Characterization of cDNAs Encoding Small and Large Subunits of ADP-Glucose Pyrophosphorylases from Watermelon (Citrullus vulgaris S.)

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Three cDNA clones encoding ADP-glucose pyrophosphorylases were isolated from a full red fruit cDNA library of watermelon (Citrullus vulgaris S.). Sequence analyses indicated that one clone, wms1, corresponds to the small subunit, and two clones, wml1 and wml2 (a partial gene), are the large subunits of AGPase. The presumed AGPase proteins encoded by wms1, wml1, and wml2 have 526, 526, and 481 amino acids, respectively. The protein sequences have the conserved amino acids important for the substrate or regulator binding site, with some variation.

Developmental changes in the amounts of wms1, wml1, and wml2 transcripts in fruits were measured by northern blot analysis. Their expression levels decreased from the small green to medium green stages, then increased in accordance with fruit ripening, which was different from those of tomato and oriental melon.

Key words: ADP-glucose pyrophosphorylase; isoforms; developmental expression; watermelon (Citrullus vulgaris S.)

ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) is known to catalyze the formation of ADP-glucose from glucose-1-phosphate and ATP. This reaction is an essential step in starch biosynthesis.\(^1\)

AGPase is a heterotetrameric enzyme with two small subunits and two large subunits, which are encoded by different genes.\(^2,3\) In higher plants, the two kinds of subunit have some similarity in size and amino acid sequence,\(^4,5\) but the presence of the two subunits is essential for normal enzymatic function.\(^5,6\) Between the two subunits, there are differences in the number of isoforms and the expression pattern. That is, the small subunit has one or two isoforms,\(^6,7\) highly conserved among the plant species and expressed in all tissues. Meanwhile, the large subunit is present as multiple isoforms and shows tissue-specific expression.\(^7,8,9\) The large subunits, even in the same plants, are less conserved and different in sensitivity to allosteric activation and inhibition by 3-phosphoglycerate (3-PGA) and Pi, respectively.\(^10\)

Starch is the most significant form of carbon reserve accumulated in storage organs in plants. Thus, most studies on AGPase have been focused on cereals and tubers of plants, such as barley, wheat, maize, and potato, which have high levels of starch. Although fruits are also important carbohydrate sinks in plants, the studies on AGPase in fruit-producing plants are very rare. In this study, we isolated three cDNAs encoding for one small and two large subunits and characterized the expression patterns in watermelon fruits.

Materials and Methods

Plant materials. Watermelon (Citrullus vulgaris S. cv. Gold Medal) was used throughout this work. Plants were grown under greenhouse conditions and their fruits were harvested at four stages of development determined by maturity and internal fruit color. Developmental stages are as follows: small green (SG, size: 6–7 cm in diameter), 10 days after anthesis; medium green (MG, size: 17–18 cm in diameter), 25 days after anthesis; mature turning (MT, full size: 23–25 cm in diameter), 40 days after anthesis; and full red (FR), 50 days after anthesis.

Construction of a cDNA library and screening. Total RNA was extracted from full red watermelon fruits using the hot phenol RNA isolation procedure.\(^12\) Poly(A)\(^+\) RNA was isolated by PolyATtract mRNA Isolation System III (Promega). A watermelon fruit cDNA library was constructed by using the Zap-cDNA synthesis and Gigapack II gold cloning kits (Stratagene) according to the manufacturer’s instructions. In vivo excision of pBluescript plasmids was done in the Escherichia coli SOLR strain. The library was screened with the radiolabeled PCR products, as described below, by standard plaque lift methods.\(^13\) After prehybridization for 1–2 hr at 42°C in 30% formamid, 5 × Denhardt’s solution, 5 × SSPE, and 100 μg/ml denatured salmon sperm DNA, filters were washed twice in 2 × SSC and 0.05% SDS for 15 min at 42°C and twice in 0.2 × SSC and 0.1% SDS for 15 min at 68°C.

PCR amplification and probe preparation. First strand cDNA was synthesized from poly(A)\(^+\) RNA by reverse transcriptase (Promega) with random hexamer as the primer. PCR was done on the first strand cDNA

\(^1\) Genebank accession number: AF032471 (wms1), AF032472 (wml1), AF032473 (wml2).

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Abbreviations: AGPase, ADP-glucose pyrophosphorylase; 3-PGA, 3-phosphoglycerate, PCR, polymerase chain reaction; wms1, an AGPase small subunit of watermelon; wml1 and wml2, two AGPase large subunits of watermelon; wms1, wml1, and wml2, genes coding for wms1, wml1, and wml2, respectively; bp, base pairs.
according to the following conditions: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C (30 cycles). For the amplification of the small subunit cDNA, the sense primer (SU2: 5'GAGA[G/A]CA[G/A]TTG[C/A]AG[C/A/T]ATG-3') and the antisense primer (SL1: 5'GTAACCAATAATCTTCCCCAGTA-3') were used. For the amplification of the large subunit cDNAs, the sense primer (LU2: 5'CC[A/C/T]ATGAG[C/T]AA[C/T/TTG[C/T]AA-3') and the antisense primers (LL1: 5'CC[A/C/J]ATN[C/G][A/C][A/C/T]GNCAT[A/G]TA[A/G/T]GG-3', LL2: 5'CC[A/G/T]AT[A/G/TC[C/T]TCCCCA[A/G]TA[A/G/JTC-3'] were used. These PCR primers were synthesized on the basis of the conserved regions of the previously reported sequences of AGPase subunits.14-19 The amplified products were subcloned into the pBluescript SK+ vector and their nucleotide sequences were analyzed. Cloned PCR fragments were used as probes for screening for full-length clones.

DNA sequencing and analyses. Nucleotide sequencing using the dideoxy chain termination method20 was done using the Sequenase 2.0 kit (United States Biochemical) for a double strand to avoid errors; that is, the sense strand with the Erase-a-Base system (Promega) for serial deletion and the antisense strand with custom-made (DNA International) oligonucleotide primers for sequencing of internal sequences. Computer analyses for the nucleotide and amino acid sequences were done by PCGENE software (IntelliGenetics Inc., Release 6.60).

Northern blot analysis. Total RNA was isolated from fruits in four developmental stages (SG, MG, MT, and FR) and fractionated on a denaturing agarose (1.0%) gel. After the transfer to the nylon membrane (Hybond-N from Amersham), filters were prehybridized at 42°C for 1-2 hr in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. The hybridization to the labeled probe with [α-32P]dCTP and random primer was done overnight in a hybridization buffer. The filters were washed twice at room temperature for 10 min in 2 x SSC and 0.1% SDS, once at 65°C for 15 min in 1 x SSC and 0.1% SDS, and twice at 65°C for 15 min in 0.1 x SSC and 0.1% SDS. To avoid cross-hybridization with other isoforms, we used a 3' untranslated region as the probe DNA. The probes were the AfIIII-XhoI fragment (260 bp) of wms1 cDNA, the PvuI-XhoI fragment (360 bp) of wml1 cDNA, and the XhoI-XhoI fragment (200 bp) of wml2, each of which corresponds to a 3' untranslated region (3' UTR) labeled with [α-32P]dCTP.

Results

Isolation of cDNA clones encoding for AGPase small and large subunits

A watermelon fruit cDNA library was constructed in λ-ZapII and screened with the 247-bp (SU2-SL1), 514-bp (LU2-L1), and 663-bp (LU2-L2) PCR products amplified by PCR reaction. Using these PCR products as probe DNA, we isolated 32, 25, and 8 clones, respectively, out of 500,000 plaques. The approximately 2-kb cDNA inserts of isolated clones were partially sequenced with PCR primers. We obtained three different cDNA clones: one designated wms1 for the small subunit and the other two wml1 and wml2 for the large subunits.

The nucleotide sequences of the three clones (wms1, wml1, and wml2) are presented and compared with each other in Fig. 1. The lengths of wms1, wml1, and wml2 cDNA clones are 1958 bp, 1994 bp, and 1691 bp, respectively. The clones have an uninterrupted open reading frame deriving 57-58 kDa polypeptides, except for wml2 that is a partial gene lacking the 5' coding region of the transit peptide. Putative polyadenylation signals were found in the 3' untranslated regions of three clones, which were located 126 bp (AGTAAA) and 26 bp (AATAAT), 92 bp (AATAAG), and 58 bp (AATAAC) upstream from the polyadenylated site for wms1, wml1, and wml2, respectively.

In the whole scales, the small subunit of wms1 has low similarity with each of the two large subunits, 36% and 46% with wml1 and wml2, respectively. The identity between the two large subunits is 59%. In the coding regions, the similarity between wms1 and each of wml1 and wml2 is 45% and 54%, respectively, and there is 69% identity between the two large subunits.

Comparison of the deduced amino acid sequences between small and large subunits

The deduced amino acid sequences of wms1, wml1, and wml2 were compared as shown in Fig. 2. The protein sequence of wms1 shows 43% and 50% similarity to those of the two large subunits, wml1 and wml2, respectively. The similarity between the two large subunit isoforms is 72%. The N-terminal regions, containing transit peptides, show no significant similarity. In the deduced amino acid sequences, the four regions are well conserved, which were previously shown to be conserved sites in the small and large subunits of AGPase.21 Site 1, which shows similarity to the ATP binding site [AF[K/G]/WFI/Q/R]GTAD[A/S][V/W/I]R for the large subunit and [NF[N/D]WFQGTADAVR for the small subunit] in glycogen synthase from E. coli is well conserved in wms1 and wml2, but less conserved in wml1. In the large subunit of wml1, the lysine residue on the third position is replaced by histidine. Sites 2 and 4 have high levels of similarity with the presumed glucose-1-phosphate (QFE[K/Q]PKG for the large subunit and EF[A/S/E][K/N]PKG for the small subunit) and the 3-PGA binding site (SGIV/V/I[K/E] H[K/A]TIXDG for the large subunit and SGIV/I/V[NIKDALIPSQ for the small subunit), respectively. Site 3, the presumed 3-PGA and Pi regulation site (C11/D[K/M]N[K/R]GI) in the large subunit, is conserved in wml1, wml2, and even in wms1.

Evolutionary relationship among plant AGPase large subunits

The deduced amino acid sequences were compared between watermelon and other plants. In the small subunit, the sequence of wms1 had a high number of identi-
Fig. 1. Comparison of the Nucleotide Sequences Among wmsl, wml1, and wml2.

This alignment was generated by Clustal W (version 1.60). An asterisk indicates identical nucleotides. Gaps were introduced to maximize identities, represented by a dash. The first and second shaded regions represent the start codon (ATG) and the stop codon (TAA for both wmsl and wml2, and TGA for wml1), respectively. Putative polyadenylation signals are represented in bold.
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Fig. 2. Comparison of the Deduced Amino Acid Sequences of wms1, wml1, and wml2.

This alignment was generated by Clustal W (version 1.60). An asterisk indicates the identical amino acid. Gaps were introduced to maximize identities, represented by a dash. The first and second boxes show the presumed ATP (site 1) and glucose-1-phosphate (site 2) binding sites, respectively. The third and fourth boxes indicate amino acids corresponding to the presumed 3-PGA and Pi regulation (site 3), and 3-PGA (site 4) binding sites, respectively. The shaded letters are important residues for substrate or regulator binding.

cal residues (approximately 90%) with the previously reported sequences of AGPase, which is consistent with the previous studies. In the large subunit, the similarity between watermelon and other plants varies from 62% to 91%. The phylogenetic tree of the plant AGPase large subunits is presented in Fig. 3. The results showed that wml1 and wml2 have a higher similarity with dicot isoflavones of potato, tomato, and sugar beet than with monocot isoflavones of barley, wheat, and maize. Both wml1 and wml2 are most similar to oriental melon mlf1 and mlf2, respectively. Also, wml1 is similar to tomato AgpL1 and potato agpS, and wml2, with tomato AgpL2 and potato aspS2. However, both wml1 and wml2 are distantly related to tomato AgpL3 expressed in leaves and to isoforms expressed in the endosperms of wheat, barley, and maize.

Expression patterns of wms1, wml1, and wml2 during watermelon fruit development

We investigated the expression patterns of watermelon AGPase genes in the process of fruit development (Fig. 4). The expression level of wms1 was observed as the highest in the small green stage, as the lowest in the medium green stage, and markedly increased in the full red stage. Both wml1 and wml2 showed a similar expression pattern as wms1, with some difference in the transcript amount. Unlike wms1, wml1 and wml2 have the
highest expression in the full red stage. The pattern of wml2 was more similar to that of wms1 in the point of increase from the medium green to mature turning stage, while the wml1 transcript was consistent during these stages.

Discussion

It has been reported that the large subunit of AGPase, in potato,\textsuperscript{14,17} tomato,\textsuperscript{10} and Arabidopsis,\textsuperscript{15} exists in at least three isoforms encoded by independent nuclear genes. In this study, we isolated cDNAs encoding two large subunits of AGPase and one small subunit from watermelon fruits. wml1 and wml2 mRNA transcripts were strongly detected in fruits as shown in Fig. 4. However, they were hardly detected in other tissues including leaves, stems, and roots (data not shown), which is consistent with that plant AGPase large subunits have the characteristic of tissue-specific expression. These results imply that watermelon might have an additional isoform of the AGPase large subunit(s), besides wml1 and wml2, which would be dominantly expressed in tissues other than fruits.

The deduced amino acid sequences of wms1, wml1, and wml2 have the sites showing homology with ATP (site 1), glucose-1-phosphate (site 2), and 3-PGA (site 4) binding sites of AGPase.\textsuperscript{21} It is known that in site 1 large subunits have a lysine residue or glycine at the third position, and small subunits have an asparagine or aspartic acid residue. In our results, wml2 also has the lysine residue, while wml1 has a histidine residue at the corresponding position. Since site 1 is presumed to be the ATP binding site, the mlf 1 isoform with histidine may represent the enzyme activity or substrate binding affinity different from that of the mlf 2 isoform.\textsuperscript{20} Site 3 that may be involved in both 3-PGA and Pi regulation is well conserved in wms1, wml1, and wml2.

The transcripts of wms1, wml1, and wml2 were detected in all developmental stages of fruits. There was the highest expression in the small green fruits for wms1, and in full red fruits for wml1 and wml2. The expression of wms1 mRNA was on the whole stronger than that of both wml1 and wml2. wml1 and wml2 genes showed some differences in expression patterns and levels during fruit development. Also, wml1 and wml2 were strongly expressed in fruits, but weakly or not at all in other tissues (data not shown). Although it is likely that the two AGPase large subunits play a role(s) in starch synthesis during watermelon fruit development, the detailed physiological roles are not clear. Probably, the different expression of wml1 and wml2 could provide AGPase with differing sensitivities to regulation.\textsuperscript{11}

The decrease in the amounts of the AGPase' transcripts from the small green to medium green stages and the following increase in accordance with fruit ripening have not been reported in the studies on fruits including tomato\textsuperscript{10} and oriental melon (unpublished data). The expressions of tomato Agp1L, oriental melon mlf1, and oriental melon mlf2 were the highest in young fruits, but sharply decreased as fruits matured. Tomato Agp1L was expressed in all developmental stages of fruits with a very low level. That is, the expressions of AGPases in watermelon fruits have the characteristic patterns, which are not in other investigated fruits, such as tomato and oriental melon. These results suggest that, in fruits, the expression patterns of AGPase could vary with plant species. It is known that the expression of AGPase genes during fruit ripening is related to starch accumulation in sink tissues or their sink strength. Generally, starch contents in fruits decrease as fruit ripens.\textsuperscript{24} However, our results show that the expression of AGPase the genes increases during the fruit development of watermelon even in the later stages. These phenomena imply that watermelon fruit may accumulate starch at the later stage.

Studies on the transcriptional regulation of AGPase through transgenic plants, differential changes of starch contents, and enzyme activity changes in ripening fruit could lead to the elucidation of relations between the expression of the AGPase gene and starch synthesis or sink strength.

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References

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