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Characterization of shrunken endosperm mutants in barley

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ABSTRACT

Despite numerous studies on shrunken endosperm mutants caused by either maternal tissues (*seg*) or kernel per se (*sex*) in barley, the molecular mechanism for all of the eight *seg* mutants (*seg*1–*seg*8) and some *sex* mutants is yet to be uncovered. In this study, we determined the amylose content, characterized granule-binding proteins, analyzed the expression of key genes involved in starch synthesis, and examined starch granule structure of both normal (Bowman and Morex) and shrunken endosperm (*seg*1, *seg*3, *seg*4a, *seg*4b, *seg*5, *seg*6, *seg*7, and *sex*1) barley accessions. Our results showed that amylose contents of shrunken endosperm mutants ranged from 8.9% (*seg*4a) to 25.8% (*seg*1). SDS-PAGE analysis revealed that 87 kDa proteins corresponding to the starch branching enzyme II (SBEII) and starch synthase II (SSII) were not present in *seg*1, *seg*3, *seg*6, and *seg*7 mutants. Real-time quantitative PCR (RT-qPCR) analysis indicated that *waxy* expression levels of *seg*1, *seg*3, *seg*6, and *seg*7 mutants decreased in varying degrees to lower levels until 27 days after anthesis (DAA) after reaching the peak at 15–21 DAA, which differed from the pattern of normal barley accessions. Further characterization of *waxy* alleles revealed 7 non-synonymous single nucleotide polymorphisms (SNPs) in the coding sequences and 16 SNPs and 8 indels in the promoter sequences of the mutants. Results from starch granule by scanning electron microscopy (SEM) indicated that, in comparison with normal barley accessions, *seg*4a, *seg*4b, and *sex*1 had fewer starch granules per grain; *seg*3 and *seg*6 had less small B-type granules; some large A-type granules in *seg*7 had a hollow surface. These results improve our understanding about effects of *seg* and *sex* mutants on starch biosynthesis and granule structure during endosperm development and provide information for identification of key genes responsible for these shrunken endosperm mutants.

1. Introduction

Shrunken endosperm in barley (*Hordeum vulgare* L., 2n = 14, HH) can be caused by mutations of genes expressed in either maternal tissues [*seg* (shrunken endosperm genetic), i.e. independent of the pollen source] (Jarvi and Eslick, 1975; Ramage and Crandall, 1981; Ramage and Scheuring, 1976) or in the kernel itself [*sex* (shrunken endosperm xenia), i.e. expressing xenia] (Eslick and Hockett, 1976). Eight *seg* mutants (*seg*1–*seg*8) that were not allelic to each other have been reported (Eslick and Hockett, 1976; Jarvi and Eslick, 1975; Ramage and Crandall, 1981; Röder et al., 2006). Anatomical studies revealed that due to the necrosis and crushing of the chalaza and nucellar projection of the pericarp during the grain-filling period, four mutants (*seg*l, *seg*3, *seg*6 and *seg*7) showed premature termination of grain filling, leading to thin and wrinkled seed (Felker et al., 1985). The other four mutants (*seg*2, *seg*4, *seg*6, and *seg*8) were characterized with abnormal endosperm growth but normal development of maternal-origin tissues (Felker et al., 1985). Although the genes responsible for the *seg* mutants have yet been identified, there have been numerous studies reports on maternal effects on endosperm development in *seg* mutants (Djarot and Peterson, 1991; Felker et al., 1983, 1984a,b, 1985). A recent report suggested that reduction of abscisic acid contents contributed to the abnormal endosperm development in *seg*8 (Sreenivasulu et al., 2010). Compared with *seg* mutants, more *sex* mutants have been identified and attracted more attention (Bosnes et al., 1987; Morell et al., 2003; Schulman et al., 1995).

As the main constituent of barley grains (60–64% of kernel dry weight), starch is the most abundant storage reserve carbohydrate and can be used as our nutrition and as a feedstock for bioethanol production in industry (Van Hung et al., 2006; Zeeman et al., 2010). Barley starch contains amylose and amylopectin in a ratio ranging from 20–

Abbreviations: ABA, abscisic acid; AGPase, adenosine 5′ diphosphate glucose pyrophosphorylase; CTAB, hexadecyltrimethylammonium bromide; DAA, days after anthesis; DWC, dual-wavelength colorimetric; GAPDH, glyceraldehyde-phosphate dehydrogenase; GBSS I, granule-bound starch synthase I; NCBI, National Center for Biotechnology Information; RT-qPCR, real-time quantitative polymerase chain reaction; SBEII, starch branching enzyme II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SD, standard deviation; *seg*, shrunken endosperm genetic; *sex* or *shx*, shrunken endosperm xenia; SEM, scanning electron microscopy; SNPs, single nucleotide polymorphisms; SSII, starch synthase II.

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30%/70–80% (Zeeman et al., 2010). Amylopectin biosynthesis in the cereal endosperm involves a set of enzymes, starch synthase (EC2.4.1.21), branching enzyme (EC 2.4.1.18), and starch debranching enzymes (EC 3.2.1.41 and 3.2.1.68) (James et al., 2003). However, the 60-kDa granule-bound starch synthase I (GBSS I, or waxy protein, EC2.4.1.242) encoded by the *waxy* gene is the only enzyme essential for amylose synthesis (Denyer et al., 2001).

Despite numerous reports on physicochemical characteristics of *seg* and *sex* mutants during endosperm development, little is known about effects of *seg* and *sex* mutants on starch biosynthesis and structure. Aiming at revealing the diversity of these mutants and providing basic data for further studying their genetically mechanism, we assessed amylose, characterized granule-binding proteins, analyzed expression of key genes involved in starch synthesis, and examined starch granule structure in *seg* and *sex* mutants.

2. Materials and methods

2.1. Plant materials

Eleven barley accessions were used in this study. They included eight shrunken endosperm mutants, two with normal endosperm, and one with waxy endosperm (Table 1). *seg*4a and *seg*4b with different accession numbers belong to the same *seg* mutant, *seg*4. The accessions with GSHO, CIho and PI numbers were kindly provided by USDA-ARS (http://www.ars-grin.gov). NA40 (CDC Candle) was from Canada and deposited at Triticeae Research Institute, Sichuan Agricultural University, China. Barley seeds were surface-sterilized and germinated on wet filter paper for 2 days. Seedlings were then transferred to sterilized compost in individual pots and grown at 24 °C day and 20 °C night with a photoperiod of 16/8 h (light/dark) for 7 days, vernalized at 4 °C for 7 days in the dark, and then transferred again to the greenhouse at the same conditions. Anthesis was considered to have occurred when the awn emerged. Spikes were harvested at 3-day intervals from 3 days after anthesis (DAA) until 27 DAA. Harvestings were conducted between 2 and 4 pm local time. Grains from the harvested spikes were removed, dehulled, frozen in liquid nitrogen, and then stored at −80 °C until RNA extraction.

2.2. Measurement of amylose

Mature grains were cut into halves and stained in 0.1% (w/v) iodine solution (1.0 g of potassium iodide and 0.1 g of iodine diluted to 100 mL with distilled water). Amylose content of grain samples was measured by the dual-wavelength colorimetric (DWC) method (Zhu et al., 2008).

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Granule-binding proteins were extracted from individual seeds of barley accessions using the procedure described by Echt and Schwartz (1981). Extracted proteins were separated by SDS-PAGE as described by Laemmli (1970) and stained with Coomassie Blue G-250.

2.4. Isolation of waxy genes

Genomic DNA was extracted from 20-day old seedlings using the hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980), and cDNA was also prepared for amplifying open reading frame. The primers for cloning the barley *waxy* genes and their promoter regions were listed in Supplementary Table 1. PCR amplification, cloning, and sequencing ware performed as described by Ma et al. (2013b).

2.5. Real-time PCR quantification

Expression levels of *waxy* and another six genes involved in starch synthesis were determined. One of those six genes is *HvCWIN1* and it encodes cell wall-bound invertase 1 (HvCWINV1) which is the primary cell wall-bound invertase responsible for providing fast dividing endosperm cells with hexoses associated with starch synthesis in barley caryopses (Weschke et al., 2003). Three of genes were *HvAGPS1a*, *HvAGPS1b* and *HvAGPS2*, and the other two were *HvAGPL1* and *HvAGPL2*; and they encode small (*S1a*, *S1b*, and *S2*) and large (*L1* and *L2*) subunits of adenosine 5′ diphosphate glucose pyrophosphorylase (AGPase), respectively. Primers used for real-time quantitative PCR (RT-qPCR) analyses were listed in Supplementary Table 1. Actin (Jiang et al., 2011), adenosine triphosphatase (ATPase, EC 3.6.1.3) (Boscari et al., 2009) and glyceraldehyde-phosphate dehydrogenase (GAPDH) (Ma et al., 2013a) were used as internal controls in these analyses. RNA extraction, cDNA synthesis, and expression analyses were carried out as described by Jiang et al. (2011).

2.6. Scanning electron microscopy (SEM) analysis

The morphology of starch granules was examined by scanning electron microscopy. Samples were transversely cut with a knife and mounted on circular aluminum stubs with double-sided sticky tape. They were examined and photographed by FEI Quanta 450 (FEI Company, Hillsboro, OR, USA).

2.7. Data analysis

Triplicate measurements were taken in determining hundred grain weight and amylose content, and the results were given as the mean ± standard deviation (SD). NCBI Blast tools were used to search target sequences from available public database (http://blast.ncbi.nlm.nih.gov). Multiple alignments were carried out using CLC Main Workbench 6.7.2 for promoter sequences and Clustal W in MEGA 5 for coding sequences (Tamura et al., 2011). RT-qPCR data were normalized by geo-metric averaging of internal control genes mentioned

Table 1
The barley accessions and their characteristics.

Name	Accession	Details	2-/6-rowed	Hulled/ hull-less	Waxy/normal	Origin or developed region	Pedigree[a]
*seg*1	GSHO 1852	Shrunken endosperm 1	2	H	N	North Dakota, United States	BGS377s *seg*1/7* BOWMAN
*seg*3	GSHO 1957	Shrunken endosperm 3	2	H	N	North Dakota, United States	BOWMAN *7/BGS379 *seg*3
*seg*4a	GSHO 1853	Shrunken endosperm 4	2	H	N	North Dakota, United States	BOWMAN *5/BGS380 *seg*4
*seg*4b	GSHO 753	Shrunken endosperm 4	2	H	N	Montana, United States	A spontaneous mutant in Compana (PI 539111)
*seg*5	GSHO 754	Shrunken endosperm 5	6	H	N	Montana, United States	A spontaneous mutant in Sermo/7* Glacier line
*seg*6	GSHO 1975	Shrunken endosperm 6	2	H	N	North Dakota, United States	*seg*6.g INGRID/3* BOWMAN
*seg*7	GSHO 2468	Shrunken endosperm 7	2	H	N	Arizona, United States	A spontaneous mutant in Ingrid (CIho 10083)
*sex*1	GSHO 2474	Shrunken endosperm xenia 1	2	H	N	North Dakota, United States	A spontaneous mutant in Carlsberg II (CIho 10114)
Morex	CIho 15773	Normal endosperm	6	H	N	Minnesota,United States	Cree/Bonanza
Bowman	PI 483237	Normal endosperm	2	H	N	North Dakota, United States	Klages//Fergus/Nordic/3/ND1156/4/Hector
NA40	NA40	CDC Candle, waxy endosperm	2	H-L	W	Canada	

[a] Pedigree information is from USDA-ARS (http://www.ars-grin.gov/).