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Isolation and Nucleotide Sequence Analysis of ADP-glucose Pyrophosphorylase gene from Chinese cabbage (*Brassica rapa* L.)

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Abstract

ADP-glucose pyrophosphorylase (AGPase) catalyzes the key regulatory step in starch biosynthesis. Two cDNA clones encoding AGPase subunits were isolated from the leaf cDNA library of Chinese cabbage (*Brassica campestris* L. spp. *pekinensis*). One was designated as *BCAGPS* for the small subunit and the other as *BCAGPL* for the large subunit. Both cDNAs have uninterrupted open reading frames deriving 57 kDa and 63 kDa polypeptides for *BCAGPS* and *BCAGPL*, respectively, which showed significant similarity to those of other dicot plants. Also, However, the deduced amino acid sequence of *BCAGPL* has a unique feature. That is, it contains two regions (R1 and R2) lacking in all other plant enzymes. This is the first report of *BCAGPL* containing R1 and R2 among plant large subunits as well as small subunits. From the genomic Southern analysis and BAC library screening, we inferred the genomic status of *BCAGPS* and *BCAGPL* gene.

Introduction

Starch is the major storage carbohydrate of photosynthetically fixed carbon in higher plants. The importance of starch as a storage compound is revealed by its wide distribution. It is present in all major organs of higher plants and, in certain tissues, can accumulate to high levels (ap Rees, 1977).

A key enzymatic step in starch biosynthesis is catalyzed by ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27). ADP-glucose pyrophosphorylase catalyzes the conversion of glucose-1-phosphate and ATP into ADP-glucose, releasing pyrophosphate as a product. ADP-glucose serves as the activated glucosyl donor in the synthesis of starch in higher plants (Preiss, 1991). AGPase is a heterotetramer composed of two small and two large subunits, which are encoded by two different genes (Morell et al., 1987). Even though there is little difference in size and sequence, both subunits are required for optimal activity (Lin et al., 1988). AGPase is considered as a main regulatory enzyme in the synthesis of starch, and is mainly activated by 3-phosphoglycerate (3-PGA) and inhibited by orthophosphate (Pi) in most organs of higher plants (Preiss, 1991). The small subunit of higher plant AGPase is highly conserved, whereas the similarity among different large subunits is lower (Smith-White and Preiss, 1992).

Most studies on AGPase have focused on crop plants accumulating high levels of starch and fruit-producing plants, such as barley (Villand et al., 1992), wheat (Ainsworth et al., 1993), potato (La Cognata et al., 1995), maize (Giroux et al., 1995), tomato (Park et al., 1998), melon (Park et al., 1998), and watermelon (Kim et al., 1998). However, there has been no effort to investigate AGPase in vegetables because the level of starch contents is low. However, carbohydrates, including starch and soluble sugars, play an important role in the taste and fermentation of vegetables (Kim et al., 1989; Park, 1995).

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Therefore, studies on AGPase of vegetables can broaden the information base on the content and composition of carbohydrates. Additionally, they can illuminate more clearly the phylogenetic linkage among plant AGPase.

Bacterial artificial chromosome (BAC) system is commonly used for developing large insert libraries, which has been a useful tool for the genomic research in plant. Chinese cabbage has 550Mb genome size. It is about 4 times as large as *Arabidopsis* genome, a model plant (Arumuganathan and Earle, 1991). Using the BAC system, we constructed the Chinese cabbage *Hin*dIII BAC library (not published) and now apply to the genome research such as physical mapping, chromosome walking, and comparative mapping.

In this study, we have isolated two cDNAs encoding for one small and one large subunit of AGPase from Chinese cabbage, which is one of the most important vegetable crop in Korea. Through a comparison of sequences, we investigated whether Chinese cabbage AGPase has a specific site or sequence, and the relationship with AGPases from other plants species. Through the genomic Southern analysis and the screening of the BAC library, we analyzed the status in genome of Chinese cabbage.

Materials and Methods

Plant materials

Chinese cabbage (*Brassica campestris* L. spp. *pekinensis* (Lour) Olsson) was used throughout this work, and was pre-trimmed to remove discolored outer leaves and roots. Plants were cultivated under greenhouse conditions. Sample materials were immediately frozen with liquid nitrogen, stored in -70℃, and then were used for isolation of total RNA.

Construction of a cDNA library and screening of *BCAGPS* and *BCAGPL* cDNAs

Total RNA was extracted from Chinese cabbage leaves using the hot phenol RNA isolation procedure as described by Verwoerd et al. (1989). Poly(A)$^+$ RNA was isolated by PolyATtract mRNA Isolation System III (Promega). A leaf cDNA library was constructed by using the Zap-cDNA synthesis and Gigapack® II gold cloning kits (Stratagene) according to the manufacturer's instruction manual. The library was screened with random-primed, [α-^{32}P]dCTP-labeled 250-bp cDNA fragment for small subunits and 620-bp for large subunit's, as described below, by using standard plaque lift methods (Sambrook et al., 2000). After prehybridization for 1-2 hr at 42℃ in 30% formamide, 5x Denhardt's solution, 5x SSPE, and 100 μg/mL denatured salmon sperm DNA, the filters were incubated with ^{32}P-labeled PCR products for 24hr under the same conditions. The filters were washed twice in 2x SSC and 0.05% SDS for 15 min at 42℃ and twice in 0.2x SSC and 0.1% SDS for 15 min at 68℃.

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 according to the following conditions: 1 min at 94℃, 1 min at 55℃, and 1 min at 72℃ (30 cycles). For amplification of the small subunit cDNA, the sense primer (SU2: 5'-AA[C/T]ATGGGNGGNTATA[C/T]AA[A/G]AA[C/T]GA-3') and the antisense primer (SL1: 5'- GT[C/T]TC[A/G]TA[A/G]TA[A/G]TCNGCNCCCAT-3') were used. For amplification of the large subunit cDNAs, the sense primer (LU2: 5'-CC[A/C/T]ATGAG[C/T]AA [C/T]TG[C/T][A/T]T[C/T]AA-3') and the antisense primers (LL2: 5'-CC[A/G/T]AT[A/G]TC[C/T]TCCCA[A/G]TA[A/G]TC-3') were used. These PCR primers were synthesized on the basis of the conserved regions of the previously reported sequences of AGPase subunits. 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.

Nucleotide sequencing

Nucleotide sequencing using the dideoxy chain termination method (Sanger, 1977) was done using the Sequenase 2.0 kit (United States Biochemical), and T3 promoter, T7 promoter and custom-made (Genotech, Korea) oligonucleotide primers, for a double strand to avoid errors. Computer analyses for the nucleotide and amino acid sequences were done by PCGENE software (IntelliGenetics Inc., Release 6.60).

Southern blot analysis

The genomic DNA of Chinese cabbage was isolated from young leaves using the urea-extraction method. Each of 10 μg genomic DNA was digested with *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Sac*I, fractionated on 0.8% agarose gel in 0.5xTBE, and then blotted onto the Hybond-N$^+$ nylon membrane filter. Hybridization was carried out with 606 bp fragment (position 373-978) of *BCAGPS* cDNA or 589

bp fragment (position 418-1006) of *BCAGPL* cDNA as probes at 42℃ overnight. The filter was washed two times for 10 minutes at 65℃ with 2x SSC/0.1% SDS and washed with 0.2x SSC/0.1% SDS at 65℃. The membrane filter was exposed to the X-ray film at -70℃ for 1-7 days.

Screening and isolation of BAC clone encoding *BCAGPS* and *BCAGPL*

Three high-density filters contained 55,296 BAC clones, which were prepared from Chinese cabbage *Hin*dIII BAC library (not published). The probe was the same one of southern blot analysis. Hybridization was carried out at 65℃ in 270 mM sodium phosphate/1 mM EDTA/3.7% SDS for overnight. Stringent washing were performed with two changes each of 1x SSC/0.1% SDS and 0.5×SSC/0.1% SDS at 65℃. Membranes were exposed to X-film for 1~4 days. Plasmid DNAs from positive BAC clones were prepared. BAC clones were cultured in 2xYT/12.5 μM Chlroramphenicol at 37℃ for 18 hrs, and then extracted by alkaline lysis method (Sambrook et al., 1989). DNA were digested with *Hin*dIII and then electrophoresed in 0.8% agarose gel/0.5× TBE, and blotted onto the Hybond-N$^+$ nylon membrane filter. Hybridization was performed using ECL direct nucleic acid labelling and detection systems(Amersham co.).

Results and Discussion

Isolation of cDNA clones encoding AGPase small and large subunits

We isolated two cDNA clones from the leaf cDNA library of Chinese cabbage. One designated *BCAGPS* for the small subunit (Figure 1) and the other *BCAGPL* for the large subunit (Figure 2).

The length of *BCAGPS* (GenBank accession number AF347697) and *BCAGPL* cDNA (accession number AF347698) clones are 1841 bp and 1844 bp, respectively. The *BCAGPS* cDNA contains a 1560 bp coding region, 30 bp 5'-UTR (untranslated region), and 251 bp 3'-UTR (Figure 1). The *BCAGPL* cDNA is composed of a 1713 bp coding region, 84 bp 5'-UTR, and 47 bp 3'-UTR (Figure 2). Both cDNA regions have an uninterrupted open reading frame deriving about 57 kDa and 63 kDa polypeptides, respectively. Putative polyadenylation signals (Joshi, 1987) were found at the positions 1719-1724 (AACAAA) or 1816-1821 (AATAAT), and 1821-1826 (AATAAA) in the 3'- UTR of *BCAGPS* and *BCAGPL*, respectively. In the whole scales, the nucleotide sequence of *BCAGPS* shows 53.7% similarity to *BCAGPL*. In the coding regions, the similarity bet ween *BCAGPS* and *BCAGPL* is 59.9%.

Comparison of the deduced amino acid sequences of small and large subunits with known AGPase poly peptides

The deduced amino acid sequences of *BCAGPS* and *BCAGPL* were compared as shown in Figure 3. The protein sequences of both subunits share a considerable homology (50.9% identity), but the N-terminal regions containing transit peptides show a lower similarity. Except for Site 2 of *BCAGPS*, the deduced amino acid sequences of both subunits contain highly conserved residues, which were

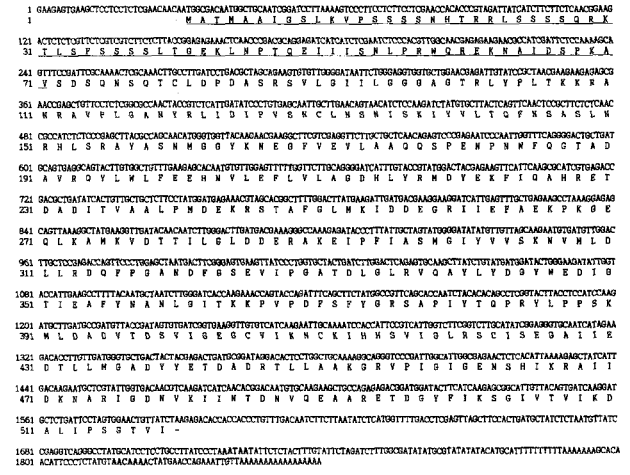


Figure 1. Nucleotide sequence and deduced amino acid sequence for the *BCAGPS* clone isolated from Chinese cabbage leaf. The putative transit peptide region is underlined. Bold letters represent the putative polyadenylation signals.

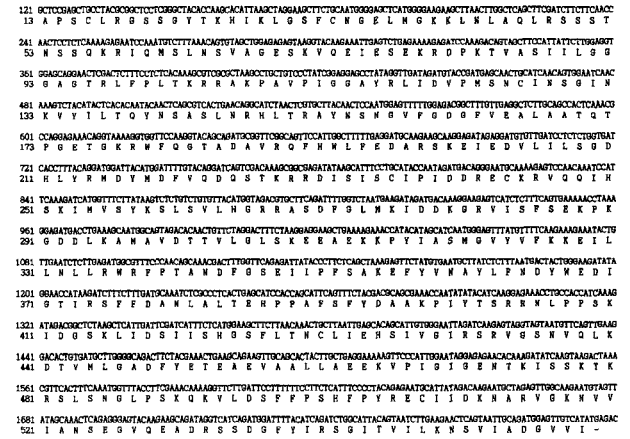


Figure 2. Nucleotide sequence and deduced amino acid sequence for the *BCAGPL* clone. Bold letters represent the putative polyadenylation signals.

previously shown to be both 3-PGA and Pi binding sites (Site 1, 2, 3, 4, 7 and 8) (Greene et al., 1998; Fu et al., 1998), a substrate binding site (Site 5) (Greene and Hannah, 1998), and a subunit interaction site (Site 6) (Ballicora et al., 1999). However, a proline residue of Site 2 in small subunit was not conserved in other plant enzymes, which have mainly an arginine residue. Also, two regions (R1 and R2) that were lacking in both subunits of other plants' AGPase are found in *BCAGPL* (Figure 3 and 4). Since the regions contain a number of charged amino acids, they might be located on the surface of a protein. However, the function of the regions is unknown and remains to be tested.

Evolutionary relationship among plant AGPase large subunits

The deduced amino acid sequences were compared between Chinese cabbage and other plants (Figure 4 and 5). The sequence of *BCAGPS* has the highest homology with *Arabidopsis atagps* (85.4% identity) and *BCAGPL* has the highest homology (90.4% identity) with *Arabidopsis ApL1*. Similarly, the phylogenetic dendrograms show that *BCAGPS* is similar in its sequence to *Arabidopsis atagps* and *Citrus agpS*, and *BCAGPL* is to *Arabidopsis ApL1*, tomato *agpL3*, and potato *agpS3* (Figure 5). In contrast, both subunits of Chinese cabbage AGPase are distinguished from the endosperm isoforms of monocots. These results suggest an evolutionary link among the dicot plants. Also, *BCAGPL* is distantly related to other isoforms of watermelon *wml1* and *wml2*, melon *mlf1* and *mlf2*, tomato *agpL1* and *agpL2*, and potato *agpS* and *agpS2*. The phylogenetic relationship among AGPase large subunits shows that the enzymes can be categorized into four branches.

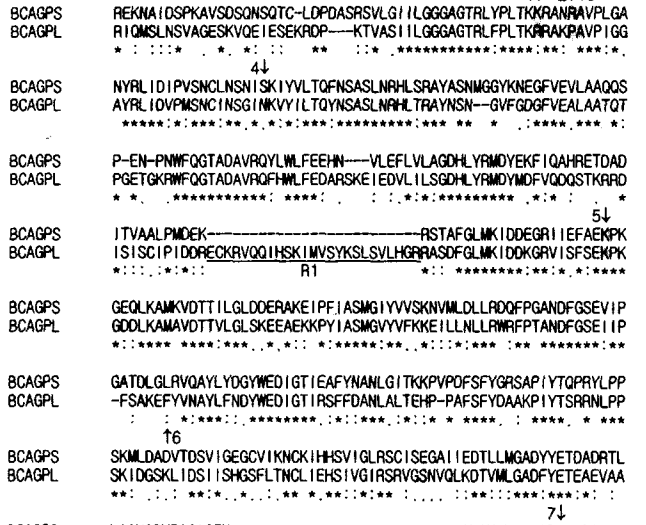


Figure 3. Comparison of the deduced amino acid sequences of *BCAGPS* and *BCAGPL*. The sequences were aligned using the Clustal X (version 1.64b) program (Thompson et al., 1994). An asterisk indicates identical amino acids. The shaded letters are important residues for substrate binding (Site 5), regulator binding (Site 1, 2, 3, 4, 7 and 8), or subunit interaction (Site 6). The regions (R1 and R2) with different sequences between *BCAGPL* and other plant enzymes are underlined.

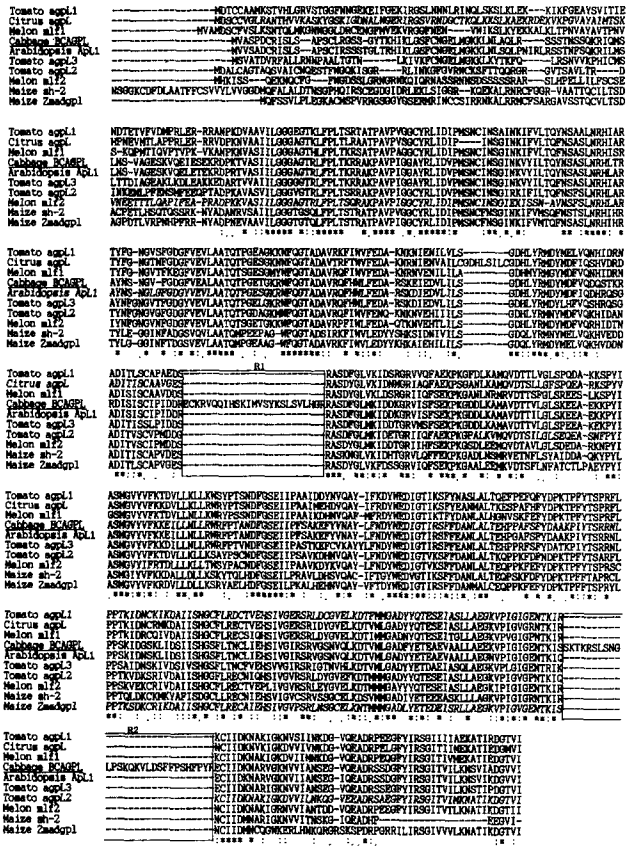


Figure 4. Alignment of the deduced amino acid sequences of the *AGP*ase large subunits gene from various species. Alignment is performed with the deduced amino acid sequences of *AGP*ase large subunits from various plants: *mlf1* (AF030383) and *mlf2* (AF030384) from oriental melon; *agpL1* (U88089), *agpL2* (U85496) and *agpL3* (U85497) from tomato; *ApL1* (X73367) from *Arabidopsis*; *sh-2* (S48563) and *Zmadgpl* (Z38111) from maize; *agpL* (AF184598) from *Citrus*; and *BCAGPL* (AF347698) from Chinese cabbage. This alignment was generated by the Clustal X (version 1.64b). Gaps are marked with dashes. The conserved amino acid residues are marked with asterisks. The regions (R1 and R2) with different sequences between *BCAGPL* and other plant enzymes are boxed.

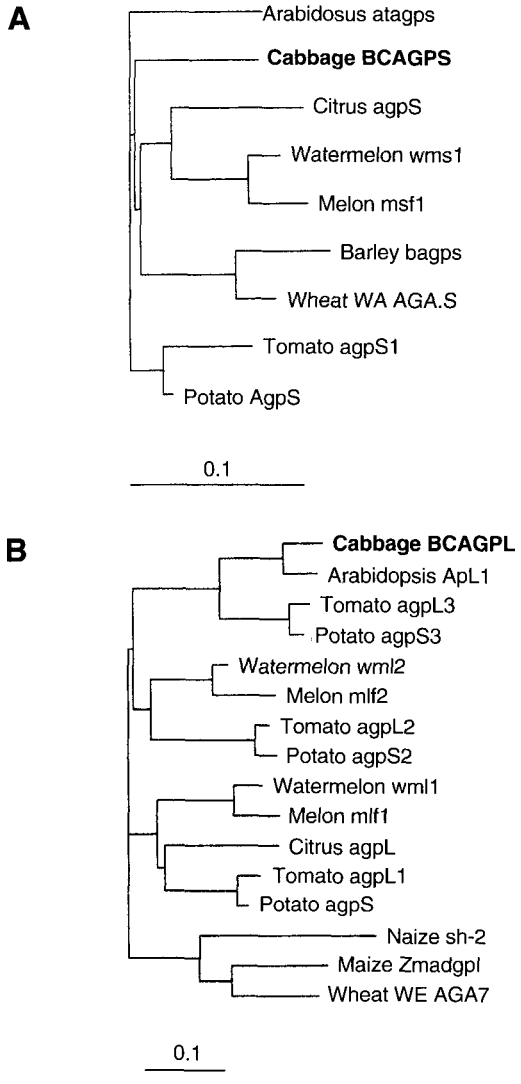
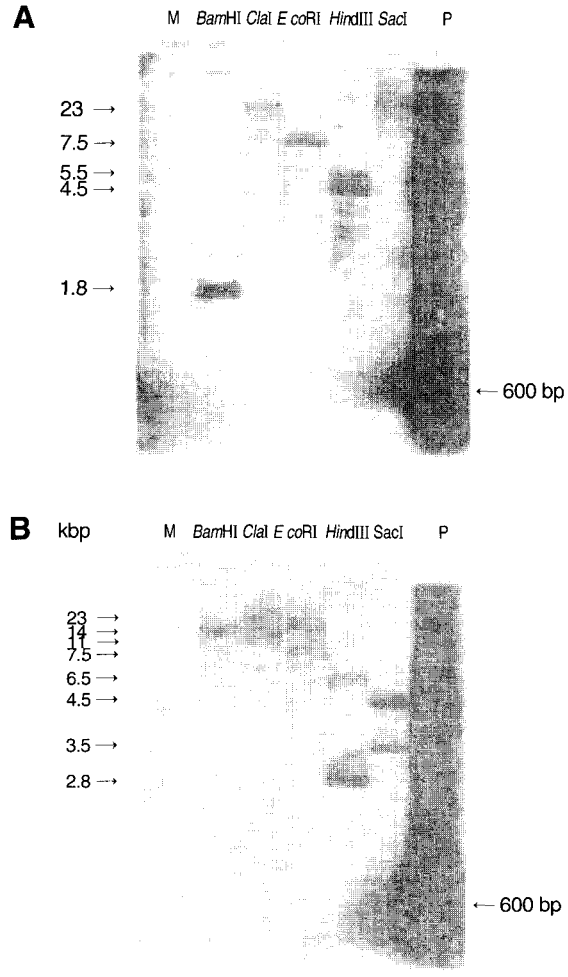


Figure 5. Phylogenetic relationship of the Chinese cabbage *BCAGPS* and *BCAGPL* genes to other plant AGPase small (A) and large subunits (B). Phylogenetic analysis is based on the deduced amino acid sequences of AGPase from various plant species mentioned in Figure 3 and other organisms, including *wms1* (AF032471), *wml1* (AF032472) and *wml2* (AF032473) from watermelon; *msf1* (AF030382) from oriental melon; *agpS1* (L41126) from tomato; *AgpS* (X55650), *agpS* (X61187), *agpS2* (X74982) and *agpS3* (X76136) from potato; *agagps* (U70616) from *Arabidopsis*; *WA AGA.S* (AF244997) and *WE* AGA7 (X14350) from wheat; *bagps* (Z48563) from barley; *agpS* (AF184597) from *Citrus*; *BCAGPS* (AF347697 from Chinese cabbage. The tree was generated by Clustal X (version 1.64b) and TreeView (version 1.6.1). The scale on the bottom shows similarity between two clusters and '0.1' means 0.1 nucleotide substitutions per site.



Fiugre 6. Genomic Southern blot analysis of *BCAGPS* (A) and *BCAGPL* (B). Ten micrograms of Chinese cabbage genomic DNA was digested with *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Sac*I for each DNA sample. Size markers (kb) are indicated on the left. A letter 'P' represents positive control of hybridization.

Genomic southern analysis

To determine the AGPase gene number in Chinese cabbage, genomic southern analyses were performed. *Bam*HI, *Hin*dIII, *Cla*I, *Eco*RI, and *Sac*I restriction enzymes were used because of the absence of those sites in probe DNAs of *BCAGPS* and *BCAGPL*. When the 606 bp *BCAGPS* fragment was used as a probe, one band was detected in *Bam*HI, *Cla*I, and *Eco*RI lane, and two bands were detected in *Hin*dIII lane (Figure 6-A), respectively. This result suggested that *BCAGPS* gene might be existed in Chinese cabbage genome as single or two copies.

In the case of *BCAGPL*, one band was detected for *Bam*HI, and two bands were detected for *Cla*I, *Eco*RI, *Hin*dIII, and *Sac*I, respectively (Figure 6-B), which suggested that *BCAGPL* might be existed as single or two copy. It has been reported that the large subunit of AGPase in dicot plants including tomato, *Arabidopsis*, potato, melon, and watermelon exists in two more isoforms encoded by different genes (Villand et al., 1993; Ballicora et al., 1998;

Kim et al., 1998; Park and Chung, 1998; Park et al., 1998). Thus, two bands might result from the existence of other isoform(s) of AGPase large subunits, but not from two copy of *BCAGPL* gene. That is, Chinese cabbage might have additional isoforms of AGP large subunits, besides *BCAGPL*, which was supported by the results of BAC clone screening as described below.

Isolation of BAC clone containing *BCAGPS* and *BCAGPL* gene

For isolation of genomic DNA and physical mapping of *BCAGPS* and *BCAGPL* gene, we were screened the BAC clone using the Chinese cabbage *Hin*dIII BAC library (not published). After first screen using high-density filters, 45 and 43 BAC clones were hybridized with *BCAGPS* and *BCAGPL*, respectively. Plasmid DNA was extracted from each positive clones of primary screens. Nine and twelve positive BAC clones were rehybridized with *BCAGPS* and *BCAGPL* probes, respectively (Figure 7-A and 7-B). Because the BAC library covers about 11-fold of genome size of Chinese cabbage, we presumed that the isolated genomic clones contain all genomic DNA existed in the genome. Also, the size of signals is similar to those of Southern blot analysis. In the case of *BCAGPL*, we isolated only the BAC clones with same size (about 6.5 kb), which strongly supported that *BCAGPL* gene exists in Chinese cabbage genome as single copy. Using these BAC clones, we are performing the physical mapping project of Chinese cabbage genome and the elucidation of genomic structure of *BCAGPS* and *BCAGPL*.

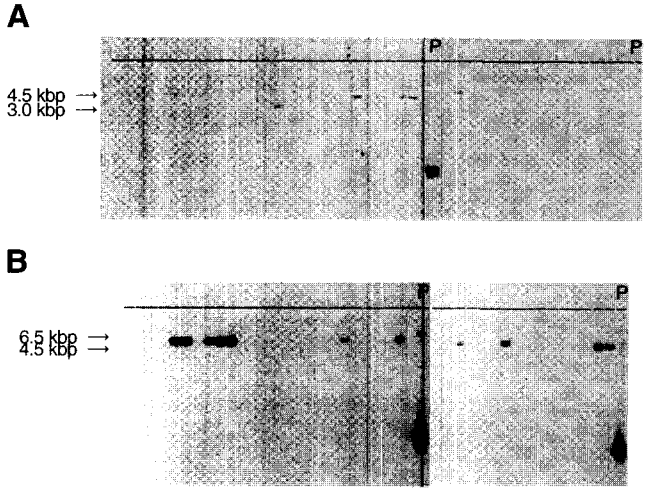


Figure 7. Isolation of BAC clones encoding *BCAGPS* and *BCAGPL*. Plasmids isolated from positive Bac clone were digested with *Hin*dIII. A letter 'P' represents positive control of hybridization. Size markers (kb) are indicated on the left.

In summary, we cloned two AGPase subunits genes (*BCAGPS* and *BCAGPL*) and analyzed the structural characteristics in Chinese cabbage, which is a dicot and vegetable plant. The deduced amino acid sequence indicated that *BCAGPL* polypeptide has unique regions (R1 and R2), which are lacking in all other plant AGPase. Through the genomic southern analysis and BAC library screening, we suggested that Chinese cabbage has single copy AGPase gene. On the basis of our results, further studies on the expression of AGPase and carbohydrate composition in Chinese cabbage will be applied to agronomy and the food industry. Also, the generation of transgenic plants and protein purification and characterization will be helpful in clarifying the role of specific characteristics.

Acknowledgments

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