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Determination of amylose content and expression analysis of the Granular-Bound Starch Synthase I (GBSS1) gene in rice grains

Oko, A. O.[1&2*], Kumar, A.[1], Lal, M. K.[1] and Sharma, S. G.[1]
[1]Division of Crop Physiology & Biochemistry, ICAR- National Rice Research Institute, Cuttack, India
[2]Department of Biotechnology, Ebonyi State University, Abakaliki, Nigeria.

Abstract

The study determined amylose content and expression level of GBSS I gene in rice grain. A total of eighteen (18) rice (*Oryza sativa* L) cultivars of *Indica* varieties were collected from the National Rice Research Institute (NRRI) Cuttack and screened for their percentage amylose contents. Three cultivars namely Bindli, Naveen and Pooja which had contrasting characters in percentage amylose were then planted and used for the expression analysis. In the first stage of grain filling Bindli had 0.27 fold expression while Pooja and Naveen had 1.72 and 1.44 respectively. There was dramatic increase in the expression levels of the gene in the middle stage of grain filling in all the three cultivars with relative folds of 10.97, 42.71 and 27.34 recorded in Bindli, Pooja and Naveen respectively. However, at the last stage of grain filling, GBSS1 expression was low compared to the second stage with expression folds of 7.12, 7.55 and 1.67 in Bindli, Pooja and Naveen cultivars respectively. On the whole, Bindli cultivar showed lowest expression of the gene (GBSS1) when compared to Pooja and Naveen.

Key words: rice gene, amylose, expression analysis, GBSS1
Correspondence:*okpanioko@gmail.com

Introduction

Starch is a glucose polymer that is an important carbohydrate and energy source in the human diet. Human caloric intake is primarily met by starch which makes up as much as 80% of the daily calories consumed in most parts of the world (Keeling and Myers, 2010). Starch comprises two glucose polymers which are amylose and amylopectin. Amylose is a linear and relatively short polymer of glucose units linked by α(1 ⟶ 4) glycosidic bonds, while amylopectin is a branched and longer polymer where glucose units are arranged linearly through α(1⟶4) glycosidic bonds, with branches emerging through α(1 ⟶ 6) glycosidic bonds occurring every twenty-four to thirty glucose units (Sajilata et. al., 2006).

The amylose content in rice grain starch is of great importance when determining the quality of the rice grain (Fasahat et. al., 2014). It affects the stability, dietary fiber content, texture, and viscosity of processed rice (Morita et al., 2002). Generally, starches with higher amount of amylose are more resistant to digestion(Hu et. al., 2004). The cooking and eating characteristics of rice which are the bases of choice for the consumers is determined by the amylose content and gelatinization temperature (Samina et. al., 2012; Oko et. al., 2012). For instance, there is good linear correlation between hardness and amylose content (Rolando et al., 2004). Also, a positive correlation exists between amylose content and length/breadth ratio in relation to elongation of cooked rice (Rachel et al., 2013). The amylose content of starches usually ranges from 15 to 35% (Oko et. al., 2012; Hamaker and Griffin, 1993). Rice low in amylose are generally

known to be sticky and moist, whereas those high in amylose are non-sticky, flaky and dry (Juliano et. al., 1965). However, deviations from this correlation exist, which include low - amylose rice that are non sticky vice versa. Also, rices containing the same amylose content may differ substantially in hardness (firmness) and stickiness (Perez and Juliano, 1979).

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Amylose/amylopectin ratio of rice cultivars is one of the three main factors that appear to explain most of the variation in glycaemic and insulinaemic responses to rice; the other two are post-harvest processing (particularly parboiling) and consumer processing (cooking, storage and reheating) (Boers et. al., 2015). Based on amylose content, milled rice is classified in "amylose group" as follows: Waxy (1 - 2% amylose), Very low amylose content (10 - 20% amylose). Intermediate amylose content (20 - 25% amylose). High amylose content (25 - 33% amylose) waxy and low-amylose starches are desired for their influence on cooking quality and seed softness, especially in rice (Liu et. al., 2009).

In general, rice cultivars are presently classified according to their amylose content into three major groups: low, medium and high amylose content cultivars (Varavinit et. al., 2003). Starch synthase is a group of important enzymes involved in the synthesis of amylose and amylopectin, and it may be further classified into two major types according to their distributions in the amyloplast, namely granule-bound starch synthase (GBSS) and soluble starch synthase (SSS). For GBSS, there are two isoforms, GBSSI and GBSSII. GBSSI, also known as the WAXY protein, is tightly bound to starch granules and has a molecular mass of 58 - 60 kDa. This enzyme provides the largest proportion of total GBSS activity (Dry et al. 1992), and is the major enzyme responsible for amylose synthesis (Wang et al., 2006). Granule bound starch synthase (GBSS) activity is an important determinant of amylose content in endosperm of low- and medium-amylose cultivars, while factors other than the enzyme activity limit amylose synthesis in the high-amylose cultivars (Umemoto and Terashima, 2002). This gene invariably influences glycemic index of rice as reported by Fitzgerald et. al., (2011).

The link between natural variation in particular starch synthesis genes and starch properties is well established in some cases. GBSS I (*waxy*gene) is primarily responsible for the synthesis of linear chains of glucose molecules found in amylose and is the most well characterised cereal grain starch synthesis enzyme. A number of SNPs in the rice *waxy*gene, at the intron/exon 1 junction site, exon 6 and exon 10, impact starch quality by affecting amylose content (Cai et. al., 1998; Chen et. al., 2008; Larkin and Park 2003).The objectives of the study were to determine amylosecontent and describe the expresson level of GBSS I gene in rice grains.

Materials and Methods

Sample and Sample Preparation

A total of eighteen (18) rice (*Oryza sativa* L) cultivars of *Indica* rice varieties were collected from the National Rice Research Institute (NRRI) Cuttack and used for this work. The rice samples were milled (10%) using Satake huller (THU-35A, kw 0.2-0.4, rpm 1900, NO 1012080, made in Japan) and miller (Satake Grain Testing Mill, Tm-05, NO 554023 made in Japan). Thereafter, the samples were ground to powder using an electric powered blender (Icon Classic -mixer grinder, C No HP/14/001/0064, made in India) to 100 mesh sizes.

Determination of Amylose content

Amylose and amylopectin contents were estimated by using Amylose/Amylopectin assay kit-the Megazyme kit (Megazyme Ireland International, Ltd., Bray, Ireland) according to the manufacturer's recommendation. Rice flour weighing 20 mg was taken into a 10 ml falcon tube and dispersed by heating with 1 ml dimethylsulphoxide (DMSO); lipids were removed by precipitating the starch in ethanol (6 ml) according to manufacturer's manual (Megazyme Ireland International, Ltd., Bray, Ireland). Precipitated starch of the sample was dissolved in an acetate/salt solution where amylopectin is precipitated by the addition of Concanavalin A (4 ml) followed by centrifugation. The amylose in the supernatant was then enzymatically hydrolyzed to D-glucose and analyzed using glucose oxidase/peroxidase reagent. The total starch was also measured in a separate aliquot of the acetate/salt solution using the same treatment. The content of amylose in the starch sample was estimated as the ratio of GOPOD absorbance at 510 nm of the supernatant of the Concanavalin A precipitated sample to that of the total starch sample

Expression Analysis

Plant material and sample preparation

Three rice (*Oryza sativa* L) cultivars namely, Bindli (a local rice grown in UP), and the NRRI released varieties Naveen and Pooja with varying amylose contents (low, medium and high) were used for studies related to expression analysis. Developing seeds of rice after flowering (i.e. at 2, 7 and 14 days after anthesis , DAA) and seedling leaves (7 to 10 days old) were harvested from rice grown under natural climatic conditions in the fields of ICAR-NRRI, Cuttack and frozen immediately in liquid nitrogen and later stored at -80^{0}C for analysis.

Differential gene expression by qRT-PCR

For quantitative expression analysis as described by Jeng et. al., (2007(modified). Samples were collected at the three stages of the developing grains as described earlier and stored at -80°C. Frozen plant tissues were homogenized using pestle/mortar in liquid nitrogen and 1 ml of Qiagenlysis buffer was added per 100 mg of tissue in 2 ml micro-centrifuge tubes. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. An aliquot of each RNA sample was analysed on 1% agarose gel for confirming the intactness of the ribosomal RNA bands. RNA sample concentrations were measured twice on NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and stored at -80°C. First-strand cDNA was synthesized using 5mg of total RNA with the PrimeScriprtTM 1st strand cDNA synthesis kit (Takara Clontech) following the manufacturer's instructions.

Expression levels of GBSS1 were normalized to housekeeping gene β-tubulin which was used as an internal control gene. The qRT-PCR reaction was performed in triplicate in 96-well optical plates using gene-specific primer pairs (FP: 5'AACGTGGCTGCTCCTTGAA3') and (RP:5'TTGGCAATAAGCCACACACA3') and SYBR Green (Takara, Clontech, Japan) Thermal cycling conditions comprised 50°C for 1 h followed by an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. All qRT-PCR reactions were preformed in the Eppendorf Master Cylcerreal Time PCR (Master Cycler ep realplex^{2}S 200-250, SL. 6300000604) followed by analysis of the dissociation curve, taking a fluorescence reading at every degree between 55 and 95°C, to ensure the amplification of only one amplicon. The data analysis was done using Eppendorf Master Cycler RealPlex software.

Results and Discussions

Amylose Screening

The data on amylose content of rice varieties screened are presented in Table 1. Amylose content of milled rice was reported to correlate positively with hardness values of cooked rice and negatively with stickiness values (Perez and Juliano, 1987). Rice varieties with high amylose content are said to have lower glycemic index. Low amylose levels are associated with cohesive, tender, and glossy cooked rice grains. On the other hand, high levels of amylose cause rice to absorb more water and consequently expand more during cooking, and the grains tend to cook dry, fluffy, and separate (Juliano, 1971). Rice starch with high amylose fraction shows higher degree of retrogradation and lower increase in consistency index, shear stress and plastic viscosities than rice starch with lower amylose content (Tukomane and Varavinit, 2008). Denardin et. al., (2012) reported that high amylose feeding results in longer satiation, gain in body weight and apparent increases in digestibility, fecal water content and nitrogen excretion, reduced fecal pH, lower postprandial blood glucose response, serum total cholesterol and triglyceride levels and pancreas weight, and higher fasting serum glucose concentration and liver weight. Panlasigui et. al., (1991) reported that the digestibility and glycemic response were significantly different among rice varieties with similar amylose content, arguing that amylose and amylopectin ratio is not the sole determinant of the rate of starch digestion and postprandial glycemic response.

Table 1: Amylose content (%) of milled rice samples of different cultivars

R i c e e S	% A en
P o a	2 2 a.±4 067 .
R a j il	2 2 a. ±1 65 .
P u s a i b	2 2 a. ±3 86 .
A j y	2 1 a. ±0 81 .
L a t	2 1 a. ±8 66 .
S a t y aa	1 8 b. ±3 02 .
L u n a aS	2 1 a. ±3 407 .
V a n a	2 0 a. ±8 51 .
A n i j	2 1 a. ±6 01 .
S a h b hn	2 1 a. ±2 67 .
P – M5 6	2 0 a. ±9 31 .
N i u	2 1 a. ±5 401 .
S w aa	2 1 a. ±7 51 .
M a m ir	1 5 c. ±0 407 .
B i in	1 4 c. ±4 82 .
C R D 6	2 2 a. ±2 406 .
K a l i –1 1g	2 1 a. ±8 34 .
N a rv	1 8 b.±2 07 .

Means with different superscripts are statistically significant difference (p <0.05)

Expression analysis of GBSS1:

Granule bound starch synthase1 (GBSS I) enzyme catalyzes one of the enzymatic steps of starch biosynthesis and is responsible for the synthesis of amylose(Me´rida et. al., 1999). GBSS1 is highly expressed in developing seeds and the activation or suppression of this gene may result in alteration of amylose biosynthesis in rice seeds. The gene is usually expressed more in high amylose rice cultivars and lower expression is found in low amylose rice varieties. The rice Bindli showed lower expression of the gene as compared to Pooja and Naveen. In the first stage of grain filling Bindli had 0.27 fold expressions as compared to Pooja and Naveen which showed 1.72 and 1.44 fold expression respectively (Fig. 1). This suggests that the expression of GBSS1 is low at the initial stages of

grain filling and so may be the accumulation of amylose. In the second stage (middle stage) of grain filling, there was a dramatic increase in the expression of GBSS1 in all the three cultivars with a relative 10.97, 42.71 and 27.34 fold expression recorded in Bindli, Pooja and Naveen respectively. This is in conformity with the findings of Hirose and Terao (2004). In wheat, GBSSI expression is high in endosperm tissue (Vrinten and Nakamura, 2000), while it is rapidly expressed at the middle or mid-late stages of seed development in amaranthus, where they are classified as late expressers alongside *SSII-3, SSIII-2* (Park and Nishikawa, 2012). As Pooja recorded the highest expression of the GBSS1 gene, it suggests that it may have more amylose than others. In fact, this is supported by the results of amylose analysis (Table 1). At the third stage of grain filling, GBSS1 expression was lower as compared to the second stage with expression folds of 7.12, 7.55 and 1.67 in the rice Bindli, Pooja and Naveen respectively, suggesting a decline in the enzyme activities, which may be due to saturation of the expected product (amylose) in the grains. In a mutant, where the amount of GBSSI and the activity of ADP-glucose pyrophosphorylase (AGPase) were higher than that in the wild type and parent mutant lines, the endosperm of the seeds contained high amylose among other traits (Fujita et. al., 2011). The amount of GBSS I and its activity together are the main factors controlling amylose synthesis (Li-jie et al., 2015). Studies of waxy mutations in wheat and other cereals have shown that null mutations in genes encoding GBSSI result in amylose-free starch in endosperm and pollen grains, whereas starch in other tissues may contain amylose (Vrinten and Nakamura, 2000).

Conclusion

This result suggests that the gene granular bound starch synthase I (GBSS1) could be the target gene when the amylose content of rice is to be modified.

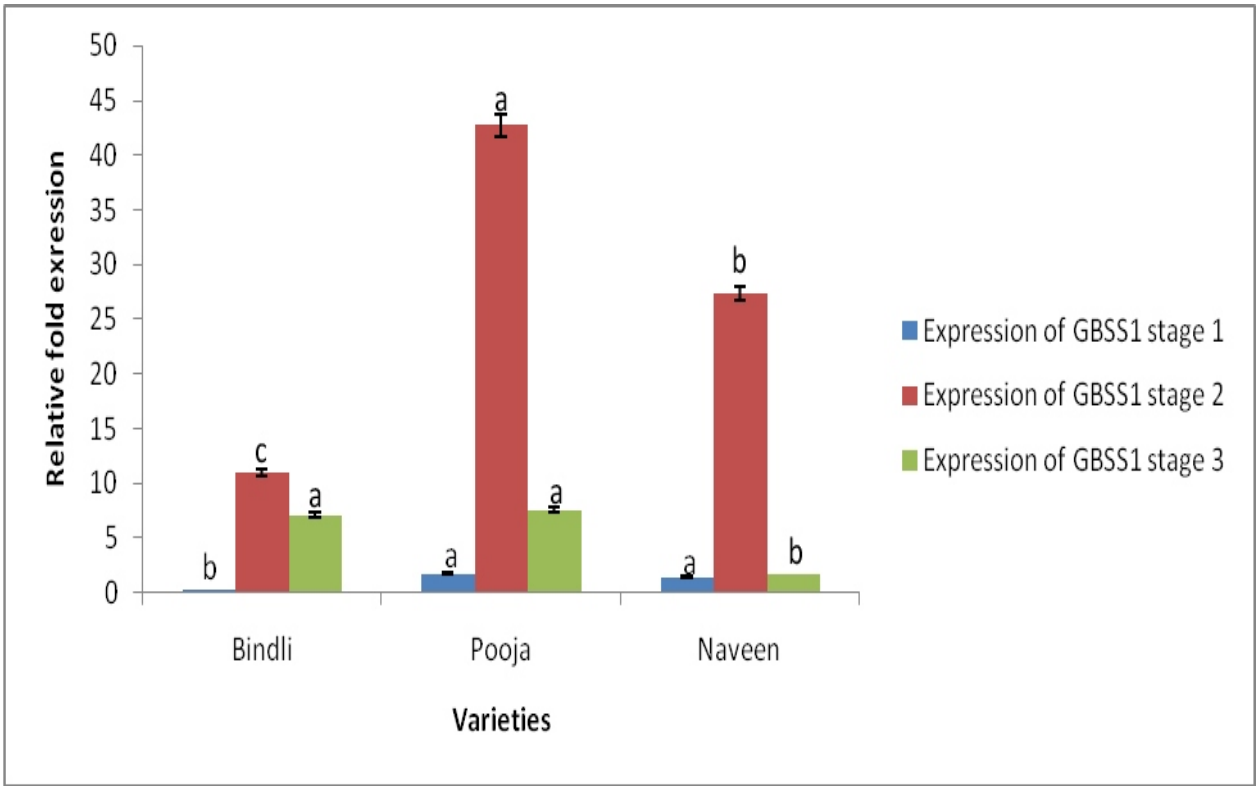

Figure 1: Expression analysis of GBSS1 in rice plants. qRT-PCR analysis of rice cultivars Bindli, Pooja and Naveen compared to the internal control *β tubulin*. Each value represents the mean ± S.E. (n = 3) Means with different superscripts are statistically significant difference ($p < 0.05$)

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