

Study on *Waxy* Gene Polymorphism and Amylose Content of Breeding Lines Derived from *Oryza rufipogon* x *Oryza Sativa* CV. MR219

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ABSTRACT

Amylose in rice is controlled by granule-bound starch synthase, a protein encoded by the *Waxy* (*Wx*) gene. This study was conducted to identify the polymorphisms in the partial *Waxy* gene for two BC₂F₇ and nine BC₄F₃, breeding lines and their parents, *Oryza sativa* cv. MR219 and *O. rufipogon*, and also the association between the polymorphism of the partial *Waxy* gene and the amylose content. Sequences of all 13 breeding lines showed that the microsatellite (CT)₁₇ located 55bp upstream of the 5'-leader intron splice site and had G at the first nucleotide of the splice donor site of intron 1 of the *Wx* gene. The amylose content analysis revealed that all the samples with similar (CT)₁₇ were in the intermediate category (20–25%), except for one BC₂F₇ line (19%). The genotype determined using the microsatellites and SNP markers supports the intermediate category of the amylose content values.

Keywords: *Waxy* gene, amylose content, *Oryza rufipogon*

INTRODUCTION

Rice is a staple food for the majority of the world's population. As a primary source of

carbohydrate, rice is the most important crop in Malaysia. The qualities of rice that appeal to human consumers are flavour, fragrance, and the texture of cooked rice (Henry *et al.*, 2006). In Malaysia, intermediate amylose levels in the rice cultivars are considered a good quality (Lim *et al.*, 1986). The amylose content in rice has been reported to vary from 15-35% (Ball *et al.*, 1998). According to Juliano (1992), the categories

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for amylose content are waxy (0-2% amylose), very low (2-12% amylose), low (12-20% amylose), intermediate (20-25% amylose) and high (25-33% amylose). Rice with low amylose levels are usually tender, cohesive and glossy, while higher amylose cultivars are dry, fluffy and separated when cooked and these percentages are mediated by the proportion of amylose to amylopectin in starch granules. The chemical structure of amylose is linear, as opposed to the highly branched amylopectin that determines the textural properties conferred by starch gelatinization (Juliano, 1971). Unnevehr *et al.* (1992) have shown that the rice in Malaysia contains amylose in the intermediate to high categories. However, local consumers prefer rice with intermediate amylose content. Hence, the high amylose content of the locally produced rice should be improved to better suit the consumers' taste. Pooni *et al.* (1992) suggested that the amylose content might be related to the effects of the maternal plant or cytoplasm, whereas Xu *et al.* (1995) reported that rice amylose content was mainly controlled by the triploid endosperm genotype without any cytoplasmic effect.

In rice, amylose is synthesized by granule-bound starch synthase (GBSS), which is also known as *Waxy* (*Wx*) protein (Jahan *et al.*, 2002). This protein is encoded by the *Wx* gene (Tan *et al.*, 1999). Non-waxy rice cultivars commonly have two different alleles at the *Wx* locus, namely *Wx^a* and *Wx^b*. The alleles encode different levels of GBSS, and is thus involved in controlling the amylose content. The *Wx^a* allele is predominant in non-waxy *indica*

cultivars, while the *Wx^b* allele is common to the non-waxy *japonica* variety (Yamanaka *et al.*, 2004).

The *Waxy* gene has a size of 5499bp and it consists of 13 exons with a 1.1 kb untranslated leader intron (Wang *et al.*, 1995; Hirano & Sano, 1991; Umeda *et al.*, 1991). A polymorphic microsatellite (CT)_n has been identified in the *Wx* gene, located 55bp upstream of the putative 5'-leader intron splice site (Bligh *et al.*, 1995). Tan and Zhang (2001) reported that the cultivars with low (CT)_n repeats (n≤14) had high amylose content, while those with high (CT)_n repeats (n≥16) had low and intermediate amylose contents. In addition, a single nucleotide polymorphism (SNP), G or T at the 5' leader intron splice site, influences the gene expression and causes variation in amylose content. In a study by Ayres *et al.* (1997), all of the strains with 18% or less amylose had the sequence AGTTTATA, while all the strains with higher proportion of amylose had AGGGTATA. These two polymorphisms have been shown to contribute to 81.2-91.2% of the amylose content (Ayres *et al.*, 1997; Tan *et al.*, 1999; Shu *et al.*, 1999) and are regarded as the molecular markers to determine amylose content.

In the present study, the samples were compared based on their partial *Wx* gene sequences, covering both the microsatellite and SNP locations. The specific primer pairs (Waxy-F and Waxy-R) were used for polymorphisms detection in the selected breeding lines from BC₂F₇ and BC₄F₃ generation.

MATERIALS AND METHODS

The Plant Materials

The wild parent *O. rufipogon* (IRGC105491) and the high yielding cultivar *Oryza sativa* cv. MR219 were crossed bred to develop transgressive variants with MR219 being the recurrent parent. The two BC₂F₇ and nine BC₄F₃ variants used in the present study were selected based on the field performance (Bhuiyan, 2010). BC₂F₇ lines G19 and G33 have a mean amylose content of 22.6 and 19.0% (intermediate and low), respectively (unpublished data from Parviz Fasahat). *Wx* gene was only 800Kb away from the grain weight QTL on chromosome-6 (unpublished data from Ngu Mee Siing). Therefore, the nine breeding lines from the population BC₄F₃ (8-1, 10-5, 16-20, 9-12, 11-17, 15-5,

22-9, 29-5 and 29-7) were selected based on their grain weight data and also the level of introgression from *Oryza rufipogon* at the grain weight QTL region. Breeding lines 8-1, 10-5, 9-12, 15-5 and 16-20 have low or similar grain weight as compared to the recurrent parent, MR219. The breeding lines 11-17, 22-9, 29-5 and 29-7 have high grain weights comparable to that of the recurrent parent, MR219. The breeding lines have different levels of introgression from *O. rufipogon* for grain weight QTL on chromosome 6 (Table 1), but only the breeding line 11-17 carried grain weight QTL.

Amylose Content Analysis

The amylose contents of the nine BC₄F₃ samples were determined by the UPM-

TABLE 1

The mMean amylose content (%), number of (CT)_n repeats and single nucleotide polymorphism (SNP) for the 13 samples

Samples	Designation	Level of introgression for grain weight QTL (%)	100 Grain-weight (g)	Amylose content mean (%)	(CT) _n repeats	SNP
MR219	MR219	0	2.85	25.3	17	G
<i>Oryza rufipogon</i>	<i>Oryza rufipogon</i>	100	n/a	25.1	17	G
G19	R17-1-83-3-B-B	n/a	n/a	22.6	17	G
G33	R14-3-66-4-B	n/a	n/a	19.0	17	G
8-1	UKMRC6-8	50	2.75	20.2	17	G
9-12	UKMRC6-9	25	2.78	22.5	17	G
10-5	UKMRC8-10	0	2.73	21.4	17	G
11-17	UKMRC8-11	100	3.03*	20.6	17	G
15-5	UKMRC11-15	25	2.88	22.8	17	G
16-20	UKMRC13-15	0	2.80	21.4	17	G
22-9	UKMRC14-22	100	2.96	23.4	17	G
29-5	UKMRC19-29	25	2.97	20.9	17	G
29-7	UKMRC19-29	25	2.93	21.3	17	G

* Duncan's Multiple Range Test P < 0.05

BERNAS Food Analysis Laboratory, Universiti Putra Malaysia, Serdang, Malaysia. The samples were ground through a sieve and defatted by refluxing with methanol for 6 hours in a Soxtec extraction unit (Soxtec™ 2050, FOSS Analytical, Denmark). After defatting, flour was spread and left for two days at room temperature to allow evaporation of residual methanol. Meanwhile, 0.1 gram of the defatted samples was transferred into a 100 ml volumetric flask. Nine ml of 1M sodium hydroxide solution was added to each sample. After adding 1 ml ether, the mixture was heated in a boiling water bath for 10 min. The samples were covered and allowed to stand at room temperature overnight. The volume was adjusted with distilled water and mixed. Then, the amylose content was determined with FIA (flow injection analyser) (FOSS Co., Sweden). The sample was injected into a carrier stream of diluted sodium hydroxide and mixed with the iodine colour reagent and an acetate buffer. Finally, the colour of the iodine-starch complex was measured in a flow cell.

The Wx Microsatellite Allele and Wx Gene Analysis

The leaf samples of the breeding lines were collected at the age of 14 days. DNA was extracted following modified chloroform based on the DNA extraction protocol by Murray and Thompson (1980) and QIAGEN plant minikit.

The PCR amplification of the *Wx* microsatellite was performed using the primer pairs, namely, Waxy-F (5'-

ACCATTCCTTCAGTTCTTTGTCT-3') and Waxy-R (5'-TAGCATGTATGAGACTACTTGTA-3'). These primer pairs have been reported previously and flanked the beginning of exon 1 and the beginning of intron 1 (Prathepha, 2003). The volume of PCR reaction mixture was 30 µL, containing 25 ng DNA template, 0.2 mM of each primer pair, 1.5 mM MgCl₂, 0.1 mM dNTPs and 1.5 U Taq DNA polymerase (Intron). The PCR profile included pre-denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min 30 sec. The final extension was at 72°C for 5 min. Meanwhile, the PCR amplified products were analyzed on 1.5% agarose gel at 90 V for 1 h and visualized under UV light after staining with ethidium bromide (AlphaImager™ 2200). The sizes of the fragments were determined by comparing them with the mobility of 100 bp DNA ladder (Biolabs). The PCR products were then purified using QIAquick Purification Kit (QIAGEN). The purified PCR products were sent to First BASE Laboratories Sdn. Bhd. for sequencing. For each sample, the PCR and forward/reverse sequencing reactions were repeated three times for sequence confirmation. The results were analyzed using Sequence Scanner and ClustalW (2.0.12) multiple alignment tool (<http://www.ebi.ac.uk/Tools/clustalw2>).

RESULTS

Amylose Content Analysis

The amylose content analysis showed that G33 had the lowest amylose content (19%)

among the 13 samples, while the other 12 samples contained intermediate amylose content ranging from 20.2% to 25.3% (Table1).

The Wx Microsatellite Allele and Wx Gene Analysis

The amplified PCR products of the 13 samples were approximately 250 bp in length, as shown in Fig.1. Based on the DNA sequences, both the microsatellites and SNP were successfully detected (Fig.2). All the 13 samples have the same genotype, (CT)₁₇ at the SSR locus, and nucleotide G at the SNP locus.

DISCUSSION

The amylose content of rice is the most important determinant of the cooking quality affecting the textural and organoleptic

properties (Juliano, 1971). The textural qualities such as grain cohesiveness, tenderness and glossiness are affected by starch gelatinization properties of rice. The degree of water absorbed by starch granules on heating is affected by the amylose content and its linear structure. Higher amylose content facilitates extensive hydrogen bonding, contributing to the crystallinity of structure which are more resistant to swelling on heating but associated with more water absorption, greater grain expansion during cooking but upon cooling, grains become dry, fluffy and separate. In contrast, starch granules with less amylose gelatinize rapidly and are associated with greater grain gelatinization, stickiness, and glossiness.

The amylose content analysis showed that all the 13 samples could be categorized

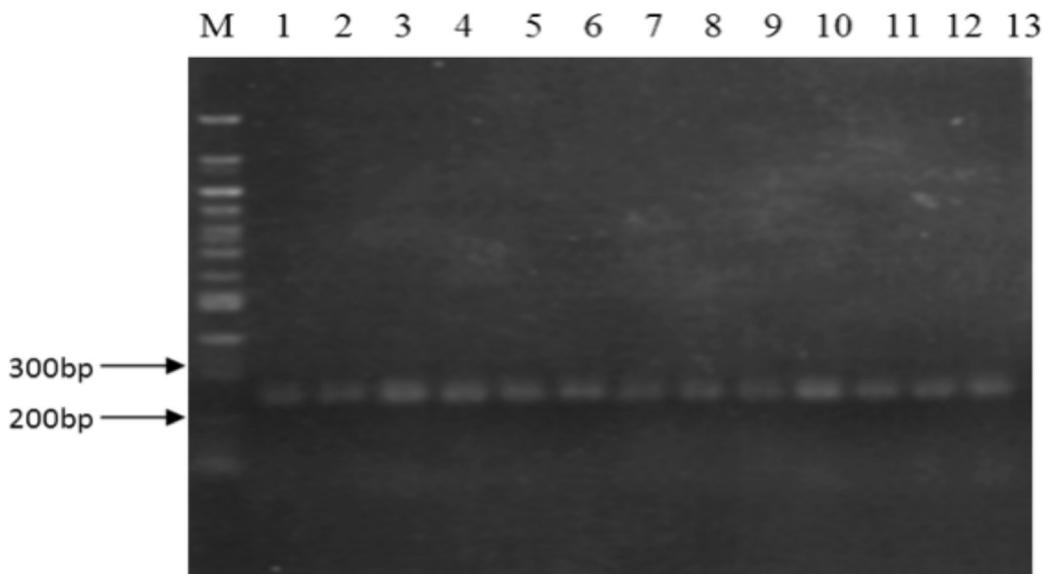


Fig.1: Amplified PCR products of 13 samples. M=100bp ladder. Well 1 to 13 = 8-1, 10-5, 16-20, 9-12, 11-17, 15-5, 29-5, 22-9, 29-7, G33, G19, *Oryza sativa* cv. MR219 and *O. rufipogon* (IRGC105491)

as intermediate (20-25%), except for G33. MR219 and *O. rufipogon* have the mean values above the top and bottom range values of the intermediate category. These results correspond to the findings of some previous studies, whereby the rice cultivars with (CT)_n repeats, $n \geq 16$ would have low to intermediate amylose contents (Tan & Zhang, 2001), and the rice cultivars with base G at the SNP location had more than 18% amylose content (Ayres *et al.*, 1997). Jayamani *et al.* (2007) reported that the accessions with (CT)₁₇ and (CT)₁₈ had both the AGGTATA and AGTTATA sequences. The microsatellite classes (CT)₁₇, and (CT)₁₈ had similar levels of amylose content (approx. 21%) that were subdivided into 17T/17G and 18T/18G haplotypes, and recorded 20.0/24.3 and 19.7/25.3% amylose content, respectively. Prathepha and Baimai (2004) also reported that non-glutinous rice with intermediate (20-25%) and high amylose content (25%) had the base G at the SNP locus.

Wx gene was very near to a grain weight QTL on chromosome-6 (800kb of physical distance according to the rice genome automated annotation database, <http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/>) and could increase the amylose content of the BC₄F₃ breeding lines in the process of population development. However, there is no increase in the amylose content or *Wx* gene polymorphism observed for BC₄F₃ breeding lines with different grain weights. Therefore, these breeding lines, though having introgression from *O. rufipogon* and higher grain weight, have

intermediate level of amylose content which is preferred by local rice consumers. In this study, there was no correlation observed between *Wx* gene and grain weight QTL on chromosome 6.

MR219 and *O. rufipogon* (IRGC105491) had very similar amylose content (25.3 and 25.1%, respectively). As a wild rice species, *O. rufipogon* is expected to have higher amylose content as compared to cultivated rice. Nonetheless, the *O. rufipogon* accession used in this study might have been inter-crossed with *O. sativa* and then gone through many generations of selfing resulting in the lower amylose content. Prathepha (2008) reported that the mean values of amylose content of 212 *O. rufipogon* accessions from Thailand ranged from 12.5-28.1%, and the rice-to-wild gene flow could have a significant impact on the wild populations.

In addition, the amylose content of rice also has a bearing on the consumers' health in relation to the mediating glycemic response after ingestion, which is measured as glycemic index (GI) (Brand-Miller *et al.*, 2009; Foster-Powell *et al.*, 2002). Rice varieties have been classified as either low-, moderate- or high-GI, depending on the amylose content (Juliano & Goddard, 1986; Behall & Howe, 1995). Some studies indicate that higher amylose contents result in lower glucose and insulin responses (Hallfrisch & Behall, 2000), whereas others suggest that the amylose content alone may not be a good predictor of glycemic response (Panlasigui *et al.*, 1991).

Intermediate levels of amylose content in all the breeding lines showed that there is no correlation between the *Wx* gene and grain weight QTL. Thus, determining the amylose content in the breeding lines helps to produce rice which can be favourable to Malaysian consumers as well as worldwide consumers.

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