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水稻蜡质基因引导区的两个 SSR 序列与直链淀粉含量的相关性

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摘要: 分析了蜡质基因引导区的两个简单重复序列(SSR)$(CT)_n$ 和$(AATT)_n$在 74 份水稻材料中的多态性及其与直链淀粉含量(AC)的关系。这些材料包括了籼稻(*Oryza sativa* L. ssp. *indica*)、粳稻(*O. sativa* ssp. *japonica*)和普通野生稻(*O. rufipogon*), 其 AC 值覆盖了栽培稻 AC 分布的整个范围。以$(CT)_n$作标记检测到 8 个等位基因, 粳稻品种趋于含有重复数目较多($n \geq 16$)的等位基因, 重复次数较少($n \leq 14$)的等位基因只出现在籼稻中。$(AATT)_n$ 检测到 2 个等位基因, 野生稻中少数植株表现出杂合性。分析表明 AC 与这两个 SSR 序列基因型高度相关, 高 AC (>22.0%)品种具有(CT)重复次数较少($n \leq 14$)的等位基因; 相反, 除了糯米外, 所有低或者中等 AC 的品种都有(CT)重复数较多($n \geq 16$)的等位基因。具有重复次数较多的$(AATT)_6$等位基因的品种多为高 AC, 具有重复次数较少的$(AATT)_5$等位基因的品种多为低或中等 AC。不同 SSR 基因型品种间 AC 差异极显著。虽然目前还不能确定这两个 SSR 序列在直链淀粉合成中的直接功能, SSR 变异与 AC 间近乎完全的相关性可作为分子标记直接用于水稻的品质改良。

关键词: 微卫星 DNA; 稻米品质; 直链淀粉含量
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Correlation of Simple Sequence Repeat (SSR) Variants in the Leader Sequence of the *waxy* Gene with Amylose Content of the Grain in Rice

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Abstract: Variation of two simple sequence repeats (SSRs) in the leader region of the *waxy* gene was analyzed in a sample of 74 accessions, including *Oryza sativa* L. ssp. *indica*, *japonica* and wild rice (*O. rufipogon*) representing a wide distribution range of amylose content (AC) in cultivated rice. Eight alleles were detected in the $(CT)_n$ motif and two alleles were resolved in the $(AATT)_n$ motif. The distribution of the alleles of the two SSRs was quite uneven as detected by the $(CT)_n$ motif. The repeat numbers of the two SSR motifs, $(CT)_n$ and $(AATT)_n$, appeared to be inversely related such that the total length of this region was maintained. AC of the varieties was highly correlated with the length of SSRs. Differences in AC among the various SSR genotypes were statistically highly significant as analyzed using genotypes of both SSR motifs. Although the SSR variation did not seem to have obvious function in the synthesis of the starch synthase encoded by the *waxy* gene, the almost perfect correlation between the two SSRs and AC level could be used for quality improvement in rice breeding programs.

Key words: microsatellite DNA; rice quality; amylose content

The *waxy* gene (abbreviated as *Wx*) encodes the granule bound starch synthase (GBSS, EC 2.4.1.11)[1]. Several alleles of the rice *Wx* gene have been identified, cloned and completely sequenced[2,3]. It has been reported that the function of the *Wx* gene is largely responsible for the amylose content (AC) of the endosperm[4,5], which is one of the main determinants for cooking and eating quality of rice[6].

Microsatellites are sequences of tandem repeats (also referred to as simple sequence repeats (SSRs)) with the length of repeats in the range of a few (usually 2 to 4) base pairs (bp). It has been observed that SSRs are

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widely distributed in plant and animal genomes, and are highly polymorphic among species of many organisms[7]. Utilization of SSR markers in genome mapping has been extensively explored in many organisms including crop plants, such as rice, wheat, maize, barley, tomato, soybean, *Brassica* species and tropical forest trees[8]. There has also been evidence indicating that repeat number of SSRs may affect the protein-binding and transcriptional activation of the genes[9].

High level of polymorphism has been reported in the $(CT)_n$ motif in the leader sequence of the rice *Wx* gene. Bligh *et al*[10] found 4 alleles for the $(CT)_n$ motif with repeat number ranging from 8 to 20. Ayres *et al*[11] detected 8 alleles for the same (CT) motif after screening 92 American cultivars. They also found that most of the variation in AC of the endosperm could be explained by the $(CT)_n$ variants.

There is another SSR motif $(AATT)_n$ in the leader sequence of the *Wx* gene residing in the first intron at 182 bp downstream of the $(CT)_n$ repeat, according to the sequence provided by Wang *et al* (GenBank accession number X65183). There has not been reported study in the literature on the variation of the $(AATT)_n$.

In this study, we investigated the diversity of the two SSRs described above using a sample of 74 rice cultivars representing a broad range of rice germplasm, including *indica*, *japonica* and the common wild rice with the widest range of AC distribution. We also investigated the correlation between the SSR variants and AC in this sample.

1 Materials and Methods

1.1 Plant materials and DNA extraction

A total of 66 varieties of cultivated rice (*Oryza sativa* L.) and 8 accessions of the common wild rice (*O. rufipogon*) were used in the study (Table 1). Cultivar IR841-85-1-1-2 was kindly provided by Dr. G. S. Khush, Basmati 370 by Dr. M. Jackson, and Pusa Basmati and Kasuri by Dr. M. Mohan. Eight accessions of the common wild rice were collected from Dongxiang County of Jiangxi Province. All the rice materials were planted in the rice-growing season of 1997 in the Experimental Farm of Huazhong Agricultural University, Wuhan.

Leaves of young seedlings were harvested from the field grown plants and ground to fine powder with mortar and pestle under liquid nitrogen. DNA was extracted according to published method[12].

Table 1 The rice accessions, their SSR genotypes and amylose contents (AC)

Accession	Name	Source	Subspecies	AC	$(CT)_n$	$(AATT)_n$
1	02428	China	*japonica*	10.4	18	5
2	Akihikari	Japan	*japonica*	14.3	18	5
3	IR74	IRRI	*indica*	15.4	18	5
4	Nanjing 11	China	*indica*	25.1	14	6
5	Simiao	China	*indica*	13.7	18	5
6	Xin Simiao	China	*indica*	25.3	14	6
7	IR36	IRRI	*indica*	27.3	14	6
8	Zidao	China	*indica*	18.0	17	5
9	Indonesia Paddy	Indonesia	*indica*	24.9	11	6
10	Zaoshajing	China	*japonica*	15.2	19	5
11	Balilla	Italy	*japonica*	14.2	18	5
12	Lunhui 422	China	*indica*	12.5	18	5
13	Dullar	India	*indica*	27.6	11	5
14	Shengli Xian	China	*indica*	27.1	14	6
15	Hunan Ruanmi	China	*indica*	12.9	18	5
16	CP231/SL017	USA	*indica*	15.2	18	5
17	Della	USA	*indica*	18.9	20	5
18	Zhenshan 97	China	*indica*	27.2	11	6
19	Minghui 63	China	*indica*	15.8	18	5
20	Maoya	Thailand	*indica*	14.0	18	5
21	Suyu Nuo	China	*japonica*	0.0	16	5
22	Basmati 370	IRRI	*indica*	19.9	18	5
23	Guichao 2	China	*indica*	26.8	14	6
24	Teqing	China	*indica*	27.1	14	6
25	Xiang'ai	China	*indica*	27.2	14	6
26	Guang B	China	*indica*	26.9	14	6
27	V20	China	*indica*	28.3	14	6
28	Maxie	China	*indica*	27.9	14	6
29	Ce 64	China	*indica*	24.9	11	5
30	Wuyujing 2	China	*indica*	15.6	18	5
31	Wuyujing 3	China	*indica*	13.4	18	5
32	9311	China	*indica*	14.5	20	5
33	IR841-85-1-1-2	IRRI	*indica*	17.3	18	5
34	Zhenzhuai	China	*indica*	25.9	14	6
35	Wase Aikoku	Japan	*japonica*	0.0	18	5
36	Zhachanglong	China	*indica*	24.9	14	6
37	IR26	IRRI	*indica*	27.1	14	6
38	Tetep	Vietnam	*indica*	27.5	8	5
39	Qiandaijing	Japan	*japonica*	14.6	17	5
40	IR24	IRRI	*indica*	14.8	18	5
41	Jixue Nuo	China	*indica*	0.0	18	5
42	Yifang Nuo	China	*indica*	0.0	18	5
43	Haomei	China	*indica*	20.4	18	5
44	Zhuziqing	Japan	*japonica*	18.7	19	5
45	Basmati 245	IRRI	*indica*	19.2	17	5
46	Mawei Zhan	China	*indica*	22.9	14	6
47	IR72	IRRI	*indica*	24.1	11	5
48	Starbonett	USA	*indica*	19.3	20	6
49	Ye Ao Simiao	Thailand	*indica*	28.2	14	6
50	Dull MH	China	*indica*	0.0	14	6
51	Taihu Nuo	China	*indica*	0.0	19	5
52	Yue Nong 2	China	*indica*	12.7	18	5
53	Xiang Wan	China	*indica*	10.3	18	5
54	Hunan Wan	China	*indica*	9.6	18	5
55	Jianzhen 2	China	*indica*	18.2	18	5
56	Gui 630	Guyana	*indica*	9.6	18	6
57	Gongcheng 3	China	*indica*	13.2	18	5
58	Guanglu'ai 4	China	*indica*	25.7	14	6
59	E Wan 5	China	*japonica*	12.9	18	5
60	Zhonghua 8	China	*japonica*	16.7	18	5
61	Yuchi 231-8	China	*indica*	23.6	14	6
62	Hanfeng	China	*japonica*	16.6	18	5
63	Puqi Ainangu	China	*indica*	27.4	14	6
64	IR34	IRRI	*indica*	27.9	14	6
65	IR8	IRRI	*indica*	28.7	14	6
66	Nipponbare	Japan	*japonica*	12.2	18	5
67	R04	China	Wild	—	11	6
68	R07	China	Wild	—	11	6 5
69	R08	China	Wild	—	11	6
70	R09	China	Wild	—	11	6
71	R13	China	Wild	—	11	6 5
72	R14	China	Wild	—	11	4 5
73	R23	China	Wild	—	14	6
74	R25	China	Wild	—	11	6

—, AC was not analyzed because of no fertile flowers under the long day conditions in Wuhan.

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  1 GGCTTCACGC AACGGCGCTA CAAATGACCA CCCACACCAC CATCTCTCAC CATTCCTTCA
 61 GTTCTCTGTC TATCTCAAGA CACAAATAAC TGCAGTCTCT CTCTCTCTCT TCTCTGCTTC
121 ACTTCTCTGC TTGTGTTGTT CTGTTGTTCA TCAGGAAGAA CATCTGCAAG GTATACATAT
181 ATGTTTATAA TTCTTTGTTT CCCCTCTTAT TCGAGTCGAT CACATGCAT CTTTCATTGC
241 TCGTTTTTCC TAACAAGTAT TCTCATACAT GCTAATTTCT GTAAGGTGTT GGGCTGGAAA
301 TTAATTAATT AATTAATTGA CTTGCCAAGA TCCATATATA TGTCCTGATA TTAAATCTTC
361 GTTCGTTATG TTTGGTTAGG CTGATCGATG TTATTCTAGA GTCTAGAGAA ACACATCCAG
421 GGGTTTCCAG CTAGCTCCAC AAGATGGTGG GCTAGCTGAT CTAGATTTGA AGTCTCACTC
481 CTTCTTAATT ATTTGATATT AGATCATTTT TAATATT
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Fig. 1. Partial sequence of the leader region of the *waxy* gene.
The repeat motifs are in darkened bold letters, and the primers for 484/485, MX4 and MX21 are underlined in solid, dashed and dotted lines, respectively.

1.2 SSR assay

Three pairs of SSR primers were used for PCR amplification (Table 2), all of which amplify the 5′ sequence of the *Wx* gene (Fig. 1)[2, 13]. The primer pair 484/485 was directly adapted from Bligh *et al*[10]; and the other two primer pairs, MX4 and MX21, were adapted from Xiong *et al*[14]. MX4 and 484/485 tagged the same $(CT)_n$ motif, and MX21 tagged the $(AATT)_n$ motif at 182 bp downstream of the (CT) repeat[13]. PCR was carried out essentially as described in Wu and Tanksley[15] except that 10 μL reaction volume was used. Product of sequencing reaction of M13 DNA was used as the molecular weight marker in the polyacrylamide gel electrophoresis.

Table 2 Primer sequences for the amplification of the SSR motif in the leader region of the *waxy* gene

Primer	Sequence	Targeted motif	Reference
MX4	F: GACAAAGAGCCACCCACACC	$(CT)_n$	Xiong *et al*[14]
	R: TTTCCAGCCCAACACCTTAC		
MX21	F: TGCATCTTTCATTGCTCGTT	$(AATT)_n$	Xiong *et al*[14]
	R: ACCCCTGGATGTGTTTCTCT		
484/485	F: CTTTGTCTATCTCAAGACAC	$(CT)_n$	Bligh *et al*[10]
	R: TTGCAGATGTTCTTCCTGATG		

1.3 Measuring AC

Measurements for AC of the rice varieties were as described previously in Tan *et al*[16]. The analysis for each variety was repeated four times and the average of the four replications was used as the measurement for each variety.

2 Results

2.1 Distribution of the SSR alleles

Screening of 74 accessions with the 484/485 primer pair resolved eight alleles with the repeat numbers ranging from 8 to 20 (Fig. 2), which was the same as the results reported by Ayres *et al*[11]. The distribution of the alleles was quite uneven. Three varieties had the allele $(CT)_{20}$, 3 varieties had allele $(CT)_{19}$, 29 varieties had allele $(CT)_{18}$, 3 varieties had allele $(CT)_{17}$, 1 variety had allele $(CT)_{16}$, 22 varieties had allele $(CT)_{14}$, 12 varieties had allele $(CT)_{11}$ and 1 variety had allele $(CT)_8$. Four of the 8 alleles, $(CT)_{19}$, $(CT)_{18}$, $(CT)_{17}$ and $(CT)_{16}$, were found in *japonica* varieties; all the alleles, except $(CT)_{16}$, were detected in *indica* varieties; and 2 alleles, $(CT)_{14}$ and $(CT)_{11}$, were observed in the wild rice accessions (Table 3). Thus, *japonica* varieties appeared to be preferentially associated with larger repeat numbers, whereas shorter repeats, alleles $(CT)_{14}$, $(CT)_{11}$ and $(CT)_8$ occurred only in *indica* varieties (Table 1).

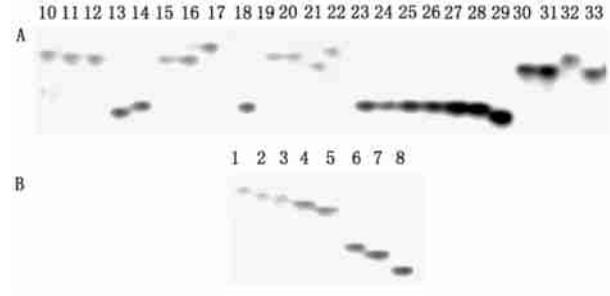

Fig. 2. SSR genotypes of the selected accessions amplified using the 484/485 primers targeting the $(CT)_n$ motif.
Panel A shows the various SSR genotypes amplified from the accessions. The numbers on top of panel A correspond to the accession numbers in Table 1. Panel B illustrates the deduced alleles that are as follows: 1, $(CT)_{20}$; 2, $(CT)_{19}$; 3, $(CT)_{18}$; 4, $(CT)_{17}$; 5, $(CT)_{16}$; 6, $(CT)_{14}$; 7, $(CT)_{11}$; 8, $(CT)_8$.

Only 5 alleles were detected by the MX4 marker in the same 74 accessions, although MX4 tagged the same $(CT)_n$ motif as did 484/485[2, 13]. This was largely due to the higher molecular weight of the PCR products amplified by this marker, which could not be well separated in the electrophoresis.

Three alleles were detected by the marker MX21 (Fig. 3). All three alleles were observed in the wild rice accessions but only two alleles were detected in cultivated rice varieties. According to the sequences of Cai *et al*[5],

Table 3 The average of the amylose contents (AC) value associated with each of the SSR genotypic classes

SSR motif and repeat number	Number of non-waxy variety	Number of waxy variety	Amylose content[1)]	Significance[2)]
(CT)				
8	1	0	27.5±0.0	A
11	5	0	25.7±1.6	A
14	20	1	26.6±1.6	A
16	0	1	—	—
17	3	0	17.3±3.8	B
18	26	3	14.3±2.8	B
19	2	1	17.0±2.5	B
20	3	0	17.6±2.7	B
n≤14	26	1	26.4±1.5	A
n≥16	34	5	15.0±3.1	B
(AATT)				
5	36	5	16.2±4.5	B
6	24	1	25.5±4.0	A

1) Only the non-waxy varieties were included in the calculation. 2) The groups were assigned using Duncan's multiple range test.

Fig. 3. SSR genotypes of the selected accessions amplified using MX21 primers targeting the $(AATT)_n$ motif.
Panel A shows the various SSR genotypes amplified from the accessions. The numbers on top of panel A correspond to the accession numbers in Table 1. Panel B illustrates the three alleles and the genotypes detected in the eight wild rice accessions.

the two alleles observed in the cultivated rice were $(AATT)_5$ and $(AATT)_6$, and the third allele can be deduced to be $(AATT)_4$. Two accessions of the wild rice *O. rufipogon*, R07 and R13, were heterozygous for alleles $(AATT)_5$ and $(AATT)_6$, and R14 was heterozygous for alleles $(AATT)_4$ and $(AATT)_6$ (Fig. 3b). The remaining varieties and accessions were homozygous for either $(AATT)_5$ or $(AATT)_6$.

The repeat numbers of the two SSR motifs, $(CT)_n$ and $(AATT)_n$, appeared to be inversely correlated such that the total length of this region was maintained (Table 1). For example, when the $(CT)_n$ motif had a larger number of repeats, e.g., $n \geqslant 16$, the $(AATT)_n$ motif would have a smaller number of repeats ($n=5$). Conversely, when the $(CT)_n$ motif had a smaller number of repeats, i.e., $n \leqslant 14$, the $(AATT)_n$ motif would have a larger number of repeats ($n=6$).

2.2 Correlation between the *Wx* alleles and AC

AC of the varieties was clearly correlated with the length of SSRs according to the data in Table 1. For the $(CT)_n$ motif amplified using 484/485 primer pair, all the varieties of high AC (e.g. > 22%) had alleles with smaller numbers of repeats, e.g., $n \leqslant 14$; and conversely, all varieties of low to intermediate AC, except waxy rice varieties, had alleles with larger numbers of repeats, i.e., $n \geqslant 16$. Results of one-way ANOVA showed that the differences in AC among the various SSR genotypes were statistically highly significant; the genotypes of $(CT)_n$ repeats could explain 81.2% of the AC variation among the varieties used in this study.

For the $(AATT)_n$ motif amplified with the MX21 marker, majority of the varieties with high AC had the $(AATT)_6$ allele, whereas most of the varieties with low AC had the $(AATT)_5$ allele (Table 1). One-way ANOVA showed that the AC difference among the $(AATT)_n$ genotypes were statistically highly significant; SSR genotypes could account for 72.9% of the AC variation among the rice varieties.

The situation for the waxy varieties was somewhat complicated. Five of the 6 waxy varieties had $n > 16$ for the $(CT)_n$ repeats, but one waxy variety was observed to have $(CT)_{14}$ (Table 1).

3 Discussion

The most important outcome from this study is the nearly perfect correlation between SSR genotypes and AC among the varieties analyzed in this study. Microsatellite instability (MSI) caused by malfunction of DNA repair system has been reported to be associated with cancer susceptibility in mammals[17-19]. Thus, one question about the high level of polymorphism of the two SSR loci is the possible role of the SSR variants in controlling of the AC level or other functions of the *Wx* gene. According to the *Wx* gene sequence provided by in literature[2,5,13], the $(CT)_n$ motif is located in the first exon of the leader sequence not far from the transcription start site, and the $(AATT)_n$ motif is in the intron of the leader sequence. Several researchers reported incomplete splicing of intron 1 in cultivars of intermediate AC, which decreased expression of *Wx* gene[4,5]. Their results clearly showed that the aberrant splicing is the cause for the reduced AC, although the pattern of the splicing differed from one cultivar to another. Their results also showed that all the patterns of aberrant splicing involve nucleotides at or near either the donor or receptor site of splicing, whereas both of the SSR motifs are located at least a hundred nucleotides away from these sites. Thus, it is not clear whether these two SSR motifs have any direct role in specifying the AC.

We previously observed that, in addition to AC, another two traits of the rice endosperm, namely, gel consistency and gelatinization temperature, are also controlled by the *Wx* gene or tightly linked region[16]. Thus, high

correlation of the SSRs with AC also implies high correlation of the SSRs with these two traits. It should also be interesting to understand the molecular nature of gel consistency and gelatinization temperature in future studies.

All the three traits are important components of the starch quality of the rice endosperm. Thus, regardless of the role of the SSRs in the biosynthesis of the starch synthase, these two SSRs are very useful markers for marker assisted selection for grain quality improvement in rice breeding programs.

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