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Creation of a high-amylose durum wheat through mutagenesis of starch synthase II (*SSIIa*)

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ABSTRACT

In cereal seeds, mutations in one or more starch synthases lead to decreased amylopectin and increased amylose content. Here, the impact of *starch synthase IIa* (*SSIIa* or SGP-1) mutations upon durum starch was investigated. A screen of durum accessions identified two lines lacking SGP-A1, the A genome copy of SGP-1. The two lines were determined to carry a 29 bp deletion in the first exon of *SSIIa*. The SGP-A1 nulls were crossed with the durum variety 'Mountrail' and F_5 derived SGP-A1 null progeny lines were treated with EMS. From each EMS population, one SGP-B1 null mutation was recovered with each being a missense mutation. Each of the SGP-1 nulls was found to have large increases in amylose content and reduced binding of SGP-2 and SGP-3 to the interior of starch granules. RNA-Seq was used to examine the impact the loss of SGP-1 has upon other starch biosynthetic genes. Significant increases in transcript levels of several starch biosynthetic genes were observed in SGP-1 nulls relative to Mountrail. The resultant high amylose durums may prove useful in the creation of value added pasta with increased firmness and reduced glycemic index.

1. Introduction

In bread wheat (*Triticum aestivum* L.), amylose accounts for approximately 25% of starch with amylopectin accounting for the other 75% (reviewed in Tetlow, 2006). The "waxy" proteins (granule bound starch synthase I) encoded by the *Wx* genes are solely responsible for amylose synthesis after the production of ADP-glucose by ADP-glucose pyrophosphorylase (AGPase) (reviewed in Tetlow, 2006). In contrast, amylopectin synthesis involves a host of enzymes such as starch synthases (SS) I, II, III, IV, starch branching enzymes (SBE) I and II, and starch de-branching enzymes (reviewed in Tetlow, 2006).

Several starch biosynthetic proteins remain bound to the interior of starch granules with a subset of these proteins designated the starch granule proteins (SGPs). Using SDS-PAGE, Yamamori and Endo (1996) separated the SGPs from bread wheat starch into SGP-1, SGP-2, SGP-3 and WX. The SGP-1 fraction was further resolved into SGP-A1, SGP-B1, and SGP-D1 and the associated genes localized to the homoeologous group 7 chromosomes (Yamamori and Endo, 1996). SGP-1 proteins are isoforms of SSII encoded by the genes *SSIIa-A*, *SSIIa-B*, *SSIIa-D* on the short arms of group 7 chromosomes (Li et al., 1999).

A survey of hexaploid wheat germplasm identified lines lacking SGP-A1, SGP-B1, or SGP-D1 (Yamamori and Endo, 1996) which were crossed to create an SGP-1 null (Yamamori et al., 2000). The SGP-1 null had a 29% increase in amylose content (37.3% null vs. 28.9% wild-type), deformed starch granules, reduced starch content, and reduced binding of SGP-2 and SGP-3 to starch granules. These SGP-1 mutations were later shown to reduce starch binding without impacting SGP protein expression levels (Kosar-Hashemi et al., 2007). Lafiandra et al. (2010) reported that SGP-1 null lines created from crosses between the durum (*Triticum turgidum ssp. durum*) cultivar 'Svevo' and hexaploid SGP-A1/B1 null lines (Yamamori and Endo, 1996) had an 89% increase in amylose content compared to Svevo (43.6% SGP-1 null vs 23% wild-type) as well as reduced binding of SGP-2 and SGP-3. Elimination of *SbeIIa* in durum through RNA interference also resulted in increased amylose ranging from +29% to +200% (24% wild-type vs. 31–75% *SbeIIa* RNAi lines) (Sestili et al., 2010b). The very high amylose results observed by Sestili et al. (2010b) may not be due solely to *SbeIIa* expression reduction since *SbeIIa* mutants have amylose level increases similar to those of *SSIIa* mutations (28% *sbeIIa* versus 23% wild-type) (Hazard et al., 2012).

Abbreviations: FSP, flour swelling power; SGP, starch granule protein.

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To date a detailed expression profile of starch synthesis genes in an SGP-1 null background has not been reported. RNA-Seq is an emerging method that allows for gene expression analysis at the transcript level and, compared to other methods, has a greater sequencing sensitivity, a large dynamic range, and the ability to distinguish between differing alleles or isoforms of an expressed gene (Marioni et al., 2008). RNA-Seq is therefore an ideal method to determine the effect a null SGP-1 genotype has on expression of other starch synthesis genes.

Cereals with high amylose content are desirable because they have more resistant starch. Resistant starch is starch that resists break down slowly and thus acts more like dietary fiber while promoting microbial fermentation (reviewed in Nugent, 2005). The SGP-1 null alleles (Yamamori and Endo, 1996) were introgressed into the Italian bread cultivar 'N11' and whole grain was prepared into bread which was found to have increased resistant starch and decreased glycemic index relative to standard whole wheat breads (Hallstrom et al., 2011). Similarly, products prepared from *ssIIa* barley have been shown to have a reduced glycemic index and improve bowel health indices (Bird et al., 2008; King et al., 2008). Rats fed *SbeIIa* RNAi seeds with an amylose content of ~80% also showed significant improvements in bowel health indices (Regina et al., 2006).

In addition to the positive impact of increased amylose upon glycemic index, higher amylose might enhance durum product quality. Pasta that is firmer when cooked is often preferred as it resists overcooking and high amylose should increase pasta firmness. Our goal here was to develop a high-amylose durum line through the creation of *SSIIa* mutations and to examine the effect an SGP-1 null genotype has on the expression of other starch biosynthetic genes.

2. Materials and methods

2.1. Creation and screening of a mutagenized durum wheat population

Durum wheat accessions obtained from the USDA National Small Grains Collection (NSGC, Aberdeen, ID) and the International Center for Agricultural Research in the Dry Areas (ICARDA) were screened for SGP-1 mutations using SDS-PAGE of SGPs (see below). From the 200 NSGC *Triticum durum* core collection accessions screened, one line, PI 330546, lacked SGP-A1 and none lacked SGP-B1. From the 55 ICARDA *T. durum* accessions screened, one line, IG 86304, lacked SGP-A1 and none lacked SGP-B1. These two lines were crossed independently with the cultivar "Mountrail" (PVP 9900266) (Elias and Miller, 2000) and progeny lines advanced via single seed descent to the F_5 generation. Seed from lines homozygous for the SGP-A1 null trait that had seed and plant characteristics similar to Mountrail from each cross were then treated with ethyl methanesulfonate (EMS) as described in Feiz et al. (2009) with the exception that 0.5% EMS was used and plants were advanced two generations in the greenhouse to the M_1:M_2 generation. Seed from 294 Mountrail/PI 330546 M_1 lines and 196 Mountrail/IG 86304 M_1 lines were pre-screened for potential *SSIIa-B* mutations using the flour swelling power (FSP) test reported by Martin et al. (2008) who found that increased amylose content is associated with decreased FSP.

2.2. Starch extraction

For each selected low flour swelling power (FSP) genotype and parental controls, four seeds were ground in a Braun coffee mill (Proctor Gamble, Cincinnati, OH) for 10 s and then placed in a 2 ml microcentrifuge tube along with two 6.5 mm zirconia balls and agitated for 30 s in a Mini-beadbeater-96. The zirconia balls were removed from the microcentrifuge tubes and 1.0 ml of 0.1 M NaCl was added to the whole grain flour and samples were incubated for 30 min at room temperature. After 30 min, a dough ball was made by mixing the wet flour using a plastic Kontes Pellet Pestle (Kimble Chase, Vineland, NJ) and the gluten ball was removed from the samples after pressing out the starch. The liquid starch suspension was then transferred to a new pre-weighed 2.0 ml tube and 0.5 ml ddH_20 was added to the remnant starch pellet in the first tube. The first tube was vortexed, left to settle for 1 min and the liquid starch suspension transferred to the second tube. The starch suspension containing tubes were centrifuged at 5000 g and the liquid was aspirated off. Next, 0.5 ml of SDS extraction buffer (55 mM Tris-Cl pH 6.8, 2.3% SDS, 5% BME, 10% glycerol) was added, the samples were vortexed till suspended, and then centrifuged at 5000 g. The SDS buffer was aspirated off and the SDS buffer extraction was repeated once more. Then, 0.5 ml of 80% (w/v) CsCl was added to the starch pellets, samples were vortexed till suspended, and centrifuged at 7500 g. The CsCl solution was aspirated off and the starch pellets were washed twice with 0.5 ml ddH_20, and once in acetone with centrifugation speeds of 10,000 g. After supernatant aspiration, the starch pellets were left to dry overnight in a fume hood.

2.3. SDS-PAGE of starch granule proteins

To purified starch, 7.5 μl of SDS loading buffer (SDS extraction buffer plus bromophenol blue) was added per mg of starch. Samples were heated for 15 min at 70 °C, vortexed, centrifuged for 1 min at 10,000 g, and then 40 μl of sample was loaded in a 203 × 203 × 0.75 mm 10% (w/v) acrylamide gel prepared using a 30% acrylamide/0.135% piperazine diacrylamide (PDA) w/v stock solution. The gel had a 4% w/v acrylamide stacking gel prepared using a 30% acrylamide/0.8% PDA w/v stock solution. Gels were run at 25 mA/gel for 45 min and then 35 mA/gel for 3 h and proteins were visualized by silver staining.

2.4. PCR screening for mutations in SSIIa-A and SSIIa-B

Leaf tissue from M_2 plants suspected of having *ssIIa-B* mutations and parental lines was collected from two-three leaf stage seedlings and stored at −80 °C. DNA was isolated from seedlings as previously described (Feiz et al., 2009). Coding regions of *SSIIa-A* and *SSIIa-B* were amplified and sequenced from duplicate DNA samples using previously described primers and PCR conditions (Shimbata et al., 2005; Sestili et al., 2010a). Amplicons were analyzed for single nucleotide polymorphisms using Seqman Pro in the Lasergene 10.1 Suite (DNASTAR, Madison, WI). The two durum high amylose (DHA) SGP-1 double mutants discovered were DHA175, from the Mountrail/PI 330546 cross and DHA55, from the Mountrail/IG 86304 cross.

2.5. Differential scanning calorimetry

For random SGP-A1 wild type and SGP-A1 null F_5 derived lines from Mountrail/PI 330546, DHA175, and DHA55 differential scanning calorimeter (DSC) analysis was carried out using a Pyris 7 Diamond DSC (Perkin Elmer, Norwalk CT, USA) as previously described (Hansen et al., 2010). Three biological replicates were run in triplicate for each genotype. Amylose was determined via DSC using the method described in Polaske et al. (2005).

2.6. Colorimetric amylose determination and total starch determination

Purified starch from three biological replicates of SGP-A1 wild type and SGP-A1 null F_5 derived lines from Mountrail/PI 330546, DHA175, and DHA55 was used for amylose analysis. Amylose was