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Genetic diversity and expression analysis of granule bound starch synthase I gene in the new world grain amaranth (*Amaranthus cruentus* L.)

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ABSTRACT

We investigated the expression patterns of a *granule bound starch synthase I* (*GBSSI* = *Waxy*) gene at different developmental stages of storage and non-storage organs in *Amaranthus cruentus*. *GBSSI* transcripts were strongly expressed in the middle and mid-late stages of seed development and thereafter expression decreased. In addition, this gene was expressed in all non-storage organs tested (the leaf, stem, petiole and root) and showed a tendency to increase during plant development. Therefore, our results indicate that the amaranth *GBSSI* gene exhibits late expression in the perisperm, and that it is expressed in both storage and non-storage tissues. We also investigated the genetic diversity of *GBSSI* among 37 strains of amaranth grains originating from New World. A comparison of the *GBSSI* coding sequence revealed an extremely high level of sequence conservation, and a single nucleotide polymorphism between the sequences of non-waxy (Type I) and waxy (Type II) phenotypes was detected. This indicates that a G–T polymorphism in exon 10 (a nonsense mutation) was a unique event in the evolution of the *GBSSI* gene in amaranth grains.

1. Introduction

The genus *Amaranthus* includes about 60 species distributed in many areas of the world (Saunders and Becker, 1984). Species originating in the New World were cultivated as an ancient grain crop 5000–7000 years ago (Sauer, 1967). Ancient amaranth grains still used to this day include *Amaranthus caudatus*, *Amaranthus cruentus*, and *Amaranthus hypochondriacus*. Their cultivation is expanding in Central and South America, Africa, and some parts of Asia (Saunders and Becker, 1984). In Japan, amaranth grain was first introduced in the 1980s from Rodale Research Institute, United States, and a new amaranth grain cultivar, 'New Aztec', was developed in 2001 using 'Mexico line', which is a high-yielding and semi-dwarf line selected in Japan from *A. cruentus* genetic resources introduced from Mexico (Katsuta et al., 2001). Currently, this new grain, mostly *A. cruentus*, is cultivated in some areas of Japan, such as Iwate, Akita, and Nagano prefectures.

In recent years, amaranth has gained attention as a food and fodder crop with a high seed protein content, balanced amino acid composition, and high lysine content (Zheleznov et al., 1997). A seed of the amaranth grain contains 50–60% starch, which is of two types with differing properties responsible for non-waxy and waxy phenotypes (Okuno and Sakaguchi, 1981; Saunders and Becker, 1984). The amylose content of the amaranth grain starch is in the range of 7–14% for the non-waxy phenotype and 0% for the waxy phenotype (Okuno and Sakaguchi, 1981; Sugimoto et al., 1981; Konishi et al., 1985).

Granule bound starch synthase I (*GBSSI*), also known as the waxy protein, is responsible for amylose synthesis in the perisperm starch of amaranth grains, similar to the endosperm starch of some cereal species (Okuno and Sakaguchi, 1981; Konishi et al., 1985; Park et al., 2009). *GBSSI* is encoded at the *Waxy* loci in cereals. We previously sequenced and cloned *GBSSI* gene of *A. cruentus* (Park et al., 2009). This gene product contains an open reading frame (ORF) of 1821 bp corresponding to a polypeptide of 606 amino acid

Abbreviations: AS-PCR, allele-specific polymerase chain reaction; CTAB, cetyl trimethyl ammonium bromide; DAF, days after fertilization; *GBSSI*, granule bound starch synthase I; I2/KI, iodine solution; ORF, open reading frame; qRT-PCR, quantitative real-time PCR; RT-PCR, semi quantitative RT-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism.

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residues, and consists of 13 exons interrupted by 12 introns. *GBSSI* mutations essentially eliminate or reduce the amylose content of the starch through disrupted expression or decreased functioning of the *GBSSI* gene. Naturally occurring *GBSSI* mutants have been identified in cereals, and the mechanisms have been well characterized in rice, maize, wheat, barley, and foxtail millet. In maize and foxtail millet, many spontaneous *GBSSI* mutations caused by transposable elements have arisen (Fedoroff et al., 1983; Wessler and Varagona, 1985; Fukunaga et al., 2002). In rice, nucleotide substitutions resulting in aberrant splicing at the 5′-end of intron 1 occur (Wang et al., 1995; Cai et al., 1998; Hirano et al., 1998). In wheat and barley, there are major deletions in the *GBSSI* loci (Vrinten et al., 1999; Domon et al., 2002). Recently, we characterized the waxy phenotype of three amaranth grain species (Park et al., 2010). The *GBSSI* mutation comprised a one-base insertion and one-base substitution, which created an internal termination codon in the three *GBSSI* genes, suggesting the involvement of a nonsense mutation in *A. cruentus* and *A. hypochondriacus* or a frameshift mutation in *A. caudatus*. Although the role and mutations of *GBSSI* in the amaranth perisperm has been well studied, the expression pattern of this gene in storage and non-storage organs of amaranth remains unclear.

The amylose content of the seed and plant tissues in amaranth is thought to play important roles in palatability and starch quality as in cereals. Therefore, an understanding of the expression patterns of *GBSSI* is important for starch processing and the edible quality of agronomic crops. In addition, for efficient utilization of genetic resources and to develop strains with diverse starch composition, a detailed analysis of the genetic diversity of amaranth grains from a variety of regions is needed.

In this study, we analyze the expression patterns of *GBSSI* at different developmental stages of the seed and in different tissues using quantitative real-time PCR (qRT-PCR) analysis. In addition, we investigate genetic diversity in the *GBSSI* gene using accessions of *A. cruentus* collected from the New World, and discuss the evolution of this gene. Finally, this information is used to develop an allele-specific PCR (AS-PCR) marker for genetic differentiation of the non-waxy and waxy phenotypes in amaranth grains.

2. Materials and methods

2.1. Plant material

For expression analysis, plants of *A. cruentus* (accession no. Ames 22004; non-waxy phenotype) were grown in a glasshouse. The grains were harvested several times during crop maturation and divided into five developmental stages, namely initial (<0.0002 g), early (0.0003–0.0005 g), middle (0.0005–0.0007 g), mid-late (0.0007–0.0009 g), and late (0.001–0.0015 g), based on their external morphology, fresh weight and size (Electronic Supplementary Material [ESM] Fig. 1). Leaf, petiole, stem and root samples were taken from seedlings at the four- and six-leaf stages.

For the analysis of genetic diversity, 37 strains of *A. cruentus* were used (Table 1). All accessions were obtained from the collection in the USDA-ARS, National Genetic Resources Program. This collection originated in the New World, including Guatemala, the USA, and Mexico. The plants were grown in a glasshouse for 90 days and the grains were collected several times during maturation. The waxy and non-waxy phenotypes were distinguished by staining with iodine solution (I_2/KI); the grains of non-waxy phenotypes stained blue, whereas waxy phenotypes stained reddish brown (ESM Fig. 2). The phenotype was also determined by observation of the perisperm's appearance, which was either translucent (non-waxy) or opaque (waxy). The strains analyzed comprised 23 non-waxy and 14 waxy phenotypes (Table 1). Leaf tissues from two-week-old seedlings were harvested for genomic DNA isolation. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. Microscopic observations

Developing seeds of *A. cruentus* Ames 22004 were hand-sectioned (sections nominally 100 μm thick) using a Microslicer (DTK-1000, Dosaka, Japan). Cross-sections were stained with an iodine solution (0.1 g resublimated iodine and 0.2 g KI dissolved in 30 mL distilled water) and observed under a light microscope (Olympus BX51) or a dissection microscope (Olympus SZ).

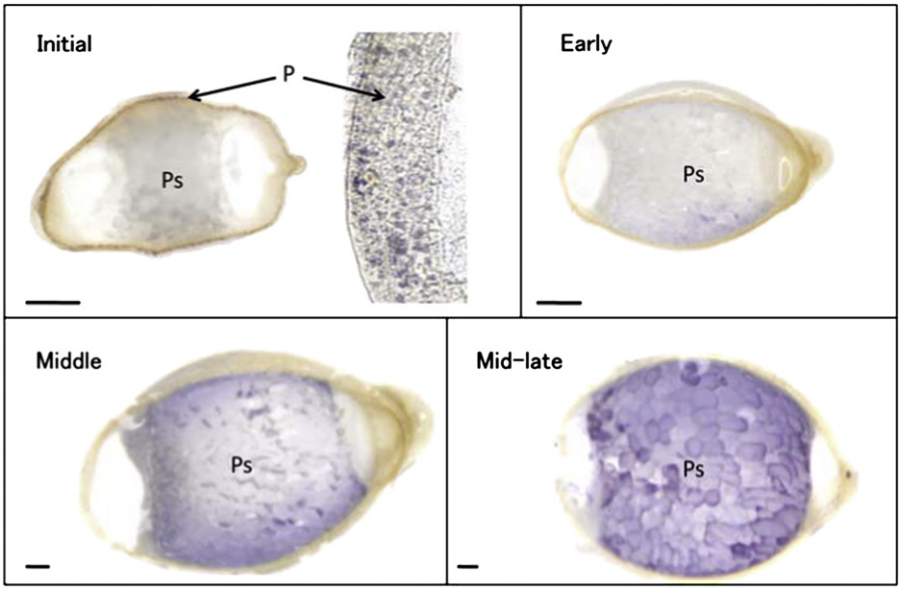

Fig. 1. Starch granules in cross-section of developing seed (initiation, early, middle, and mid-late) as shown by I/KI staining. *P*, pericarp; *Ps*, perisperm. Bars = 0.1 mm.