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QUANTITATIVE ANALYSIS ON AMYLOSE CONTENT BY DNA MARKERS THROUGH BACKCROSS POPULATIONS OF RICE (*Oryza sativa* L.)

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ABSTRACT

Amylose content is one of the important traits of grain quality properties of rice. Recently, a polymorphic microsatellite sequence closely linked to the Wx gene was reported. For this purpose, the genotype of Hoa lai and Khao Dawk Mali 105 with low amylose content (16.8% and 19.2%, respectively) was crossed with genotype IR64 with medium amylose content (20-25%), and 120 BC_2F_2 were developed. These BC showed normal distribution for amylose content. The parental genotypes were analyzed with 20 SSR primers in chromosome 6. One primer pair for the locus wxF-R showed association with amylose content. This was further confirmed through selective genotyping. The co- segregation data on the molecular marker (wxF-R) and amylose content on 120 BC_2F_2 were analyzed by means of a single marker linear regression approach. Significant regression suggested linkage between wx and a QTL for amylose content. The results showed that this marker linked to QTLs accounted for the R^2 value of 0.179. This suggested that the wx linked to QTLs contributed to 17.9% of the total in AC content among the BC_2F_2 (IR64 / Hoa Lai). The R^2 value of 0.197 suggested that the wx linked to QTLs contributed to 19.7% of the phenotype variation in AC content among the BC_2F_2 (IR64 / Khao Dawk Mali 105. This marker might be useful in marker-assisted breeding to improve rice genotype with intermediate and low amylose.

***Key words**: Amylose content, backcrossing, microsatellite marker, QTL analysis*

INTRODUCTION

Amylose content (AC), an important determinant of rice starch quality would be attracted by rice breeders. The starch is the major component of rice endosperm and consists of amylose and amylosepectin. The amylose is a relatively less- branched α (1,4) – linked glucose polymer. Starch synthase, which proceduces the (1,4) linkage, is essential for synthesizing linear glucose polymers while the branching enzyme generates the 1,6 linkage in amylopectin.

The improvement in amylose content in rice has been a major concern of plant breeders. The ratio of amylose to total starch, measured as amylose content, varies from cultivar to cultivar, it means 18-32% in indica rice and 10-22% in japonica. The high amylose levels are usually associated with dry, fluffy, and separate cooked rice grain (Juliano 1985). Therefore, improvement of rice starch quality, especially reduction of the amylose level in high yielding rice with poor quality is extremely important for breeding quality rice. The amylose content is usually higher in endosperm starch in indica rice than that of japonica rice has been explained by the presence of two types of waxy alleles, wx^a and Wx^b (Sano 1984). Wang et al. (1995) reported that AC in rice endosperm was related to the post-transcriptional regulation of the *Wx* gene. Genetic studies with rice revealed that a major gene and QTLs were detected for AC in chromosome 6 and 5 (He et al. 1999) and the major gene in chromosome 6 explained 91.1% of the total variation, it should be an allele of *wx* (*Wx*) and QTL qAC-5 explained 11.8% of the total variation, the interval of RG573-C624, a chimeric antisense construct, which contained a 756-bp antisense waxy (*Wx*) gene DNA fragment from rice and the *gus* A coding sequence, both fused to the 3.1-kb rice *wx* promoter, was efficiently introduced into several elite rice cultivars (Liu et al. 2003). However, identification of the gene remained to be discovered. Microsatellite polymorphisms in three genes, the *wx* gene encoding

granule-bound starch synthase I, the SBE gene encoding starch branching enzyme I and the SSS gene encoding soluble starch synthase I were studies for 56 accessions of waxy rice (Bao et al. 2002). This microsatellite might be useful in marker-assisted breeding to improve rice grain quality.

In the present study, the establishment of two BC populations, and its molecular linked map, were use to analyze and identify amylose content polymorphism. Parents and progenies were surveyed by available markers including: *wx* to be most promising of all the above classes of marker therefore used them extensively for study of polymorphism between the parents and subsequently BC_2F_2 representing intermediate, high and low amylose. Marker was identified that showed an association with amylose. Subsequent analysis identified a QTL for amylose, which was assigned in chromosome 6.

MATERIALS AND METHODS

Plant material

Three rice varieties express different amyloses as IR64 (intermediate: 24.5%), Hoa Lai (low: 16.9%), Khao dawk mali 105 (low: 19.2%). Two BC_2F_2 populations of IR64 / Hoa Lai and IR 64/ Khao dawk mali 105 were used this experiment.

For the evaluation of amylose content in endosperm, all 150 BC_2F_2 lines and the parents were grown in the field in 2002 and 2003. After anthesis, all lines were covered to pre-harvest. Grain samples were milled. Amylose content per 100mg of starch granules was colorimetrically determined as described by Miura et al. (1994.)

DNA Extraction for PCR Analysis

DNA suitable for PCR analysis was prepared using a simplified miniscale procedure (Lang 2001). A piece of young rice leaf (2cm) was collected and placed in a labeled 1.5 ml centrifuge tube in ice. The leaf was ground using a polished glass rod in a well of a Spot Test Plate (Thomas Scientific) after adding 400 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 25mM EDTA, 300mM NaCl and 1% SDS). Grinding was done until the buffer turned green which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400 µl of the extraction buffer was added and mixed into the well by pipetting. Around 400 µl of the lysate was transferred to the original tube of the leaf sample. The lysate was deproteinized using 400 µl of chloroform. The aqueous super-natant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. DNA was air-dried and resuspended in 50 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). An aliquot of 1 µl is sufficient for PCR analysis.

Microsatellite analysis

The primers used for amplifying are microsatellites *wxF-R*. Each 25µl amplification reaction consisted of 10mM of Tris-HCL (pH=8.0), 10xPCR buffer (10mM Tris pH 8.4, 50mM KCl, 1.8mM $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit of *Taq* DNA polymerase in a total volume of 20 µ0mM of PCR buffer 0.1mM of dNTPs, 200nM of primers, 0.5 units of *Taq* polymerase, and 20ng of genomic DNA. All DNA amplifications were performed on a PTC 100 thermal cycler under the following conditions: 5min at 94^{o}C, followed by 1minute at 94^{o}C, 60 s at 55^{o}C and 60S at 72^{o}C for 35 cycles and 7 min at 72^{o}C for final extension. An aliquot of 10 µl of the PCR product was routinely taken for gel electrophoresis to determine if amplification was successful. When the primers detected an amplicon length polymorphism, the samples were readily scored. The PCR products or the DNA fragments produced by restriction digestion were resolved electrophoretically on 1.2% agarose gel in 1 X TBE buffer.

The amplification products were mixed with an equal volume of formamide dye (98% formamide, 10mM of EDTA pH 8.0, 0.1% bromophenol blue and xylene cyanol). After being denatured at 94^{o}C for 3 min and immediately chilled on ice, 5µl of the sample was run for through a 5% polyacrylamide gel for 2 hours using due by silver staining

Amplification products were obtained using *wx* primers, developed by Blight et al. 1995, and Ayres et al. 1997, having the following sequences:

wxF 5'-TTTGTCTATCTCAAGACAC-3'

wxR 5'-TTGCAGATGTTCTTCCTGATG–3'

QTL analysis

Single marker QTL analysis using linear regression was applied with the model by Tinker (1996). Marker allele with present band was coded 1 and absent band coded 0 for conducting regression analysis

Analysis of variance (ANOVA) was performed with the SAS program version 6.04 (SAS OHIO US)

RESULTS & DISCUSSION

Amylose content in BC_2F_2

One hundred twenty BC_2F_2 were developed from IR64 / Hoa Lai and 118 individual plants from IR64 / Khao dawk Mali 105. Amylose content varied 19.2% and 16.9% for Khao Dawk mali 105 and Hoa Lai, respectively. Amylose of IR64 was noticed as 24.5 %.

The BC_2F_2 of IR64 / Hoa Lai offered a range of amylose from 17.5% to 25.0%. A frequency distribution curve was set up based on the data, with σg^2 = 1.20**, P=<0.01, heritability value of 0.758 (figure 1)

The BC_2F_2 of IR64 / Khao Dawk Mali 105 offered a range of amylose from 18.0% to 24.0%. A frequency distribution curve was set up based on the data, with σg^2 = 0.81**, P=<0.01, heritability value of 0.746 (figure 2)

Frequency distribution of amylose content in BC_2F_2 showing a good fit to the normal distribution

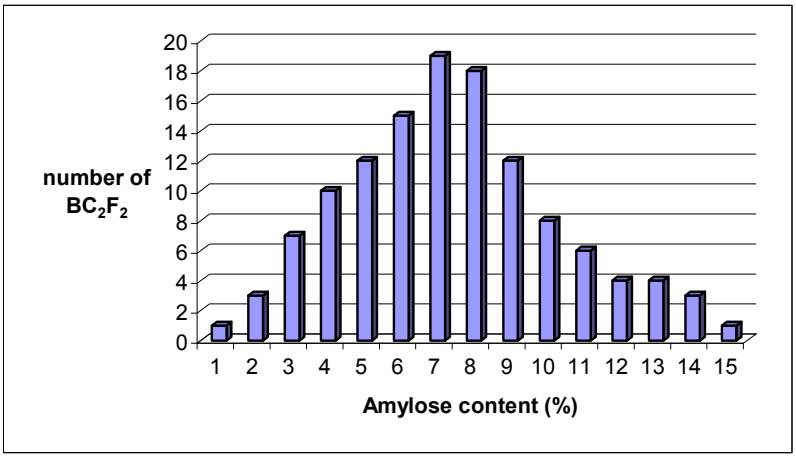

Figure 1 Frequency distribution of amylose content in BC_2F_2 of IR64 / Hoa Lai

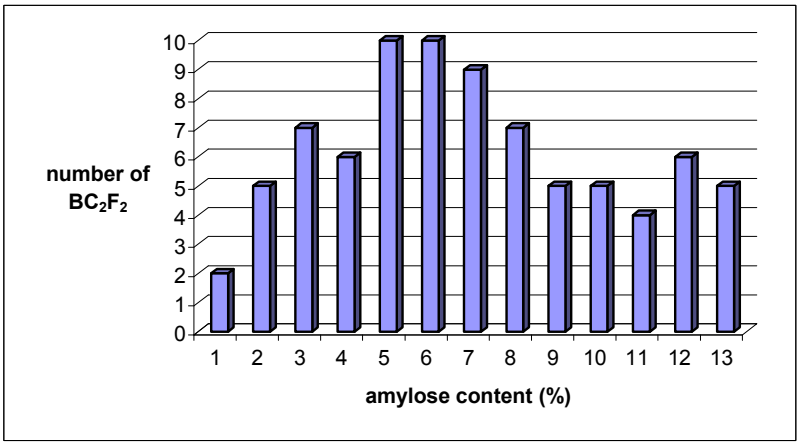

Figure 2: Frequency distribution of amylose content in BC_2F_2 of IR64 / Khao Dawk Mali 105

Microsatellite markers survey in parents

Three SSR markers, which are being successfully employed in marker-assisted selection for genotypes containing amylose different contents. Two markers detected waxy gene in IR64 / Hoa Lai. Then one marker wx detected the gene in both two BC_2F_2 populations.

This is confirmed an association between the wx marker and amylose content. To further confirm this association, we carried out selective genotyping of individual BC_2F_2 to the two populations (figure 3). The result revealed that out of the 52 BC_2F_2 belonging to similar amylose content in IR64. Sixty eight individuals of BC_2F_2 offered the same content in Khao Dawk Mali 105.

For BC_2F_2 of IR 64/ Hoa lai, wx marker was also used to survey. The result revealed that out of 54 belonging to high amylose pool, 7 offered similar content of IR64, 6 offered low amylose pool, 4 expressed a profile similar to Hoa Lai. This is confirmed an associatiation between the wx marker and amylose content. Subsequently, all 112 BC_2F_2 were genotyped using the above SSR primers, and data on segregation of the marker were recorded to conduct QTL analysis.

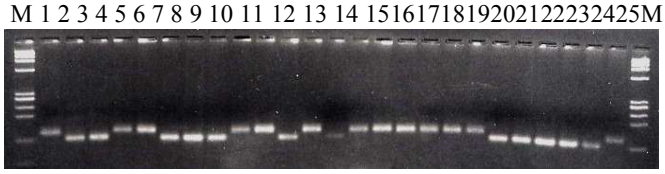

Figure 3: Polymorphism in PCR – amplified microsatellite marker in IR 64 / Hoa lai BC population. Lane 1:IR 64, lane 2: HoaLai, lanes 3-25: individuals of BC_2F_2 with Primer wxF-R

QTL analysis and gene effects

IR64 / Hoa lai

Polymorphism of marker and marker segregation obtained 12% in the parents IR64 and Hoa lai at locus *wx*. The expected segregation ratio would be 40% homozyotes and 60% heterozygotes in BC_2F_2 population. Resulting in an allele frequency of 12.0% for IR64, 28.58% for Hoa lai, and 60% for both parents. Hoa lai was possible as elite parent due to skewed allele frequencies at 34 (22.6%) of marker loci. Skewing toward the adapted elite parent could be explained by selection in the BC_1 and BC_2 during population developed but skewing toward Hoa lai was not expected.

IR64 / Khao Dawk Mali 105

Polymorphism of marker and marker segregation obtained 95% in the parents IR64 and Khao dawk Mali 105 at locus *wx*. Three SSRs were used to evaluate the BC_2F_2. The expected segregation ratio would be 98% homozyotes, 2% heterozygotes. Resulting in an allele frequency was observed as 47.6% in IR64, 51.58% in Khao dawk Mali 105.

From amylose content (AC) data of BC_2F_2 with normal distribution (figure 1), the data on genotypes of these BC_2F_2 at the locus *wx*, helped us analyze QTLs by using single marker linear regression approach. The regression of amylose content conferring to *wx* marker was highly significant indicating a linkage between molecular marker and QTL for amylose content (table 1). The R^2 value of 0.179 suggested that the *wx* linked to QTL contributed to 17.9% of the total in AC content among the BC2F2 (IR64/ Hoa Lai) and. The R2 value of 0.197 suggested that the wx linked QTL contributed to 19.7% of the total in AC content among the BC_2F_2 (IR64 / Khao Dawk Mali 105). These results that marker wxF-R may either be tightly linked to a QTL with a small effect or closely linked to a QTL with a large effect (Melchinger 1998). This is the same result by He et al. (1999), a major gene and a QTL were detected for AC in chromosome 6 and 5. The major gene in chromosome 6 that explained 91.1% of the total variation should be an allele of *wx*. The QTL: qAC-5, explained 11.8% of the total variation.

Table 1: Regression analysis of amylose content on wxF-R marker for IR 64 / Hoa Lai

Source	Degree of freedom	Mean squares	F value	P value
Regression	1	574.9135	527.488	0.0000
Residual	148	1.089909		
Total	149			

Table 2: Regression analysis of amylose content on wxF-R marker for IR 64/ Khao Dawk Mali 105

Source	Degree of freedom	Mean squares	F value	P value
Regression	1	770.0013	*****	0.0000
Residual	149	0.13414		
Total	150			

One locus involved in associated to amylose content was identified in these populations. One QTL was identified by *wx*, with R^2= 17.8% and 19.7% for IR64 / Hoa Lai and IR64 / Khao dawk Mali 105, respectively.

This experiment achieved a considerable enhancement of an available detection of amylose content in BC_2F_2 population. Plant breeders for effective selection can apply the identification of a major gene locus for amylose located nearby a microsatellite marker. Other microsatellite markers could be use to trace the flow of genes or quantitative traits loci of interest in rice and to make predictions about the outcome of crossing and selection program that will increase the future efficiency of cultivar development.

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SUMMARY IN VIETNAMESE

Phân tích di truyền số lượng tính trạng amylose bằng dấu chuẩn phân tử trên quần thể hồi giao của cây lúa (*Oryza sativa* L.)

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Hàm lượng amylose là một trong những tính trạng quan trọng nhất quyết định đến phẩm chất cơm mềm hay cứng. Gần đây, người ta rất quan tâm đến một microsatellite marker (SSR) liên kết rất chặt với gen *Wx* (waxy). Kiểu gen của Hoa Lài và Khao Dawk Mali thuộc dạng amylose thấp (16,8% và 19,2%, theo thứ tự). Chúng được chọn làm bố mẹ để lai với IR64 (giống có amylose trung bình: 20-25%). Sử dụng 120 cá thể BC_2F_2 của tổ hợp IR64 / Hoa Lài, IR64 / KDML. Dạng phân bố chuẩn được ghi nhận trong kiểu hình amylose của cả hai quần thể BC. Sử dụng 20 SSR primer trên nhiễm sắc thể số 6 để xem xét đa hình trong bố mẹ và con lai. Có một cặp mồi định vị tại locus wxF-R kết hợp rất chặt với tính trạng amylose. Số liệu ghi nhận thong qua kết qủa phân tích QTL theo ANOVA với marker đơn, mô hình tuyến tính cho thấy:

17,9% biến thiên kiểu hình tính trạng amylose được giải thích bởi QTL trên nhiễm sắc thể số 6.

Locus *wx* liên kết với QTLs trong quần thể hồi giao BC_2F_2 (IR64 / Hoa Lài) có giá trị R^2 là 0,179 và trong quần thể hồi giao IR64 / Khao Dawk Mali là 0,197. Đây là kết qủa rất tốt cho phép các nhà chọn giống ứng dụng marker này để chọn lọc kiểu gen có hàm lượng amylose trung bình như mong muốn.