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Utility of DNA Markers for the Detection of Amylose Content in Sri Lankan Rice (*Oryza sativa* L.) Varieties

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ABSTRACT

Grain quality improvement of rice is considered as prioritized research area in rice breeding programs in Sri Lanka. Amylose Content (AC) is a key determinant of end quality of rice. Amylose Content is controlled by *waxy* gene located in chromosome 6, through encoding the enzyme; granule bound starch synthase (GBSS). Gelatinization Temperature of rice is controlled by the alkali degeneration locus (*alk*) encoding a soluble starch synthase IIa (SSIIa) gene. This study explains the feasibility of two DNA markers, RM 190 and RM 314 for the detection of polymorphism in amylose content and gelatinization temperature using 18 Sri Lankan rice varieties containing different levels of amylose content. Results indicated that RM 190 was significant in distinguishing genetic variation of rice varieties with low amylose content in rice endosperm over the intermediate and high amylose content. Therefore, Simple Sequence Repeat marker (SSR), RM 190, would be useful in predicting the amylose content of the variety when they are used in rice breeding programs followed by Marker Assisted Selection.

KEYWORDS: Amylose content, Gelatinization temperature, Microsatellite markers, *Oryza sativa*

INTRODUCTION

Rice is one of the most important staple food crops of more than one-half of population with approximately 95% of production in Asia (Bhattacharjee *et al.*, 2002). Starch is the major component of the rice endosperm consists of amylose, linear molecule composed of α (1,4)-linked glycosidic chains and amylopectin, highly branched glucan with α (1,6) glycosidic bonds that connect linear chains. Amylose content (AC) (Juliano, 1985), Gel consistency (GC) (Cagampang *et al.*, 1973), and Gelatinization Temperature (GT) (Little *et al.*, 1958) are the three physiochemical characteristics of starch, involve in determination of eating, cooking and processing quality of rice.

Amylose content is considered as the most important predictor of sensory quality in rice. High AC leads for dry, fluffy, separated cooked rice but become hard upon cooling (Juliano, 1985). While low AC leads for moist, sticky and glossy rice grain after cooking. AC is the ratio of amylose amount present in endosperm to total starch content, which is vary from cultivars to cultivars, 18-32% in indica rice and 10-22% in japonica rice. Rice samples are commonly categorized into several AC classes as follows; glutinous/ waxy (AC-0-5%), low AC (<20%), intermediate AC (21-25%) and high AC (>25%) (Kongseree and Juliano, 1972).

Prior genetics studies regarding rice, discovered major gene and QTLs responsible for AC in rice endosperm. These QTLs locates in chromosome 6 and 5 and the major gene, *waxy*, that locates in chromosome 6, explained 91.9% of the total variation of AC (He *et al.*, 1999). *Waxy* gene plays a key role in amylose synthesis by encoding the enzyme, granule bound starch synthase (GBSS) (Tan *et al.*, 1999 and Fan *et al.*, 2005). Amylose content polymorphism in rice has been explained by waxy gene alleles, wx^a and wx^b (Sano, 1984). They are associated with the amount of GBSS and AC in rice endosperm. Presence of wx^a allele is responsible for synthesis of higher content of GBSS, and thus higher AC, than in the presence of wx^b. Not only that AC in rice but also associated with the post transcriptional regulation of the waxy gene (Wang *et al.*, 1995).

Gelatinization temperature (GT) of rice starch defined as the range of temperature at which nearly all the starch granules start to swell irreversibly in hot water with simultaneous loss of birefringence and crystallinity (Virmani, 1994). Low GT rice needs less energy input during cooking than high GT rice (Bao et al., 2004). GT broadly categorized into three classes: low (<70 ^{0}C), intermediate (70-74 ^{0}C) and high (>74 ^{0}C) (Kongseree and Juliano, 1972). GT of rice is controlled by the alkali degeneration locus (*alk*) on chromosome 6, encoding a soluble starch synthase IIa (SSIIa) isoform (Fan *et al.*, 2005 and Bao *et al.*, 2006).

Improvement of AC and GT, and development of cultivars with improved agronomic traits while maintaining the desired AC and GT through breeding programs are major concerns of rice breeders. But measuring of quality traits such as amylose content and gelatinization temperature are time consuming and expensive. Theretofore, this study was carried out to detect a suitable DNA marker which is closely linked with AC and GT in order to facilitate rice breeding programs.

MATERIALS AND METHODS

Plant Materials

Both mature seeds and husked and polished grains of 18 different rice varieties were collected separately for each sample from Rice Research and Development Institute, Bathalagoda (Table 2). Rice samples were collected within 3-4 months after harvesting.

Analysis of Amylose Content

Initially rice samples were husked and polished prior to milling. Twenty whole-milled rice kernels of 18 rice samples were ground separately in an Udy cyclone mill (sieve mesh size 60). Amylose content per 100 mg was determined through measuring blue value of rice varieties as described by Juliano (1971). 100 mg of rice sample was put into a 100 ml volumetric flask and 1 ml of 95% ethanol was added. Then 1 ml of 1 N NaOH was added. Then content was boiled for 20 min at boiling temperature to gelatinize the starch. After cooling the content, the volume was made up to 100 ml. 5 ml of starch solution was pipetted out into a 100 ml volumetric flask. 1 ml of 1 N acetic and 2 ml of iodine solution (0.2 g iodine and 2.0 g potassium iodide in 100 ml aqueous solution) were added. Then volume was made up to 100 ml with distilled water and solution was stood for 20 min after shaking. Finally absorbance of the solution was measured at 620 nm with JENWAY 6305 spectrophotometer (Hemson International Pvt. Ltd.).

The standard curve was prepared using 40 mg of potato amylose to calculate amylose content of rice varieties through absorbance values. Weighed 40 mg of potato amylose was put into a 100 ml of volumetric flask and 1 ml of 95% ethanol and 9 ml of NaOH were added and content was heated for 20 min at boiling temperature. After cooling the content volume of the solution was made up to 100 ml using distilled water. Then 1, 2, 3, 4, and 5 ml of amylose solution were pipetted out into 100 ml flasks. Then 0.2, 0.4, 0.6, 0.8 and 1 ml of 1 N acetic acid were added to the flasks respectively. Finally 2 ml of iodine solution was added to each flask and volumarized up to 100 ml with distilled water. Solutions were stood up to 20 min after shaking. Then absorbance values were measured at 620 nm. Measured absorbance values were plotted at 620 nm against the concentration of anhydrous amylose (mg).

Analysis of Gelatinization Temperature

GT was indirectly measured on rice by the alkali spreading value (Little et al., 1958). Husked and polished rice samples were used for the analysis. Selected duplicate sets of six milled grains without cracks of each sample were put into petridishes. 10 ml of 1.7% KOH was added and grains were spread in the petridish to provide enough space. Constant temperature at 30 ^{0}C was maintained to ensure better reproducibility. After 24 hrs, GT was identified based on the score given from 1 to 7, by visual appearance of the gelatinization. Then the gelatinization temperature was identified based on the score value of the sample reported by Dela Curz and Khush (2000).

Genomic DNA Extraction and Quantification

DNA was extracted from two-three weeks old leaves from germinated seeds. 3 cm tender leaf piece was ground with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris HCl, 0.5 M EDTA) in a micro centrifuge tube separately for each variety. Then homogenate was incubated at 70 ^{0}C for 15 min under room temperature. Tube was centrifuged at 15000 rpm for 15 min immediately after the incubation. 100 µl of isopropanol was added into a new eppendorf tube. Then supernatant was transferred into the isoproponol containing tube and mixed gently. Tube was kept under 4 ^{0}C for overnight and centrifuged at 15000 rpm for 15 min at 4 ^{0}C. The supernatant was removed and DNA pellet was washed with 150 µl of 70% ice cold ethanol by centrifuging for 10min at 15000 rpm at 4 ^{0}C. Finally supernatant was removed and pellet was air dried for 2 hrs in the dark room. After the removal of all the ethanol traces DNA was dissolved in 300 µl of 1/10th TE buffer (10 mM Tris, 1 mM EDTA) and stored at -20 ^{0}C.

Selection of SSR Primers

SSR primers that are linked with AC and GT were selected by QTL maps developed by Fan *et al.* (2005) and Tabkhkar *et al.* (2012). Accordingly two SSR primers; RM 190, RM 314 (Table 1) were selected for PCR amplification.

Table 1. DNA sequences of SSR primers

Primer	Sequence
RM 190	
Forward	5' TTTGTCTATCTCAAGACAC 3'
Reverse	5' TTGCAGATGTTCTTCCTGATG 3'
RM 314	
Forward	5' CTAGCAGGAACTCCTTTCAGG 3'
Reverse	5' AACATTCCACACACACACGC 3'

PCR Amplification

PCR amplification was performed in a 12 µl reaction volume which consisted of 5 µl of diluted DNA, 1.5 µl of 10X PCR buffer, 1.2 µl of dNTPs (2.5 mM), 0.9 µl of primers (40 pmol), 0.12 µl of *Taq* DNA polymerase and 3.28 µl distilled water.

Amplification was done with a BioRad (My Cycler™) thermal cycler with following parameters: 95 ^{0}C for 5 min followed by 35 cycles of 95 ^{0}C for 1min, 52 ^{0}C for 30 sec (for RM 190) or 57 ^{0}C for 30 sec (for RM 314), 72 ^{0}C for 1 min and final extension at 72 ^{0}C for 5 min.

Amplified PCR products were detected under agarose gel electrophoresis using 3.8 % gel stained with ethidium bromide.

After the electrophoresis, the gels were visualized under UV transilluminator and photographs were taken using a digital camera.

Statistical Analysis

The banding patterns of primers were observed and categorized by appearance of alleles. The Mann-Whitney test was performed to detect whether markers show association between AC/GT and allele type using Minitab 15.

RESULTS AND DISCUSSION

Analysis of Amylose Content and Gelatinization Temperature

Amylose content and Gelatinization Temperature was measured in 18 rice varieties that represented Sri Lankan and exotic rice.

According to the analysis At 405 and Basmathi 370 were categorized as low AC rice varieties while At 306, At 402, Bg 406, Ld 365, Ld 408 and Pusa basmathi were categorized as intermediate AC rice varieties. Other rice varieties were categorized as high AC containing rice varieties based on AC categories reported by Kongseree and Juliano (1972).

GT of used rice varieties was appeared in three categories: high, intermediate and low as indicated in Table 2.

Genotyping by RM 190

Ayres *et al.* (1997), Bergman *et al.* (2001), Lang and Buu (2004) and Chen *et al.* (2008) suggested that RM 190 primer was suitable for use as a marker for the detection of AC polymorphism during breeding process. It flanks major QTL regarding AC, *ac6a* (Fan *et al.*, 2005 and Tabkhkar *et al.*, 2012). Therefore RM 190 primer was mainly used in this study for the detection of AC polymorphism in Sri Lankan rice varieties.

Table 2. Characteristics of studied rice varieties

No.	Variety	AC (%)	Allele type produced by RM 190	GT
1	At 303	28.72	A	High
2	At 306	23.93	B	High
3	At 308	28.03	A	In
4	At 402	21.54	A	High
5	At 405	13.29	B	Low
6	Bg 250	26.15	A	In
7	Bg 300	26.41	A	In
8	Bg 305	27.35	A	High
9	Bg 358	25.21	A	H/I
10	Bg 379-2	31.88	A	High
11	Bg 403	31.88	A	Low
12	Bg 406	21.41	A	High
13	Bg 407-H	26.84	A & B	Low
14	Basmathi 370	16.24	B	In
15	Ld 356	25.3	A	In
16	Ld 365	21.67	A	High
17	Ld 408	21.79	A	Low
18	Pusa basmathi	22.09	A	Low

GT; In: Intermediate, H/I: High/Intermediate
Allele type produced by RM 190; A allele- amplified short DNA fragment, B allele- amplified long DNA fragment

With the RM 190 primer PCR amplification produced two distinguishable alleles. Both alleles were between 100-150 base pairs (bp). Except in Bg 407-H, all the other rice varieties had one allele, either A or B (A allele- amplified short DNA fragment, B allele- amplified long DNA fragment) (Figure 1).

All the low AC rice varieties produced B allele. But At 306, containing intermediate AC also produced B allele which appeared to be an exception compared to others. All the other intermediate and high AC rice varieties produced A allele.

Genotyping by RM 314

RM 314 had produced three distinguishable alleles. But phenotypic variation of AC and GT in rice varieties was unable to explain through the genetic variation of the RM 314 alleles.

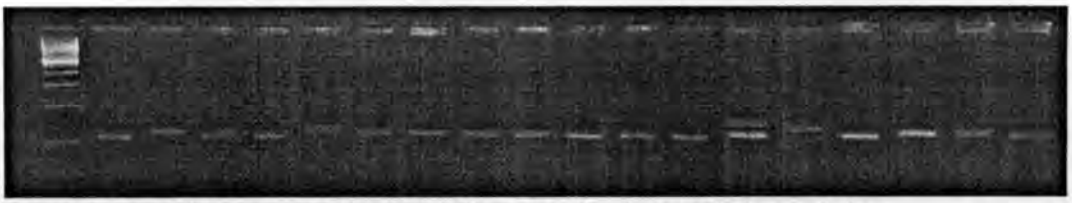

Figure 1. PCR profile generated by RM 190 marker for the tested rice varieties
Lane1- 100 bp ladder, 2-19: accordance with the varieties numbered as in table 2. Respectively 1-18.

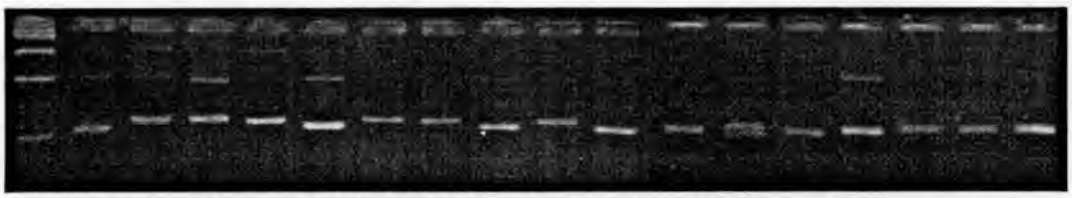

Figure 2. PCR profile generated by RM 314 marker for the tested rice varieties
Lane 1- Easy ladder (Bio line), 2-Pusa basmathi, 3-At 405, 4-Basmathi 370, 5-At 402, 6-Bg 406, 7-Ld 408, 8-Bg 250, 9-Bg 305, 10-Ld 356, 11-At 303, 12-Bg 300, 13-Bg 407-H, 14-At 308, 15-Bg 379-2, 16-At 306, 17-Bg 358, 18-Bg 403

Bg 407-H, the hybrid rice variety, also expressed heterozygosis nature through producing two allele by RM 314.

Statistical Analysis of Genotypes

In order to detect whether there is relationship between marker genotype and amylose content, the pooled amylose content under 2 allele categories were compared by Mann-Whitney test.

Table 3. Analysis of genotypes by Mann-Whitney test

Primer	Median AC(%) for allele types		p value
	A	B	
RM 190	16.239	25.470	0.0041

Accordingly, RM 190 marker is significant ($p<0.01$) distinguishing low AC rice varieties from intermediate and high AC rice varieties. Marker, RM 314 did not show any association between AC / GT and the genotype.

CONCLUSIONS

AC was determined in 18 rice varieties using blue value method. Results indicated that all used varieties were laid among Low AC, Intermediate AC and high AC. SSR marker, RM 190 distinguished two types of alleles among tested varieties and the association between marker genotype and AC was highly significant. According to our results no relationship between RM 190 and GT was found. RM 314 is unlikely to be applied to detect the polymorphism of AC and GT in rice. Accordingly, it can be concluded that RM 190 has the potential to be used in distinguishing low AC rice varieties over the intermediate and high AC varieties.

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