Taq DNA polymerase. The resulting DNA fragment were gel-purified (QIAquick Gel Extraction Kit, QIAGEN) and cloned into plasmid pUC-T. Sequence analysis was performed using MegaBACETM 1000 (Amersham Pharmacia Biotech).

4. cDNA isolation and characterization

The resulting cDNA fragment was used as probe to isolate *Oryza sativa GPT* (*OsGPT*). Hybridization was carried out at 65 ℃ for 16 h in 5× SSC, 1× Denhardt's, 0.2% SDS, 50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 100 μg/ml denatured salmon sperm DNA, and 2 ng/ml of DNA probe that had been labeled with [$\alpha - {}^{32}P$]dCTP using the random priming method. The membranes were washed under high stringency conditions. For sequencing, the positive clone was converted to pTriplEx2, and the plasmid was prepared using GFX™ Micro Plasmid Prep Kit (Amersham Pharmacia Biotech). Sequence analysis was performed using MegaBACE™ 1000.

5. Genomic DNA blot analysis

Rice genomic DNA (10 μg) was digested with restriction enzymes, *DraI*, *EcoRI*, *EcoRV*, *HindIII*, *ScaI* and *XbaI*, separated on 0.8% agarose gel, and blotted onto a nylon membrane (Amersham Pharmacia Biotech). The probe was prepared by random priming of the 455bp cDNA fragments stringency used for hybridization and washing was the same as that described above. After washing the blots were analyzed using Typhoon-8600 (Amersham Pharmacia Biotech).

6. RNA gel blot analysis

Total cellular RNA (20 μg) prepared from various rice tissues were separated on 1% formaldehyde-agarose gels, and transferred onto Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech). The probe was prepared by random priming of the present *OsGPT* CDS. Stringency used for hybridization and washing was the same as that described by the manufacture's instructions of the nylon membrane. After washing the blots were analyzed using Typhoon-8600.

RESULTS AND DISCUSSION

1. Cloning of the full-length *OsGPT* cDNA

The cDNA obtained was amplified using degenerate primers. A single band about 450 bp long was obtained, purified and subcloned. Several clones were partially sequenced and used as probes for screening of a cDNA library prepared from rice immature seeds. A single type of cDNA clone was isolated and the clone containing the insert of 1684 bp was sequenced. The *OsGPT* cDNA clone contained an ORF of 1164 bp that encodes a peptide of 387 amino acids with a calculated molecular mass of 42.0 kDa and an isoelectric point of 9.7. Putative polyadenylation signal was found at the position of 1570 – 1575

(AATAAA) in the 3' - UTR. The deduced protein sequence was highly hydrophobic and had a transit peptide consisting of 70 amino acid residues (Fig.1).

The deduced amino acid sequences of rice and other plants were compared. The sequence of *OsGPT* had the highest homology with maize *GPT* (91% identity). Hydrophobicity distribution analysis of the deduced amino acid sequences of the *GPTs* revealed approximately six membrane-spanning regions for the monomeric proteins as shown in Fig.1.

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AtGPT   MVLSVKQTLSPKIG----LFRRNPSSSLGRSPVSLSFPSTELPKRTVLAVSKPLHLSSS-   55
PsGPT   MISSLRQPSISISGSDVVLRKRHATLIQLRPQSFSPFSSREKSQRSVVSTKKPLHLACLG   60
ZmGPT   MIPSVRLSPGPAAFSGSSLRSKLPSIPSISSLKPSKYVVSSLKPLYLAPLDGPRTAELK-   59
OsGPT   MLPAVKLSPGPVAFAGTNLRSRSASVSSVSSLKPSKFVVSPLRPLYLAPLDGPRAAGQK-   59
StGPT   YAFSTNFDPLQQNLWGSKPPISSLSIKDIDFKQCDKHNILSKKPLYISAVLSGFGHADE-   59
                   : .                 :            .          : .

                                 ↓ transit peptide cleavage site
AtGPT   --------------LRAKSPVVRCEAYEADRSEPHPIGDDAAAAETKSEAAKKLKIGIYF   101
PsGPT   VGNFGSVKNFESEASFGQSDLVKCGAYEADRSEVE------GGDGTPSEAAKKVKIGIYF   114
ZmGPT   --------------SRRQPLEFRCSASAADDKESK-----TQVVPVQSEGAQRLKISIYF   100
OsGPT   --------------AQRQPLEFRCAASAADDKESK-----TEVVPVRSEAAQKLKISIYF   100
StGPT   -----------SKEFKSRDPLVQCNAYEASQPQSIP-IDIEFGQEAQAAATQKLKIGLYF   107
                             :   .:* *  *.   :               . : .::::**.:**

            I                            II
AtGPT   ATWWALNVVFNIYNKKVLNAYPYPWLTSTLSLAAGSLMMLISWAVGIVETPKTDFDFWKT   161
PsGPT   ATWWALNVVFNIYNKKVLNAYPYPWLTSTLSLACGSLMMLISWATRIAEAPKTDLEFWKT   174
ZmGPT   ATWWALNVIFNIYNKKVLNAFPYPWLTSTLSLACGSAMMLFSWATRLVEAPKTDLDFWKV   160
OsGPT   ATWWALNVIFNIYNKKVLNAFPYPWLTSTLSLACGSAMMLVSWATRLVEAPKTDLDFWKV   160
StGPT   ATWWALNVVFNIYNKKVLNAFPFPWLTSTLSLAAGSLMMLVSWATKIAETPKTDFDFWKA   167
        ********:***********:*:**********.** ***.***.  :.*:****::***.

AtGPT   LFPVAVAHTIGHVAATVSMSKVAVSFTHIIKSGEPAFSVLVSRFILGETFPTSVYLSLIP   221
PsGPT   LFPVAVAHTIGHVAATVSMSKVAVSFTHIIKSGEPAFSVLVSRFILGETFPVPVYLSLLP   234
ZmGPT   LFPVAVAHTIGHVAATVSMSKVAVSFTHIIKSAEPAFSVLVSRFFLGETFPIPVYLSLLP   220
OsGPT   LFPVAVAHTIGHVAATVSMSKVAVSFTHIIKSAEPAFSVLVSRFLLGETFPVPVYLSLLP   220
StGPT   LFPVAVAHTIGHVAATVSMSKVAVSFTHIIKSGEPAFSVLVSRLLG-ETFPLPVYLSLLP   226
        ********************************.**********::   **** .*****:*

        III
AtGPT   IIGGCALSALTELNFNMIGFMGAMISNLAFVFRNIFSKKGMKGKSVSGMNYYACLSMLSL   281
PsGPT   IIGGCALAAVTELNFNMIGFMGAMISNLAFVFRNIFSKKGMKGKSVSGMNYYACLSILSL   294
ZmGPT   IIGGCALAAVTELNFNMVGFMGAMISNLAFVFRNIFSKRGMKGKSVSGMNYYACLSIMSL   280
OsGPT   IIGGCGLAAVTELNFNMVGFMGAMISNLAFVFRNIFSKRGMKGKSVSGMNYYACLSIMSL   280
StGPT   IIGGCGLAAITELNFNLIGFMGAMISNLAFVFRNIFSKKGMKGKSVGGMNYYACLSMMSL   286
        *****.*:*:******::*********************:*******.*********::**

         IV
AtGPT   LILTPFAIAVEGPQMWVDGWQTALATVGPQFVWWVVAQSVFYHLYNQVSYMSLDQISPLT   341
PsGPT   AILTPFAIAVEGPAMWAAGWQTALSEIGPQFIWWVAAQSIFYHLYNQVSYMSLDEISPLT   354
ZmGPT   VILTPFAIAMEGPQMWAAGWQKALAEVGPNVVWWIAAQSVFYHLYNQVSYMSLDQISPLT   340
OsGPT   VILTPFAIAMEGPQMWAAGWQKALAEVGPDVVWWVAAQSVFYHLYNQVSYMSLDEISPLT   340
StGPT   LILIPFAIAVEGPQVWALGWQNAVSQIGPNFIWWVVAQSVFYHLYNQVSYMSLNEISPLT   346
         ** *****:***  :*. ***.*:: :**:.:**:.***:*************::*****

                   V                    VI
AtGPT   FSVGNTMKRISVIVSSIIIFRTPVQPVNALGAAIAILGTFLYSQVNITLP 391
PsGPT   FSIGNTMKRISVIVSSIIIFHTPIQPVNALGAAIAVFGTFLYSQAKQ--- 401
ZmGPT   FSIGNTMKRISVIVSSIIIFHTPVRAVNALGAAIAILGTFLYSQAKA--- 387
OsGPT   FSIGNTMKRISVIVSSIIIFHTPVRPVNALGAAIAILGTFLYSQAKQ--- 387
StGPT   FSIGNTMKRISVIVSSIIIFQIPIQPINALGAAIAILGTFLYSQAKQ--- 393
        **:*****************: *::.:********::*******.:
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Fig.1 Alignment of deduced amino acids of *OsGPT* with other plant *GPTs*

Arabidopsis thaliana (AtGPT, AB005232), *Pisum sativum* (PsGPT, AF020814), *Solanum tuberosum* (StGPT, AF020816), *Zea mays* (ZmGPT, AF020813). The Sequences were aligned using the CLUSTAL program. The locations of the putative membrane-spanning α-helices are indicated (I to VI). The arrow indicates the transit peptide cleavage site. Identical (*) and conserved (.:) residues are marked.

2. Southern blot analysis

The genomic DNA isolated from 14d-old rice leaves was digested with *DraI*, *EcoRI*, *EcoRV*, *HindIII*, *ScaI* and *XbaI*, and then separated on 0.8 % agarose gel. The digested DNA fragments were hybridized with the *OsGPT* 455 bp cDNA fragment probe, and washed at high stringency (0.1 × SSC with 0.1% SDS, 65 ℃). The genomic blot pattern showed one band in each DNA sample, which indicated that the gene for *GPT* is present as a single copy in the rice genome (Fig.2).

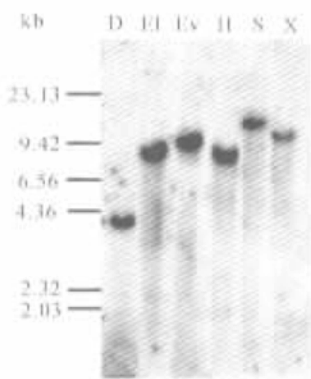

Fig.2 Southern blot analysis of genomic rice DNA
DNA was digested using the following restriction enzymes: D, *Dra*I; EI, *EcoR*I; Ev, *EcoR*V; H, *Hind* Ⅲ; S, *ScaI*; X, *Xba*I. The positions of *Hind* Ⅲ λ-DNA size markers are also indicated.

3. Analysis of *OsGPT* mRNA

The expression patterns of *OsGPT* gene in different organs were investigated. As shown in Fig.3, the transcripts of *OsGPT* could not be detected in leaves and stems; however, high levels of *OsGPT* steady state mRNA were present in roots, immature seeds, and in developing seeds up to 15 DAF.

Transcripts of the *OsGPT* gene were almost lacking in photosynthetic tissues but are abundant in heterotrophic tissues such as roots and developing seeds. This is in line with the proposed function of the *GPT* protein in these tissues utilizing Glc6P as a precursor for starch synthesis in rice.

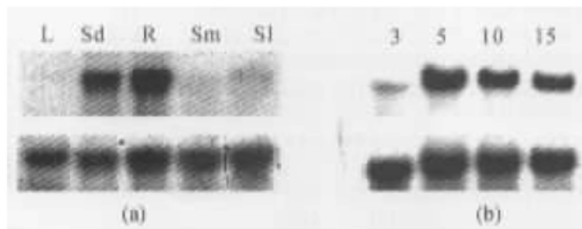

Fig.3 Northern blot analysis of *OsGPT*
(a) RNA gel blot analysis of *OsGPT* RNA from leaves (L), immature seeds (Sd), roots (R), Stems (Sm) and 7d – old seedlings (Sl); (b) RNA gel blot analysis of *OsGPT* RNA of developing rice seeds. The seeds were harvested at the indicated days after flowering. Lower panel shows the normalization of total RNA levels in each sample by the hybridization of 18S rRNA on the same blot with a tomato 18S rDNA probe.

4. Linkage of *OsGPT* Gene to a QTL for rice 1000-grain weight

This *OsGPT* gene located between two EST of S1461 and S21348 on the rice genome clone of AP004656, and the distance from *OsGPT* gene to S1461 was only 4000bp. When used as EST (S1461 and S21348) markers on the rice EST Map (Wu *et al*., 2002), identified a locus on chromosome 8 (Fig. 4). Transport of carbon from cytoplasm to amyloplast is essential for starch accumulation and grain weight in developing endosperm of rice. Given the essential physiologic role, genes controlling carbon transport are candidates for QTLs controlling starch content and grain weight. If alleles at one or more transporter or sensor loci indeed were responsible for a QTL effect on 1000-grain weight, tight genetic linkage would be observed between the QTL and the candidate gene locus. Therefore, the position of *OsGPT* loci was compared with the positions of QTLs for 1000-grain weight. On chromosome 8, a QTL for 1000-grain weight was linked to markers *V*115 and *V*150 derived from Xiao *et al*. (1995) and Lu *et al*. (1996). The *GPT* locus was tightly linked to the same markers (Fig.4) and therefore to this QTL. This linkage suggested that *GPT* alleles might play an important role in controlling grain starch content and 1000-grain weight. Further work is required to confirm this important role, because linkage analysis cannot exclude the possibility that the gene controlling the QTL is linked, but functionally unrelated to, the candidate gene locus *GPT*.

It was reported that the key enzyme for starch synthesis, ADP-glucose pyrophosphorylase, was mainly present in cytosol in the endosperms of rice and other cereals (Sikka *et al*., 2001; Denyer *et al*., 1996; Thorbjornsen *et al*., 1996). In maize, it is assumed that the Brittle-1 protein serves as an ADP-glucose/adenylate transporter, which would thus represent an alternative route to provide the plastids with a precur-

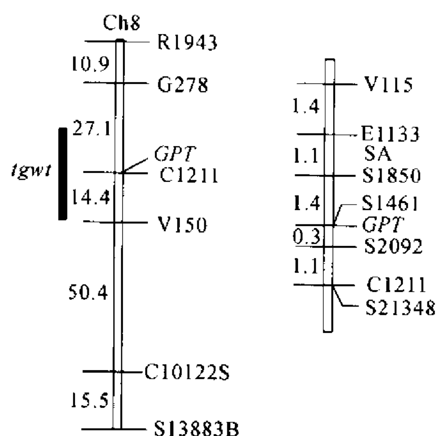

Fig.4 Mapping of *OsGPT* Loci

OsGPT loci and anchor RFLP markers (JRGP – RFLP, 2000) are indicated to the right of the chromosome 8. Kosambi values (cM) are indicated left of the chromosome. Position of a QTL for 1000 – grain weight (*tgwt*), shown to the left of the linkage groups, is derived from Xiao *et al*. (1995) and Lu *et al*. (1996), based on anchor RFLP markers with known genetic distance to QTL.

sor for starch biosynthesis (Sullivan *et al*., 1995). The expression of ADP-glucose/adenylate transporter in rice, and how the activities of both proteins are coordinated in rice seed development still remain to be determined.

In summary, we cloned the *OsGPT* gene and investigated the expression patterns of *GPT* gene in different tissues and developing seeds in rice. The *OsGPT* gene maps on chromosome 8 of rice and is linked to a quantitative trait locus for 1000-grain weight. The fact that *OsGPT* is mainly expressed in heterotrophic tissues suggests that Glc6P can be imported via *GPT* into non-green plastids such as endosperm amyloplasts for starch synthesis in rice.

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