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CLONING OF SMALL SUBUNIT ADP-GLUCOSE PYROPHOSPHORYLASE ISOFORMS FROM SWEET POTATO AND THEIR APPLICATIONS FOR GENETIC ENGINEERING OF STARCH IN PLANTS

Jung M. BAE, Chee H. HARN, Jang R. LIU

Plant Cell and Molecular Biology Research Unit, Korea Research Institute of Bioscience & Biotechnology (KRIBB), P.O.Box 115, Yusong, Taejon, 305-600, Korea

INTRODUCTION

ADPglucose pyrophosphorylase (AGPase) regulates the biosynthesis of starch through the allosteric enzyme property in higher plants. It catalyzes the reversible synthesis of ADPglucose and inorganic phosphate from glucose-1-phosphate and ATP. ADPglucose is then used as a glucosyl donor in the biosynthesis of starch.

The studies with mutant and transgenic plants identified the regulatory role of AGPase in starch biosynthesis. In maize, the mutant *sh2* or *bt2* contains only 5-15% AGPase activity, producing only 25-30% of the starch content of the wild type (3). The transgenic potato plant produced by AGPase antisense cDNA construct almost caused the failure of the production of AGPase, resulting in the abolition of starch formation in tubers (11). The E.coli AGPase mutant, showing reduced sensitivity to the allosteric inhibitor, PPi was used to induce the overexpression of AGPase. The overexpression of AGPase caused higher accumulation of starch in transgenic tobacco, potato and tomato (18).

The AGPase in higher plants is known to be a heterotetramer, consisting of small and large subunits. Two subunits can be distinguished by a slight difference of molecular weights and immunological properties(5,8,9,12,14,15,17). The cDNAs of each subunit have been isolated from various plants, including *Arabidopsis* leaf, maize endosperm, wheat and barley endosperms, potato tuber, rice endosperm and bean cotyledon (,1,2,10,13,19,20,22).

The genes encoding large subunits are represented by multifamilies in several plants, such as *Arabidopsis*, potato, barley, rice and wheat. And the expression pattern of the large subunit is highly tissue-specific, restricted to either leaf or root and endosperm in barley and wheat (13,19,21). The mutant study confirms the tissue-specific expression of large subunit isoforms. In the maize mutant sh2, defective in large subunit of AGPase in the endosperm, there was a second transcript encoding the large subunit of maize embryos (4).

The study of small subunit isoforms has just been initiated. Like the situation in the large subunit, the expression in the small subunit is organ-specific. Two cDNAs from bean showed a differential expression pattern according to the organ. One was expressed in the developing cotyledons and leaves, while the mRNAs of the other were detected only in cotyledons (22). In the maize, the cDNA isolated from leaf was different from the one in the

endosperm in the nucleotide sequence and both were expressed in distinct tissue-specific patterns (4,16)

To further characterize the functional role of the isoforms of each subunit, more isoforms of each subunit need to be isolated and analysed at the molecular and biochemical level. In fact, three different large subunit AGPase coding regions from potato have been identified. All three AGPase isoforms were expressed in the tuber tissue of potato, and were found to be 67-71% homologous to each other (6). Multiple small subunit isoforms expressed in the same tissue have not been reported.

In order to understand the roles of AGPase isoforms during development of the tuberous root and leaf in sweet potato, two isoforms of small subunit AGPase were isolated from sweet potato tuberous root and leaf libraries. The expression pattern of the two isoforms was investigated with different tissues of sweet potato. The differential expression pattern of the two isoforms by light or sucrose treatment was also elucidated. Transgenic plants were generated with two isoforms, transgenic tobacco by sense-strand cDNAs and transgenic sweet potato by anti-sense strand cDNAs to manipulate starch content.

Cloning of novel isoforms of small subunit AGPase

The cDNAs of two small subunit AGPase isoforms were isolated from sweet potato tuberous root and leaf cDNA libraries. At the nucleotide level, psTL1 and psTL2 showed 84.1% identity (cf. EMBL Nucleotide Sequence Database accession numbers Z79635 for psTL1 and Z79636 for psTL2). Deduced amino acid sequence comparison between psTL1 and psTL2 showed a high degree of identity (90.6%) while the identity dropped to 60% in the putative transit peptide region (Fig. 1). The putative mature proteins

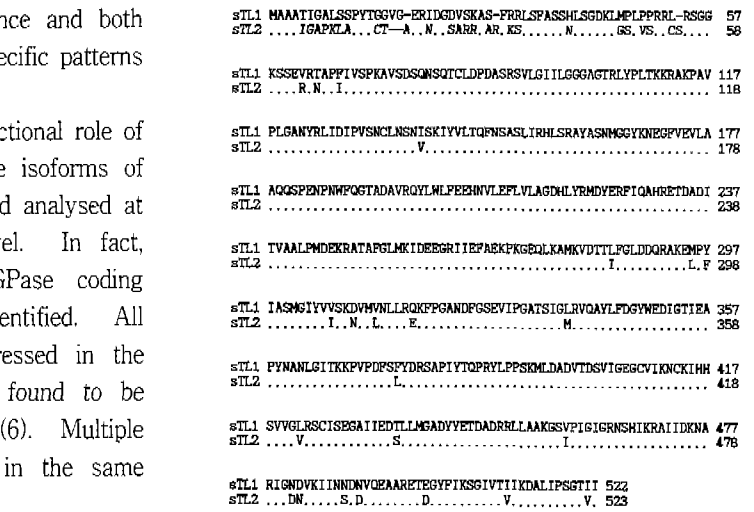

Fig. 1. Comparison of deduced amino acid sequences of sweet potato ADPglucose pyrophosphorylases sTL1 and sTL2 subunits. Identical amino acids in the sTL2 sequence are indicated by dots.

reveal an identical length of 449 amino acids and an identity of 96%. Both isoforms share a high level of sequence identity with known small subunit AGPase polypeptides of other plants. For example the amino acid sequence of psTL1 showed 87.6%, 80.2%, and 84.5% identity with small subunits of potato, rice and wheat respectively. It thus appears that psTL1 and psTL2 encode the small subunit AGPase polypeptides, designated as IbAGP-sTL1 and IbAGP-sTL2 (These will be abbreviated as sTL1 and sTL2). To elucidate the genomic organization of isoforms, Southern blot analysis was performed with sweet potato genomic DNA. Isoform-specific probes were prepared by PCR amplification of 5′ end regions of psTL1 and psTL2. The hybridization bands detected by sTL1- or sTL2-specific probe were different in size and numbers (Fig 2). The sTL1-specific probe detected a single band of *EcoRI* (2.9kb), *HincII* (0.7kb) and *HindIII* (4.3kb) digested DNAs, respectively. The sTL2-specific DNA hybridized with several different sized fragments

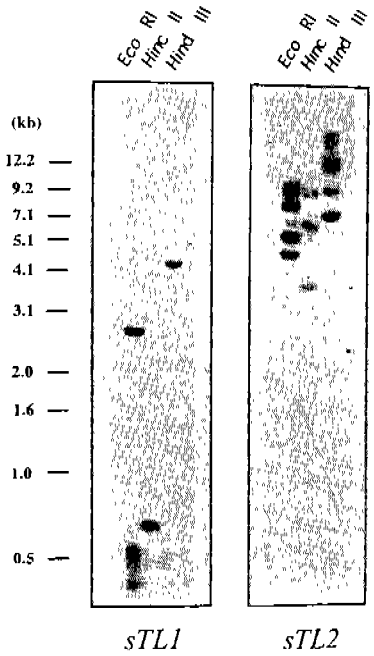

Fig. 2. Genomic Southern analysis of *sTL1* and *sTL2*. Genomic DNA was extracted from stem tissue of sweet potato, digested with *EcoRI*, *HincII*, and *HindIII* and hybridized with sTL1- or sTL2-specific DNA fragment (120bp).

of *EcoRI*, *HincII* and *HindIII* digested DNAs and no band was in common with those detected by the sTL1-specific probe. These data suggest that the genes of the two isoforms are likely to be different genes rather than alleles of the same gene. The data also indicate more copy numbers for the *sTL2* gene than the *sTL1* gene. At present, exact copy numbers of genes corresponding to sTL1 and sTL2 are not known. The result of Southern blot analysis suggests low copy number genes for sTL1 and sTL2 in sweet potato.

The expression patterns of two isoforms according to various organs were elucidated by northern analysis (Fig 3A). The highest expression of *sTL1* gene was observed in tuberous root whereas the transcript level was low in the stem. Relatively strong expression was also observed in the leaf. The *sTL2 gene*

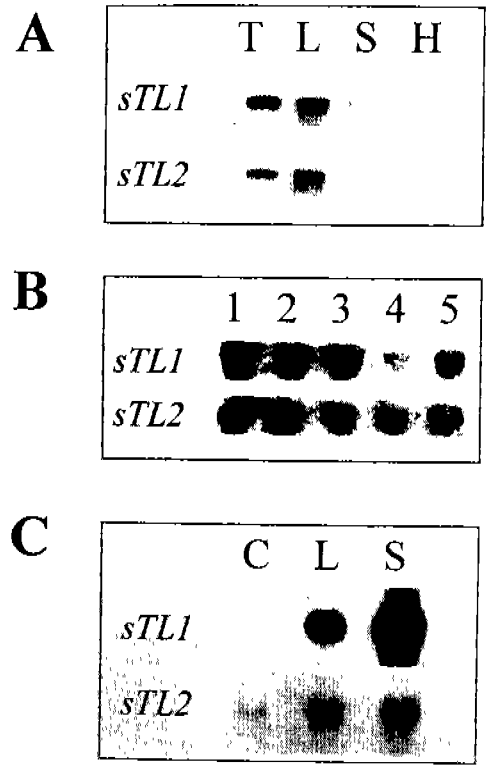

Fig. 3. Expression pattern of *sTL1* and *sTL2*. Probe was sTL1- or sTL2-specific DNA fragment (120bp). A. Expression of *sTL1* and *sTL2* in different sweet potato organs. Poly (A)$^+$ RNA (1ug) from each sample was loaded in each lane. RNA was extracted from T(tuberous root), L(leaf), S(stem) and H(hairy root). B. Expression of *sTL1* and *sTL2* in sweet potato leaves of different developmental stages. Total RNA (80ug/lane) was loaded in each lane. RNA was extracted from leaves of the following lengths; lane1, 1-2cm; lane2, 3-4cm; lane3, 5-6cm; lane4, 7-8cm; lane5, more than 10cm. C. Accumulation of *sTL1* or *sTL2* mRNA by 6% sucrose or light treatment. Total RNA was extracted from detached leaves incubated in water supplemented with 6% sucrose(S) under dark or in water under light(L) for 24 hours. Control(C) RNA was extracted from the leaves incubated in water under dark for 24 hours. 50ug of each samples was loaded in each lane.

was also expressed in the tuberous root, leaf, and stem whereas the highest level of transcript occurred in the leaf. Neither *sTL1* nor *sTL2* was expressed in the hairy root. Although *sTL2* as well as *sTL1* were expressed in the same organs such as the tuberous root, leaf and stem, the steady-state transcript level was different. The transcript level of *sTL1* was always higher than that of *sTL2*, indicating sTL1 is the major AGPase polypeptide in the tuberous root, leaf and stem.

The expression profiles of *sTL1* and *sTL2* were checked with various developmental stages of leaves (Fig 3B). The initial transcript level of *sTL1* and *sTL2* were slightly decreased when the leaves reach to 8-9cm length, but the overall expression levels of both isoforms remained relatively constant during the leaf development, indicating *sTL1* and *sTL2* are actively expressed throughout the various leaf developmental stages. The transcript level of *sTL1* was elevated enormously by 6% sucrose and moderately by light while the expression of *sTL2* was hardly affected (Fig 3C). It thus appears that *sTL1* but not *sTL2* is a sucrose-inducible and light- responsive gene although both genes are normally expressed in the leaf. This result suggests that two isoforms probably play a distinct role at various developmental stages of sweet potato.

Generation of transgenic tobacco plants expressing sweet potato AGPase mRNA

Transgenic tobacco plants were generated with small subunit of sweet potato AGPase cDNAs. AGPase vectors of pMBP1-sTL1 and pMBP1-sTL2 were constructed, where the *sTL1* and *sTL2* cDNAs are transcribed in a sense orientation under CaMV 35S promoter. The PCR data showed that ten transgenic lines were transformed with *sTL1* and seven lines were transformed with *sTL2*. In addition, two tran

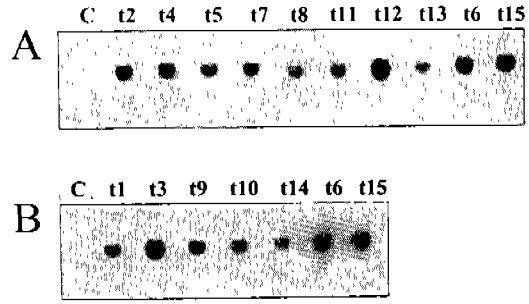

Fig. 4. Northern blot analysis of total RNA isolated from nontransformed and transformed plants. Total RNA was extracted from buds' of transformed or control plant lines. Equal amount of each sample was loaded in each lane. C: nontransformed plant; t1-t15: transformed plants by *sTL1* and/or *sTL2*. A. *sTL1* cDNA was used as a probe; B. *sTL2* was used as a probe.

sgenic lines were integrated by both *sTL1* and *sTL2*. The integrated *sTL1* and/or *sTL2* were actively transcribed and accumulated high levels of sweet potato AGPase mRNA (Fig. 4A and B). The levels of transcripts in transgenic plants were higher than those of nontransgenic plants. Although all the transgenes were under the control of CaMV 35S promoter, transcript levels were variable among transgenic lines. Transgenic lines of t3 and t12 showed highest levels of transcripts while the mRNA accumulation was low in the lines of t10, t13 and t14. Transgenic lines of t6 and t15 transformed by both *sTL1* and *sTL2* showed medium-high levels of transcripts rather than doubled amount of expression. Interestingly, the overall transcript levels of the *sTL1*-transformed plants were higher than those of *sTL2*-transformed plants. Highly expressed lines of t3 and t12, and a medium-high expressing line of t9 were selected for further analysis of enzyme activity and protein levels.

The enzyme activity was measured by coupling phosphoglucomutase and glucose-6-P dehydrogenase. It was found that there was no significant difference in the activities of AGPase

Table 1. Enzyme activities of AGPase in transgenic tobacco leaves transformed by a cDNA encoding sweet potato AGPase small subunit. T: non-trangenic; t: transgenic. t9, t3 and t12 are independent trangenic plants overexpressing transcripts of AGPase small subunit. Data points are means of three replicates with SE.

Plant	Activity (nmoles min^{-1} mg^{-1} protein)
T	53.3 ± 9.8
t9	49.7 ± 13.3
t3	47.6 ± 10.0
t12	52.4 ± 7.6

of transgenic plants and control plants (Table 1). The enzyme activities of more than 20 other trangenic plants whose transcript levels are medium-high or low-high were also investigated. None of the trangenic plants revealed any significant difference in enzyme activities comparing to control plants (data not shown). AGPase proteins from the above samples were denatured and fractionated by SDS-PAGE and hybridized with an antibody raised against AGPase small subunit from potato tuber. One major band of 50 kDa protein was detected on the immunoblot (Fig. 5). Despite the different tanscription levels of small subunit of AGPase between control and transgenic plants, immunoblot analysis of the small subunit polypeptide showed that protein levels remained unchanged in transgenic and control plants.

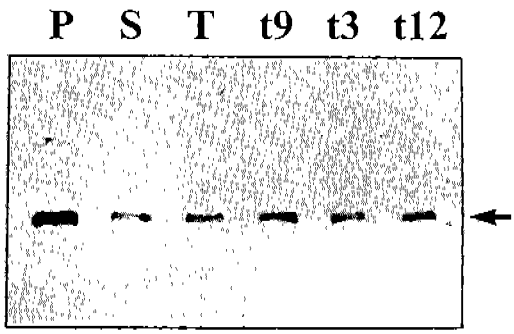

Fig. 5. Western blot of AGPase small subunit. Protein samples were isolated from following tissues of P: potato tuber; S: sweet potato leaf; T: non-transgenic tobacco leaf; t: transgenic tobacco leaf. Ten μg of protein was loaded on 12.5% acrylamide gel, transferred onto nitrocellulose and cross-reacted with antibody raised against AGPase small subunit from potato tuber. Arrow indicates the 50 kDa of small subunit AGPase polypeptides.

A partial male sterility in transgenic tobacco plants

The transgenic tobacco plants grew normally and no phenotype was identified until they began releasing the pollen grains from anthers. Flowers of the transgenic lines produced less pollen grains than those of control plants. The transgenic lines were first examined for any abnormal pollen phenotype using light microscopy. Most of the transgenic lines produced more than 50% of aborted pollen grains. Especially in two transgenic lines T4 and T27 more than 75% of the pollen grains were aborted. The aborted pollen was smaller in size, distinct from the normal rounded-shape. The normal and aborted pollen were examined by scanning electron microscopy. The pollen was collected from mature flowers of wild type and T27 transgenic plants. The size of the aborted pollen (avg. 30 μm) was estimated to be slightly smaller than that of normal pollen (avg. 26 μm). The aborted pollen was easily distinguished from the normal pollen in shape. The normal pollen was plump and rounded-shape (Fig. 6a) while the center part of aborted pollen was severely collapsed resulting in shrunken phenotype (Fig. 6b); however the exin of the aborted pollen did not appear to be significantly different from that of the normal pollen. The transverse sections of anthers from wild type, T4 and T27 were further examined by transmission electron microscopy to compare the ultrastructure of the normal pollen (Fig. 6c, f, i) and aborted pollen (Fig. 6d, e, g, h, j, and k). The aborted pollen did not contain starch granules as well as nucli or any other organelles.

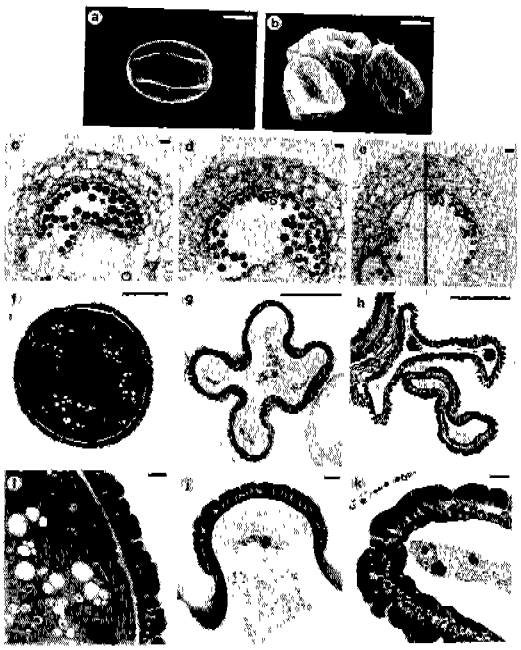

Fig. 6. Microscopic examination of normal and aborted pollen. (a and b) Scanning electron micrograph of pollen grains from wild type (a) and of an aborted pollen (b). Scale bars = 10 μm. (c, d and e) Bright-field micrographs of the cross sections of anthers of wild type (c), T4 (d) and T27 (e). Scale bars = 10 μm. (f, g and h) Transmission electron micrographs of microspores of wild type (f), T4 (g) and T27 (h). Scale bars = 10 μm. (i, j and k) Transmission electron micrographs of microspores of wild type (i), T4 (j) and T27 (k). Scale bars = 1 μm

The inserted sweet potato AGPase was strongly expressed, accumulating a high level of mRNAs in all the transgenic lines compared with the mRNA level in wild type (data not shown). The expression levels were, however, variable among nine transgenic lines. The highest transcription levels were observed in T4 and T27 (Fig. 7) while the transcription level was relatively low in T20. The fact that the pollen abortion was severest in T4 and T27 while mild in T20 suggest that the severity of the phenotype is most likely related to the transcription level of transgene.

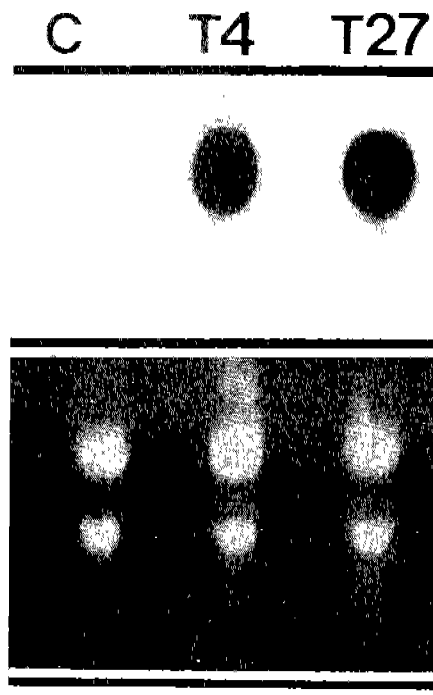

Fig. 7. Northern blot analysis of total RNA isolated from nontransformed and transformed plants. Total RNA was extracted from fully expanded leaves of transformed or control plant lines. Equal amount of each sample was loaded in each lane. C: nontransformed plant; T4 and T27: transformed plants by *sTL1*. *sTL1* cDNA was used as a probe. Lower part of each panel shows the ethidium bromide staining of RNAs

CONCLUSIONS

AGPase gene has been extensively demonstrated as the pivotal role in the starch biosynthesis of the starch accumulating sink tissues, such as potato tuber, maize endosperm or wheat endosperm. However, almost no information has been available about the role of AGPase gene in the pollen development. Recently, Lalonde et al., (1977) reported that the water-stressed wheat produced pollen grains

with little or no starch and the expression of the AGPase gene was strongly inhibited during the second phase of pollen development. In order to investigate the role of the AGPase gene in the pollen development more directly, the sweet potato AGPase small subunit cDNA was overexpressed in the transgenic tobacco plants. Most of the transgenic tobacco lines produced more than 50 % of aborted pollen grains, especially in T4 and T27, both were transformed by sTL1, more than 75 % of the pollen grains were aborted. The microscopic examination of the pollen grains revealed that the aborted pollen grains contained almost no starch granules. Since the normal pollen development depends on the sufficient supply of nutrients from the sporophytic tissues of anthers, reduced levels of the starch biosynthesis in the pollen grain or in the other floral organs which supply the nutrients for pollen development, can affect the starch biosynthesis in the pollen grains, resulting in this phenotype of no starch granules in the pollen grain. The transcript levels of AGPase small subunit were very high in bud tissues of T4 and T27 compared with control plants. Most of the transcripts were found to originate from the sweet potato AGPase gene not the endogenous tobacco AGPase gene. It thus appears that the overexpressed sweet potato AGPase mRNAs negatively affect the expression of tobacco AGPase gene, at least, in the floral organ tissues.

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