RESEARCH

The Lack of Beta-amylase Activity in Soybean Cultivar Altona *sp* $_1$ is Associated with a 1.2 kb Deletion in the 5' Region of Beta-amylase I Gene

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ABSTRACT

Previous studies have identified near-isogenic soybean [*Glycine max* (L.) Merr.] lines, one containing normal β-amylase [α-1,4-glucan maltohydrolase (EC 3.2.1.2)] activity (Altona *Sp* $_1^b$) and the other with undetectable β-amylase activity (Altona *sp* $_1$) in seeds. SDS-PAGE analysis of 50% isopropanol extracted proteins from soybean seeds revealed a prominent 56 kDa protein. This protein, which was absent in Altona *sp* $_1$, was identified as β-amylase by matrix-assisted-laser-desorption-time-of-flight mass spectrometry (MALDI-TOF-MS) and immunoblot analysis using antibodies generated against *Arabidopsis* β-amylase. Southern blot analysis showed differences in the sizes of the DNA fragments hybridizing to β-amylase probe between the near-isogenic soybean lines. A search of the soybean genome database revealed the presence of nine β-amylase genes in the soybean genome. We have isolated the β-amylase gene (GmBAM 1) by screening a genomic library of wild-type soybean and determined its nucleotide sequence. Analysis of the nucleotide sequence of the GmBAM 1 revealed a complete open reading frame that was interrupted by six introns. In contrast, the GmBAM 1 from Altona *sp* $_1$ had a 1207 bp deletion near the 5' region that included the second and third exon regions. Our results suggest that this deletion may be responsible for the lack of β-amylase activity in soybean cultivar Altona Altona *sp* $_1$.

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Abbreviations: DAF, days after flowering; MALDI-TOF-MS, matrix-assisted-laser-desorption-time-of-flight mass spectrometer; SDS, sodium dedecyl sulfate.

The enzyme β-amylase catalyzes the release of β-anomeric maltose from the nonreducing ends of starch (Robyt and Whelan, 1968; Sopanen and Lauriere, 1989). Beta-amylase is highly abundant in starch-storing organs such as seeds and tubers. In addition, less abundant "tissue-ubiquitous" forms are also found in leaves and roots of several plants (Beck and Ziegler, 1989; Ziegler, 1999). Because of their perceived role in starch metabolism and their abundance, this enzyme has been the subject of numerous investigations (Beck and Ziegler, 1989). Molecular analysis of β-amylase from barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), maize (*Zea mays* L.), soybean, sweet potato [*Ipomoea batatas* (L.) Lam.], potato (*Solanum tuberosum* L.), alfalfa (*Medicago sativa* L.), thale cress [*Arabidopsis thaliana* (L.) Heynh.] and a few other plants have been reported (Yoshida and Nakamura, 1991; Yoshida et al., 1992; Monroe et al., 1991; Totsuka and Fukazawa, 1993; Sadowski et al., 1993; Yoshigi et al., 1994; Wang et al., 1995, 1997; Wagner et al., 1996; Fulton et al., 2008). In addition to plants, β-amylase is also present in certain bacteria including *Bacillus* spp. and *Clostridum thermosulfurogenes* (Siggens, 1987; Kitamoto et al., 1988; Van Damme et al., 2001). Among plants, β-amylases share similar physiochemical

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properties; ranging from 50 to 60 kDa in molecular weight, exhibiting similar pH optima, and show significant amino acid homology (Robyt and Whelan, 1968; Beck and Ziegler, 1989; Pujadas et al., 1996).

Even though β-amylases are involved in the degradation of starch, their primary function in storage organs is not clear. Since β-amylase accumulates significantly in storage organs such as seeds and tubers, it has been suggested that they may function as seed storage proteins and vegetative seed storage proteins, respectively (Ziegler, 1999; Gana et al., 1998). Even though soybeans at maturity contain very little starch, β-amylase accumulates abundantly in soybean seeds (Adams et al., 1980). Two near isogenic soybean lines, one containing normal β-amylase activity (Altona Sp_1^b) and the other with undetectable β-amylase activity (Altona sp_1) in seeds have been characterized (Hildebrand and Hymowitz, 1980, 1981). Interestingly, the lack of β-amylase activity had no apparent effect on either the starch or the soluble sugar levels during seed development and germination (Hildebrand and Hymowitz, 1981). Based on these observations it was concluded that β-amylase has no essential role in starch metabolism of soybean seeds. In this study, we investigated the molecular basis for the lack of β-amylase activity in Altona sp_1. Our results demonstrated a 1.2 kb deletion in the 5' region of the β-amylase gene (GmBAM 1) may be responsible for the lack of β-amylase activity in soybean Altona sp_1.

MATERIALS AND METHODS

Plant Material

Seeds of soybean Altona (Sp_1^b) and Altona (sp_1) were obtained from the USDA Soybean Germplasm Collection, Urbana, IL. These soybean genotypes were grown in a greenhouse in 25-cm plastic pots containing Pro-Mix (Premier Horticulture, Rivière-du-Loup, QB, Canada) and fertilized with Osmocote Plus (ScottsMiracle-Gro, Marysville, OH) according to the manufacturer's recommendations. Seed samples were harvested at various days after flowering (DAF), separated according to size, frozen in liquid N and stored at −80°C.

Extraction and Analysis of Soybean Seed Proteins

Soybean seeds were ground to a fine powder in liquid N using a mortar and pestle. Total seed proteins were obtained by boiling 10 mg seed powder with 1 mL of sodium dodecyl sulfate (SDS) sample buffer [60 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), and 5% 2-mercaptoethanol (v/v)] for 5 min. Following extraction, the lysate was clarified by centrifugation (15,800 × *g*, 5 min), and the resulting supernatant transferred to a clean Eppendorf tube. Isopropanol-soluble proteins were obtained by adding 1 mL of 50% isopropanol to a 2 mL Eppendorf tube containing 100 mg of seed powder. Extraction was performed in a 30°C shaker for 1 h followed by centrifugation (15,800 × *g*, 5 min). A 500-μL aliquot of the supernatant was mixed with three volumes of ice-cold acetone and incubated overnight at −20°C. Precipitated proteins were recovered by centrifugation, air-dried, and resuspended in 200 μL of SDS-sample buffer. Seed proteins were resolved by SDS-polyacrylamide gel electrophoresis using a Hoeffer SE 260 minigel apparatus according to the manufacturer's recommendations (GE Healthcare, Piscataway, NJ). Resolved proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Enzyme Assay

Beta-amylase activity in soybean seed and leaf were assayed with a commercial kit Megazyme myl-3 (Megazyme International Ireland Ltd., Ireland). This assay is highly selective for β-amylase and uses the *p*-nitrophenyl-β-D-maltotrioside as the substrate. The assay was performed according to the manufacturer's recommendation.

Matrix-Assisted-Laser-Desorption-Time-of-Flight Mass Spectrometer Analysis of 56 Kilodalton Isopropanol-Soluble Protein

Isopropanol-extracted protein from Altona Sp_1^b dry seed powder was resolved by SDS-PAGE and briefly stained with Coomassie Brilliant Blue R-250. A small gel piece containing a prominent 56 kDa protein was excised with a razor blade, washed in distilled water and then destained in a 50% solution of acetonitrile (v/v) containing 25 mM ammonium bicarbonate. After a 100% acetonitrile wash, the protein contained in the acrylamide gel was subjected to digestion using 20 μL (10 μg/mL) of modified porcine trypsin in 25 mM ammonium bicarbonate (Promega, Madison, WI). Peptides resulting from tryptic digestion were analyzed using a Voyager DE-STR (Applied Biosystems, Framingham, MA) MALDI-TOF-MS. The peptides were co-crystallized with the matrix R-cyano-4-hydroxycinnamic acid. A 337 nm N laser operating at 20 Hz was used in sample ionization. Trypsin autolysis peaks of charge mass ratios 842.51 and 2211.10 served as internal standards.

Western Blot Analysis

Isopropanol-soluble soybean proteins were resolved by SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes. After transfer, the membranes were blocked with TBS (10 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% (w/v) nonfat dry milk and then incubated with polyclonal antiserum raised against purified *Arabidopsis* leaf β-amylase (a generous gift from Dr. Jonathan Monroe, James Madison University, Harrisonburg, VA) diluted 1:3000 in the blocking buffer (TBS containing 5% (w/v) nonfat dry milk). Affinity-purified goat (*Capra hircus*) anti-rabbit (*Oryctolagus cuniculus*) IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad) was used as the secondary antibody at a 1:3000 dilution in TBS-T (TBS with 0.05% (v/v) Tween-20) containing 5% nonfat dry milk. The membrane was washed four times in TBST for 10 min before and after incubation in secondary antibody. Immuno-reactive polypeptides were identified with an enhanced chemiluminescent substrate for detection of HRP (Pierce/ThermoFisher, Rockford, IL).

Genomic Southern Analysis

Genomic DNA from soybean leaves was isolated by the standard hexadecyltrimethyl-ammonium bromide (CTAB) method (Saghai Maroof et al., 1984). Ten micrograms of genomic DNA

were digested with *Xba*I and *Nde*I overnight at 37°C. The digested samples were fractionated on a 0.8% (w/v) agarose gel. After electrophoresis, the DNA was partially hydrolyzed (15 min depurination in 0.25 N HCl; 30 min denaturation in 0.4 M NaOH) before transfer to Hybond-N^+ membrane (Amersham Biosciences). After transfer, prehybridization of the filter proceeded overnight in hybridization buffer (10% (w/v) BSA, 500 mM Na_2HPO_4, 10 mM EDTA, 7% (w/v) SDS, 100 mg L^{-1} salmon sperm DNA, total volume of 20 mL) at 65°C. Hybridization was performed at 65°C for 24 h with [α-^{32}P] dCTP labeled β-amylase cDNA (Accession number X71419) which was reverse transcription-polymerase chain reaction (RT-PCR) amplified from developing seed ribonucleic acid (RNA) using primers β-amyl F 5-ATGGCCACTTCCGACAGTAACATG-3 and β-amyl R 5-TCAACCATCAACTT-TCATGTCTGTC-3. Following hybridization, the membrane was washed two times with 2x SSC, 1% (w/v) SDS, once with 1x SSC, 1% (w/v) SDS and finally two more times with 0.1x SSC, 1% (w/v) SDS. Each wash was performed for 10 min at 65°C. Hybridizing bands were detected by autoradiography, using a Cronex Lightening Plus (DuPont, Wilmington, DE, USA) intensifying screen for signal enhancement.

Ribonucleic Acid Preparation and Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was isolated from different organs (200–500 mg) using Trizol reagent (Invitrogen, Carlsbad, CA). For RT-PCR, 1 μg of total RNA was used as template and gene-specific primers were used for amplification. Gene-specific primer pairs are as follows: β-amylase-1 (Accession no. AB189842), 5′-ACCATTCTAGATATCAGAGAACTTT-GCAGC-3′ and 5′-TCAGCATTGCAGATTACAGTG-GCTGGATGC-3′; β-amylase-2 (Accession no. M92090) 5′-ACTTCAAACAAGCAGCAACTTC-TTCG-3′ and 5′-TATTAGCATAGCAGATTACGGTGGCTGC-3′; β-amylase-3 (Accession no. AJ871579) 5′-ATGGCTC-TAACACTTCGTTCTTCAACTTC-3′ and 5′-TTACA-CAAGAGCAGCCTCTTGTGTGTGC-3′; β-amylase-4 (Accession no. AJ871580), 5′-ATGGAGGTTTCGGTGATT-GGAAGCTCTCAAGC-3′ and 5′-TTAGGCTGCTTGC-ATGCTCACAGTTGAATCACG-3′; Any contaminating DNA from the template was digested by DNase treatment (Invitrogen) followed by reverse transcription and PCR using the One-Step RT-PCR kit (Qiagen, Valencia, CA). Oligonucleotides designed for a conserved region of 18S ribosomal RNA of 400 base pairs (bp) were used as controls.

Molecular Cloning of Beta-amylase

A soybean genomic library made from DNA of cultivar Williams 82 in the Lambda Fix II vector (Statagene, La Jolla, CA) was plated with *Escherichia coli* LE392 on several Petri plates (150 × 15 mm). About 150,000 recombinant plaques were transferred onto nylon Hybond-N^+ membranes (Amersham Biosciences) following the procedure of the manufacturer. A β-amylase cDNA (Accession no. X71419) was labeled with [α-^{32}P] dCTP and hybridized to the DNA on the membranes at 65°C for 24 h. Positive clones were detected on X-ray film. After four consecutive screening steps, three positive Lambda clones were identified by colony hybridization. DNA from the positive plaques was gel-purified and subcloned into plasmid vectors for subsequent sequencing.

RESULTS

Altona *sp* $_1$ Contains Low Levels of Beta-amylase Activity

Beta-amylase activity from dry seeds was determined using *p*-nitrophenyl-β-D-maltotrioside, a specific β-amylase substrate. Even though the β-amylase activity was readily detected from seed extracts of Altona (*Sp* $_1^b$) only trace amounts of activity was found in Altona (*sp* $_1$). β-amylase activity in Altona *sp* $_1$ was only about 1% of the wild-type (Fig. 1). To examine if similar differences are also present in other organs, we examined β-amylase activity in the leaves of these two soybean cultivars. Soluble proteins isolated from fully mature trifoliate leaves from nodes 5 and 6 from greenhouse grown plants were assayed for β-amylase activity. As in the case of seed, trifoliate leaves revealed substantial β-amylase activity. However, the percentage of difference in β-amylase activity between these two genotypes was less pronounced when compared to that of seed (Fig. 1). β-amylase activity in Altona *sp* $_1$ was about 27% of the wild type (Fig. 1).

An Abundant 56 Kilodalton Isopropanol-Soluble Protein is Absent in Altona *sp* $_1$

The total seed protein profile of Altona (*Sp* $_1^b$) and Altona (*sp* $_1$) were examined by SDS-PAGE. A comparison of the protein profiles revealed no obvious differences between these soybean cultivars (Fig. 2a). Both soybean genotypes accumulated similar amounts of β-conglycinin and glycinin, the two major seed storage proteins of soybean. A previous study has shown that soybean seed β-amylase is preferentially soluble in alcohol solutions (Ren et al., 1993). Therefore we extracted proteins soluble in 50% isopropanol from these two soybean genotypes. A comparison of the protein profiles showed that Altona (*sp* $_1$) was missing an abundant 56 kDa protein (Fig. 2b). This protein was surmised to be β-amylase after we performed a Western blot analysis using antibodies raised against *Arabidopsis* β-amylase. The *Arabidopsis* β-amylase antiserum showed strong cross-reactivity with the 56 kDa protein in Altona (*Sp* $_1^b$) but no cross-reacting proteins were detected from the seed extracts of *Altona sp* $_1$ (Fig. 2c). To ultimately confirm that indeed the protein missing from *sp* $_1$ was β-amylase, the protein band was excised from a 1-D gel, digested into peptide fragments and examined by MALDI-TOF-MS analysis. Employing the Mascot search engine, the empirically determined mass-to-charge ratios of the peptides were compared against known proteins in the National Center for Biotechnology Information nonredundant database. Seventeen out of 33 peptides matched with *Glycine max* β-amylase (MOWSE 192; 64:$p < 0.05$) (Table 1).

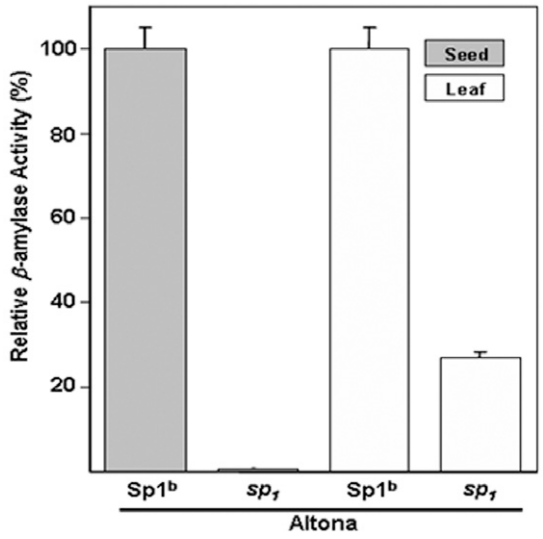

Figure 1. Beta-amylase activity in near-isogenic soybean lines. Proteins were extracted from seeds and fully expanded leaves of Altona *Sp* $_1^b$ and Altona *sp* $_1$ and the level of β-amylase activity was assayed using a commercial kit which uses *p*-nitrophenyl β-maltotrioside as the substrate. Beta-amylase activity from leaves (units/fresh weight) and seeds (units/dry weight) of the wild-type plant was considered to be 100%. Bars represent the standard error of the mean (n = 3). *Sp* $_1^b$, wild-type soybean; *sp* $_1$, β-amylase mutant.

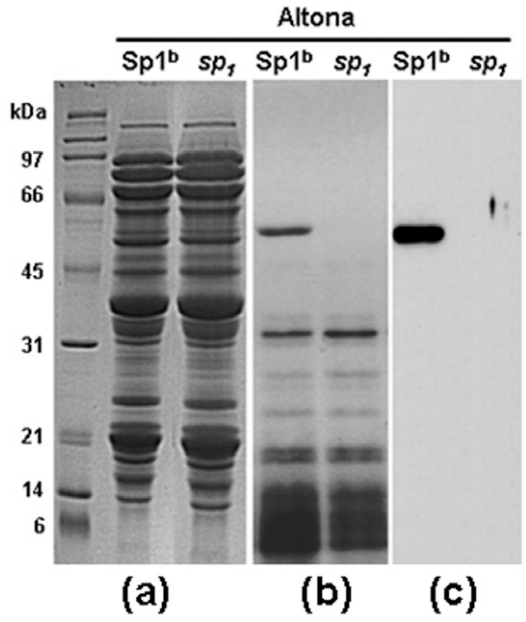

Figure 2. Total and 50%-isopropanol extracted protein profiles of near-isogenic soybean lines. Proteins were extracted from dry seeds and analyzed on 13.5% sodium dedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Panel a total proteins; Panel b and c, 50% iso-propoanol extracted proteins. Proteins in Panel a and b were visualized with Coomassie Blue R-250. Panel c, immunoblot analysis of 50% iso-propanol extracted proteins transferred to a nitrocellulose membrane and incubated with antiserum generated against *Arabidopsis* β-amylase. Immunoreactive proteins were detected by the horseradish peroxidase color development procedure. Molecular mass markers (in kDa) are shown to the left of the figure. *Sp* $_1^b$, wild-type soybean; *sp* $_1$, β-amylase mutant.

Multiple Beta-amylase Genes are Present in Soybean

A search of the soybean genome sequence database (http://www.phytozome.net/soybean) using BLAST program revealed the presence of nine β-amylase genes (Table 2). Two genes, GmBAM 2 and GmBAM 5, were located on chromosome 12 while the others were present on different chromosomes (Table 2). The BLAST analysis showed that GmBAM 1 and GmBam 2 had a high amino acid sequence similarity to *Arabidopsis* Bam 5, while soybean GmBAM 3, Gm BAM 6, GmBAM 7and Gm BAM 8 were related to *Arabidopsis* BAM3, a chloroplastic isoform of β-amylase (Table 2). To examine if soybean GmBAM 3, Gm BAM 6, GmBAM 7, and Gm BAM 8 were localized to the chloroplast, we examined for the presence of a chloroplast transit peptide using two computer programs (ChloroP and Target 1.1). Both these programs revealed the presence of a putative chloroplast transit peptide in GmBAM 3, Gm BAM 6, GmBAM 7, and Gm BAM 8. GmBAM 1 and GmBAM 2, which revealed >96% sequence similarity between them, do not contain the chloroplast transit peptide and presumably encode for cytosolic proteins.

Transcript Levels of Beta-amylase Gene Family

To analyze the transcript levels of some representative member of this gene family, we performed RT-PCR using total RNA isolated from fully mature trifoliate leaves and developing seeds at R6 stage (Fehr and Caviness, 1977). Gene-specific oligos for GmBAM 1, GmBAM 2, GmBAM 3, and GmBAM 4 was used to amplify the corresponding RNA transcripts. All the four β-amylase genes were expressed in both leaf and seed (Fig. 3). GmBAM 1 transcript was not detected in leaves and seeds of Altona (*sp* $_1$) (Fig. 3).

Southern Blot Analysis Reveals Structural Alterations in the Beta-amylase Gene in Altona *sp* $_1$

Since Altona *sp* $_1$ does not express the GmBAM 1 gene, we wanted to examine if any substantial deletion or insertion occurred in the Altona (*sp* $_1$) GmBAM 1 gene at the DNA level. When the genomic DNA was individually digested with *Xba*I or *Nde*I, the β-amylase probe hybridized with two fragments (Fig. 4a). Under stringent hybridization conditions, the β-amylase probe hybridized with 15 and 3.8 kB *Xba*I fragments and 4.0 and 2 kB *Nde*I DNA fragments in the case of Altona (*Sp* $_1^b$). The mutant shared one common DNA fragment in both the *Nde*I and *Xba*I digests, but the other fragments had different sizes when compared to the wild-type plant (Fig. 4a).

Table 1. Identification of the 56 kDa protein as β-amylase by matrix-assisted laser desorption ionization/time of flight mass spectroscopy.

Peptide†	Sequence	Observed [M+H]+	Calculated Mr
		Da	
P1	QYDWR (67– 71)	767.38	766.34
P2	SNFNIFK (430– 436)	869.48	868.44
P3	EQLLOLR (38– 44)	899.56	898.52
P4	VAGENALPR (378– 386)	926.54	925.50
P5	DGYRPIAR (324– 331)	947.54	946.50
P6	VSGIHWWYK (297– 305)	1175.63	1174.59
P7	LSMFGVTYLR (412– 421)	1186.67	1185.62
P8	GKFFLTWYSNK (260– 270)	1390.73	1389.71
P9	YNDVPESTGFFK (239– 250)	1403.69	1402.64
P10	AGHPEWELPDDAGK (225– 238)	1521.74	1520.69
P11	YNHAITPLKPSAPK (455– 468)	1536.90	1535.85
P12	MHADQDYCANPQK (442– 454)	1577.70	1576.64
P13	LLNHGDQILDEANK (271– 284)	1579.82	1578.80
P14	HHAILNFTCLEMR (336– 348)	1641.86	1640.79
P15	SGPQELVQQVLSGGWR (358– 373)	1740.96	1739.90
P16	EYLTVGVDNEPIFHGR (134– 149)	1846.96	1845.91
P17	VENHAAELTAGYYNLNDR (306– 323)	2051.04	2049.96

†The major peptides observed in the matrix-assisted-laser-desorption-time-of-flight (MALDI-TOF) mass spectral analysis of the in-gel digest of the 56 kDa protein were matched with *Glycine max* β-amylase (NCBInr GI|169913) with a statistically significant Mascot protein score of 192 (>64 required for $p < 0.05$).

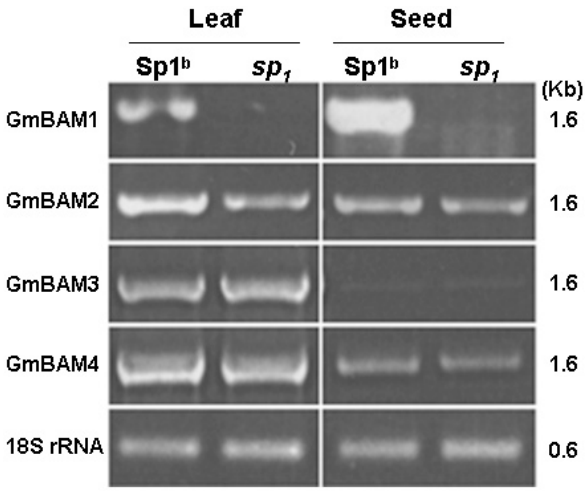

Figure 3. Transcript levels of β-amylase genes in soybean leaves and seeds. Total ribonucleic acid (RNA) (1 μg) was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for the four β-amylase genes (Materials and Methods). The 18S ribosomal mRNA was amplified as a control. The sizes of the PCR products in kb are shown to the right of the figure. The data represents three independent experiments showing similar results.

Molecular Cloning of Soybean Beta-amylase 1 Gene

To identify the molecular mechanism underlying the absence of β-amylase in the seeds of Altona *sp* $_1$ seeds, we first cloned and characterized the GmBAM 1 gene from the wild-type soybean. Accordingly, we screened a Williams 82 genomic library (Stratagene) with ^{32}P-labeled β-amylase cDNA (Accession no. X71419) at high stringency and obtained three lambda clones that revealed strong hybridization with the probe. Since all the three clones hybridized to a 3.8 kB *Xba*I fragment, one of the clones was chosen for further characterization. The DNA sequence of the 3.8 kB *Xba*I genomic fragment was determined. Analysis of the sequence revealed an open reading frame that was interrupted by six introns (Fig. 4b). The size of the smallest intron was 60 bp and the largest intron was 441 bp. The predicted protein has 496 amino acids with a theoretical molecular mass of 56 kDa. Since the 3.8 kB *Xba*I fragment lacked the promoter sequences, we sought to identify DNA fragments from the β-amylase lambda clones that contained those sequences. We performed restriction enzyme digestion of the DNA isolated from the β-amylase lambda clones and performed Southern blot analysis. Hybridization of the blot with the 527 bp *Eco*RV DNA fragment (Fig. 4b) resulted in identification of a larger DNA fragment. This fragment was subsequently cloned and its nucleotide sequence was also determined. The entire DNA sequence (4643 bp) of the GmBAM 1 was submitted to the GenBank database as accession no. DQ015707.

Second and Third Exon Regions of Beta-amylase 1 Gene are Missing in Altona *sp* $_1$

Since Southern blot analysis clearly indicated differences in the sizes of the hybridizing β-amylase DNA fragments in soybean isogenic lines, attempts were made to clone these

Table 2. Beta-amylases from soybean and their amino acid sequence similarity with *Arabidopsis* β-amylases.

Soybean†			*Arabidopsis*			
Gene	Chromosome no.	Gene ID	Gene	Chromosome no.	Gene ID	Similarity
						%
GmBAM 1	6	Gm06g45700	BAM 5	4	At4g15210	69.0
GmBAM 2	12	Gm12g11130	BAM 5	4	At4g15210	68.2
GmBAM 3	17	Gm17g16020	BAM 3	4	At4g17090	69.9
GmBAM 4	15	Gm15g10480	BAM 9	5	At5g18670	55.1
GmBAM 5	12	Gm12g32330	BAM 6	2	At2g32290	64.0
GmBAM 6	1	Gm01g41190	BAM 3	4	At4g17090	67.4
GmBAM 7	5	Gm05g05750	BAM 3	4	At4g17090	70.3
GmBAM 8	11	Gm11g04210	BAM 3	4	At4g17090	66.5
GmBAM 9	13	Gm13g28630	BAM 9	5	At5g18670	54.7

†Sequences of the β-amylase genes were obtained from soybean genome sequence database (http://www.phytozome.net/soybean).

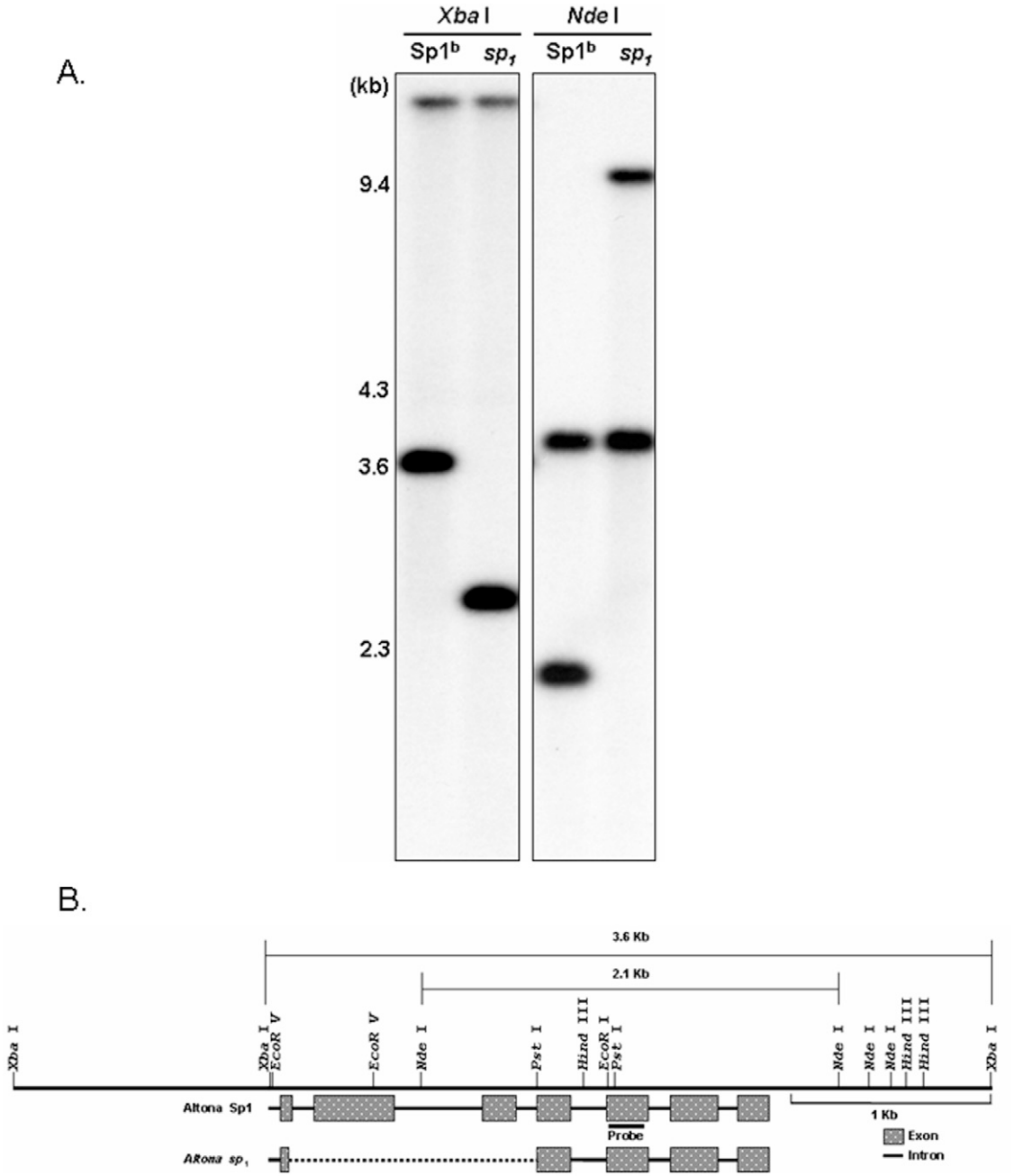

Figure 4. Organization of soybean β-amylase gene GmBAM 1. (a) Southern blot analysis of β-amylase genes. Eight micrograms of soybean genomic DNA was cleaved with restriction endonucleases *Xba*I or *Nde*I and resolved on a 0.8% agarose gel. The gel was blotted to a nitrocellulose membrane followed by hybridization with a [^{32}P]-labeled β-amylase. The positions and sizes of the Lambda-*Hind* III DNA markers (in kb) are shown to the left of the figure. *Sp* $_1$^{b}, wild-type soybean; *sp* $_1$, β-amylase mutant. (b) Restriction endonuclease map of genomic DNA and the schematic representation of soybean GmBAM 1. GmBAM 1from Altona *sp* $_1$ has a 1207 bp deletion near the 5' region that includes the II and III exon regions (represented by a dotted line). *Sp* $_1$^{b}, wild-type soybean; *sp* $_1$, β-amylase mutant.

fragments by PCR. When DNA from a wild-type plant was used as a template in a PCR reaction, a 2.6 kb DNA fragment was amplified. In contrast, using the same PCR primers (5′-ACCATTCTAGATATCAGAGAACTTT-GCAGC-3′ and 5′-TCAGCATTGCAGATTACAGT-GGCTGGATGC-3′), only a 1.4 kb DNA fragment was amplified from the β-amylase mutant. These fragments were cloned and their complete nucleotide sequences were determined. Schematic presentation of the GmBAM 1 genes from the wild-type and the mutant plants are shown in Fig. 4b. The nucleotide sequence of GMBAM 1 cDNA was used to determine the boundaries of the exons and introns in the genomic clone. The nucleotide sequence of the exon regions of the β-amylase gene was identical to the cDNA sequences. Our analysis indicated that the wild-type β-amylase gene is composed of seven exons interrupted by six introns. The β-amylase gene from Altona *sp* $_1$ revealed a 1207 bp deletion near the 5' region that included the second

and third exon regions (Fig. 4b). The DNA sequence of the GmBAM 1 from Altona *sp* $_1$ has been submitted to GenBank database as accession no. FJ610143.

Phylogenetic Analysis of Beta-amylases

In *Arabidopsis* nine putative genes encoding β-amylases (BAM 1–BAM 9) have been identified (Laby et al., 2001; Kaplan et al., 2006). Phylogenetic analysis has grouped them under four major subfamilies. BAM 5 and BAM 6 belongs to subfamily I, BAM I1 and BAM 3 to subfamily II, BAM 4 and BAM 9 to subfamily III and BAM 2, BAM 7 and BAM 8 genes to subfamily IV (Fulton et al., 2008). We have performed alignment of the amino acid sequence of soybean β-amylases with that of *Arabidopisis* β-amylases using Winstar/Megalign program. The overall sequence similarity was high among certain members while significant difference was observed between other members. GmBAM 1 and GmBAM 2 share 67 to 69% sequence similarity with *Arabidopsis* BAM 6 and BAM 5, respectively. However, the two other soybean β-amylases (GmBAM 3 and GmBAM 4), which contain plastid transit peptides share only limited amino acid identity (about 40 and 60%, respectively). Phylogenetic dendrogram analysis reveals that β-amylase gene described in this study is closely related to members of *Arabidopsis* β-amylase subfamily I which includes BAM 5 and BAM 6, GmBAM 3 was related to *Arabidopsis* BAM 3 and BAM 1 while GmBAM 4 was more closely related to *Arabidopsis* BAM 9.

DISCUSSION

To understand the molecular mechanism underlying the different levels of β-amylase activity in Altona (*Sp* $_1^b$) and Altona (*sp* $_1$), we isolated the genes (GmBAM 1) from these two soybean genotypes. The GmBAM 1 gene from the wild-type was composed of seven exons interrupted by six introns. The gene organization of soybean β-amylase is similar to that of barley (Erkkilä et al., 1998), *Arabidopsis* (Mita et al., 1995; Fulton et al., 2008), and sweet potato (Yoshida et al., 1992), and the alignment of the amino acid sequence of soybean β-amylase with other plant β-amylases reveals high homology and several conserved structural features, including the eight conserved motifs identified by Pujadas et al. (1996). However, the GmBAM 1 gene from Altona *sp* $_1$ genotype has a 1207 bp deletion near the 5' region that includes the second and third exon regions. Thus, the deletion observed in the β-amylase gene in Altona *sp* $_1$ genotype may be responsible for the low levels of β-amylase activity. However, Altona *sp* $_1$ still has small amounts of β-amylase activity when compared with the wild type. This could be explained based on the observation that there are nine β-amylase genes in soybean genome and the β-amylase activity detected in the seeds could be attributed to the products of other members of β-amylase gene family.

Extensive studies conducted on β-amylase have provided evidence for a critical role of this enzyme in the degradation of starch in plants (Beck and Ziegler, 1989). The role of this enzyme in the hydrolysis of starch reserves in germinating cereals has been extensively investigated (Ziegler, 1999) and a direct role for β-amylase in the degradation of transitory starch in potato leaves was demonstrated by antisense downregulation of chloroplast-targeted β-amylase (Scheidig et al., 2002). Two isoforms (BAM3 and BAM4) are necessary for normal starch degradation in *Arabidopsis* leaves (Fulton et al., 2008). Additionally it has been reported that chloroplastic forms of *Arabidopsis* β-amylases (BAM8 and BAM3) are needed for the protection of PSII photochemical efficiency following freezing stress (Kaplan et al., 2006). Additionally, β-amylase mutants have been used to examine the biological function of this enzyme in plants. Studies conducted with the *ram1* mutant of *Arabidopsis* suggest that β-amylase is not required for maintaining normal starch levels, rates of phloem exudation and overall plant growth (Laby et al., 2001). This is not surprising since RAM1 encodes BAM5, an isoform that is not located in the chloroplasts and thus unlikely involved in starch turnover in the leaf. Since GmBAM1 described in this study is closely related to *Arabidopsis* BAM5 it is possible that GmBAM1 may also not play a major role in starch metabolism.

Soybean seeds contain high levels of active β-amylase, yet its precise function in the soybean seed is unclear. The availability of genotypes lacking β-amylase in seeds has enabled researchers to examine the function of β-amylase (Hildebrand and Hymowitz, 1981). An examination of soybean genotypes with normal and very low β-amylase activity revealed no alteration on the total protein and oil composition of seeds, or any measurable effect on the starch and soluble sugar levels during both seed development and germination. Based on these observations it was concluded that β-amylase had no effect on starch metabolism in soybean (Hildebrand and Hymowitz, 1981). Similarly, no clear association between β-amylase activity and starch hydrolysis was seen in alfalfa, leading to the suggestion that this protein may serve a storage function (Gana et al., 1998).

Alternatively, soybean seed β-amylase may play a role in lipid mobilization during seed germination. Storage lipids in soybean seeds decrease rapidly by 3 d after germination (Wang et al., 1999). Interestingly, during this period of seed germination the β-amylase gene is downregulated (Gonzalez and Vodkin, 2007). An earlier report demonstrated that β-amylase can inhibit the activity of pancreatic lipase (Satouchi et al., 2002). Lipases present in oil bodies initiate the breakdown of triacylglycerol to free fatty acids and glycerol, and β-amylase may control the breakdown of lipids by inhibiting the activity of lipases. Further studies are required to ascertain the physiological role of this enzyme in lipid breakdown during soybean seed germination.

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