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Identification and characterization of granule bound starch synthase I (*GBSSI*) gene of tartary buckwheat (*Fagopyrum tataricum* Gaertn.)

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ABSTRACT

Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) is increasingly considered as an important functional food material because of its rich nutraceutical compounds. Reserve starch is the major component of tartary buckwheat seed. However, the gene sequences and the molecular mechanism of tartary buckwheat starch synthesis are unknown so far. In this study, the complete genomic sequence and full-size cDNA coding tartary buckwheat granule-bound starch synthase I (*FtGBSSI*), which is responsible for amylose synthesis, were isolated and analyzed. The genomic sequence of the *FtGBSSI* contained 3947 nucleotides and was composed of 14 exons and 13 introns. The cDNA coding sequence of *FtGBSSI* shared 63.3%–75.1% identities with those of dicots and 56.6%–57.5% identities with monocots (Poaceae). In deduced amino acid sequence of FtGBSSI, eight motifs conserved among plant starch synthases were identified. A cleavage at the site IVC↓G of FtGBSSI protein produces the chloroplast transit sequence of 78 amino acids and the mature protein of 527 amino acids. The FtGBSSI mature protein showed an identity of 73.4%–77.8% with dicot plants, and 67.6%–70.4% with monocot plants (Poaceae). The mature protein was composed of 20 α-helixes and 16 β-strands, and folds into two main domains, N- and C-terminal domains. The critical residues which are involved in ADP and sugar binding were predicted. These results will be useful to modulate starch composition of buckwheat kernels with the aim to produce novel improved varieties in future breeding programs.

1. Introduction

The genus *Fagopyrum* (buckwheat) belongs to the family of Polygonaceae. The demand for buckwheat is soaring for the production of functional food and in the pharmaceutical and cosmetic industries, thanks to its high nutritional value. Buckwheat is rich in essential amino acids, fiber, mineral compounds, vitamins and bioflavonoid such as rutin (Keli, 1991). Experiments with animal models have demonstrated that buckwheat flour might alleviate important diseases as diabetes, obesity, hypertension, and hypercholesterolemia (Christa and Soral-Śmietana, 2008).

In genus *Fagopyrum*, there are two cultivated species, common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.). Common buckwheat is popularized around the world for its better taste. However, tartary buckwheat is catching more attentions because it contains quercitin and more rutin than common buckwheat (Fabjan et al., 2003). Both compounds play an important protective role in the pathogenesis of multiple human diseases associated to oxidative stress such as cancer, coronary heart disease and atherosclerosis (Alía et al., 2006). Tartary buckwheat is classified as pseudo-cereal and cultivated mainly in southern China, northern India, Bhutan, and Nepal (Bonafaccia et al., 2003; Xuan and Tsuzuki, 2004). It can grow under low input conditions and be adapted to marginal lands with harsh environments (Bojka and Branka, 2002). The flour obtained from the milling of seeds of tartary buckwheat is high in starch content (around 70% Qin et al., 2010) and can be used for the production of many products, such as noodles, breads and cakes, similarly to bread wheat.

Starch consists of amylopectin and amylose. Amylopectin is a branched glucan polymer with α-1,6 glucosidic bonds that connect linear chains (Jeon et al., 2010), which is synthesized by the catalytic action of several enzymes, such as ADP glucose pyrophosphorylase (AGPase), starch synthases (SSs), starch branching enzymes (BEs), and starch debranching enzymes (DBEs). Differently, amylose is essentially linear

Abbreviations: ADP, Adenosine diphosphate; AGPase, ADP glucose pyrophosphorylase; AtGS, *Agrobacterium tumefaciens* glycogen synthase; BE, Branching enzyme; CDS, Coding sequence; DBE, Debranching enzyme; EcGS, *Escherichia coli* glycogen synthase; GP, Glycogen phosphorylase; GS, Glycogen synthase; GT, Glycosyltransferase; GBSSI, Granule-bound starch synthase I; MalP, Maltodextrin phosphorylase; NJ method, Neighbor joining method; ORF, Open reading frame; OsGBSSI, *Oryza sativa japonica* GBSSI; PCR, Polymerase chain reaction; Phyre2, Protein homology/analogy recognition engine version 2.0; RACE cloning, Rapid amplification of cDNA ends cloning; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SS, Starch synthase; FtGBSSI, Tartary buckwheat granule-bound starch synthase I; UTR, Untranslated region; UDP, Uridine diphosphate.

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and composed of 1,4-linked α-D-glucan chains (Jeon et al., 2010); AGPase and granule-bound starch synthase (GBSS) are involved in amylose production. GBSS is an enzyme that catalyzes the transfer of glucose from ADP-glucose to glucose-containing polysaccharides with α-1,4-linkages. Two GBSS isoforms were found so far, of which the GBSSI (also known as waxy protein) is mostly confined to storage tissues such as the seed endosperm (Dian et al., 2003; Smith et al., 1997; Vrinten and Nakamura, 2000). The expression of *GBSSI* gene is associated with amylose content and with technological and nutritional value of starch (Skrabanja et al., 2001; Kreft and Skrabanja, 2002). Since low or no amylose production leads to waxy endosperm, the *GBSSI* gene is also known as *waxy* gene. Because of the importance of *GBSSI* gene to influence starch quality, it has been characterized and studied in many crop plants, including cereal grain plants of the family Poaceae, such as rice (Sano, 1984), maize (Tsai, 1974), barley (Patron et al., 2002), bread and durum wheat (Fujita et al., 2001; Lafiandra et al., 2010; Miura et al., 1994; Nakamura et al., 1995) and foxtail millet (Fukunaga et al., 2002), tuber plants, such as potato (van der Leij et al., 1991), sweet potato (Kimura et al., 2000) and cassava (Salehuzzaman et al., 1993), and some important food crops and herb crops, e.g. peas (Edwards, 2002).

Although buckwheat as functional food is catching more focuses and the amylose content is one of most important component that controls its flour properties, the studies regarding buckwheat amylose have been limited to phenotypic character investigations. Though it has been reported that buckwheat amylose content in the endosperm shows a wide range of variation (Gupta et al., 2002; Mazza, 1993), this aspect has not been deepened. Molecular breeding projects are being performed to modulate amylose content with the aim to improve the starch cooking and nutritional characteristics in many crop species. However the gene information on *GBSSI* of tartary buckwheat is absent. Being *GBSSI* gene the main enzyme involved in amylose synthesis, its isolation and study in buckwheat is of fundamental importance for the improvement of starch quality and properties. In this study, we analyzed *GBSSI* gene from tartary buckwheat, which is more intensively grown and used in China than common buckwheat, thanks also to its superior nutraceutical composition.

2. Materials and methods

2.1. Plant material preparation

An accession of tartary buckwheat collected from Huidong in Sichuan province, Southwest China, was used. This accession was confirmed as an amylose containing (non-waxy) line in our previous experiment (data not shown). The mature seeds were grown in Xichang, Sichuan province, in early April. Following two months growth, at the grain filling stage, immature endosperms were collected for experimental use.

2.2. Detection of FtGBSSI protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Starch granules were purified from mature seeds and starch granule proteins were separated by SDS-PAGE following the method reported in Zhao and Sharp (1996) with some modifications (Mohammadkhani et al., 1999). Protein bands were visualized by silver staining.

2.3. Degenerate primer design

From the released *GBSSI* gene sequence data of seven species obtained from NCBI database (http://www.ncbi.nlm.nih.gov/), degenerate polymerase chain reaction (PCR) primers (DWX-F and DWX-R; Table 1) were designed. The primers were derived from conserved protein sequences ANDWHT and PSRFEP. The seven species included amaranth (*Amaranthus cruentus*; GenBank accession No. AB506113), Arabidopsis (*Arabidopsis thaliana*; GenBank accession No. AY149948.1), sweet potato (*Ipomoea batatas*; GenBank accession No. AB071604), cassava (*Manihot esculenta*; GenBank accession No. X74160), rice (*Oryza sativa*; GenBank accession No. X65183), pea (*Pisum sativum*; GenBank accession No. AJ345045), and potato (*Solanum tuberosum*; GenBank accession No. X58453).

Table 1
Primers used to amplify the sequences of *FtGBSSI* gene.

Primer name	Sequence (5′ to 3′)
DWX-F (forward)	TTGCYAAYGAYTGGCAYAC
DWX-R (reverse)	CANGGCTCAAAYCTRCTNGG
LSP	GATTTGAGGTAGCAGGGAACAAGAGCAGT
RSP	GTAGACACTGTGATGGAAGGCTATAC
F-1	TTGCCTTGCATGTGTTCATT
F-2	CTGTTCGGTTCTTTCACTGTT
F-3	CAAATGACTGGCACACTGCT
F-4	GGTTGGCTTACCGGTTGATA
F-5	CAGCTGATGTGATGGCTTTG
R-1	GTTCCCGCATTAGGTCCATA
R-2	GGTAGCAGGGAACAAGAGCA
R-3	TCAGAACCCTTCTGCTCCTC
R-4	AGATCCTGAGCCATGCAGTT
R-5	TCGAGATGCATCATGAAGAG

2.4. Total RNA extraction and first strand cDNA synthesis

Two or three immature endosperms were grinded for total RNA extraction. RNAprep Pure Plant Kit (TIANGEN, Beijing, China), specific for materials rich in polysaccharides and polyphenolics, was used. Extracted RNA was treated by DNase (TIANGEN, Beijing, China) to remove genomic DNA. RNA quality was surveyed by separation in 1.0% agarose gels, staining with ethidium bromide and observed by UV. The first strand cDNA was made from 5 μg total RNA using TIANScript RT Kit (TIANGEN, Beijing, China), and primed by Oligo$(dT)_{15}$.

2.5. PCR amplification by degenerate primers

PCR amplification by degenerate primers was done in a volume of 50 μL using rTaq™ (TaKaRa, Tokyo, Japan). Approximately 300 ng cDNA was included in the reaction mixture. The PCR conditions were: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, and the final extension at 72 °C for 5 min.

2.6. Rapid amplification of cDNA ends (RACE) cloning

The nucleotide sequences of the 5′ and 3′-ends of the *FtGBSSI* gene were amplified by the RACE method using SMARTe™ RACE cDNA Amplification Kit (BD-Clontech). 5′ and 3′ RACE PCRs were amplified with rTaq™ (TaKaRa, Tokyo, Japan) using the following gene-specific primers: LSP (5′-GATTTGAGGTAGCAGGGAACAAGAGCAGT-3′) and RSP (5′-GTAGACACTGTGATGGAAGGCTATAC-3′), respectively (Table 1). Conditions for PCR amplification were: 30 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min. PCR fragments were subcloned into pGEM-T vector and sequenced. Based on the information obtained from the sequences of the 5′- and 3′-ends, a series of gene-specific primers were designed to amplify the full-length cDNA. The reaction conditions were as follows: 5 min denaturation at 94 °C, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 7 min.

2.7. Genomic DNA exaction and full FtGBSSI gene sequencing

Genomic DNA extracted from seven-day shoots germinated from mature seeds. Extraction was performed using DNAsecure Plant Kit (TIANGEN, Beijing, China). *FtGBSSI* gene was amplified using gene-specific primers (Table 1; Fig. 3) and then sequenced. The reaction conditions were as follows: 5 min denaturation at 94 °C, followed by 30