Genotyping common SNP and a microsatellite sequence closely linked to waxy gene in rice by DNA based markers

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Abstract

The potential of different DNA based molecular markers was examined for the detection of single nucleotide polymorphism (SNP) in the waxy gene and a microsatellite (SSR) sequence closely linked to it in a collection of rice varieties. DNA was extracted from leaf samples of 68 different rice cultivars by the CTAB method and specific primers were designed for the amplification of waxy gene and SSR marker loci. After amplification of the desired fragments by polymerase chain reaction (PCR) genotyping of samples was carried out by the melting curve analysis technique. To confirm the results of the melting curve genotyping, two methods of PCR-SSCP and PBR were used. The presence of two genotypes (TT and GG) in the waxy gene observed from these two methods was perfectly consistent with the results of the melting curve analysis. The frequency of each TT and GG genotype was estimated 25 and 75 percent, respectively. Nine different genotypes including BH, HH, FF, DD, CC, EE, BB, AA and EG were found in the SSR marker site. This indicates the existence of a high genetic variability in the studied varieties. Although, there were no associations between the G-T polymorphism with the traits, the association of different patterns in SSR marker with the GW, TGW and FGN traits was significant. The results showed that the melting curve analysis technique is a fast and inexpensive method that can be used for polymorphism detection. Morever, this SSR marker site can be used as a suitable marker for detecting some economical traits in rice breeding.

Key words: Melting curve analysis, Microsatellite marker, PBR, PCR-SSCP.

INTRODUCTION

The amylose content and its gelatinization temperature of rice grain are the most important factors for the determination of rice texture, quality and processing properties (Ayres 1997). These factors are considered by plant breeding specialists to improve rice quality. Since consumers prefer high amylose content rice (over 26%), investigation on discovering variations between different varieties with high amylose content is increasing. Low Amylose content rice (1-2%) varieties are called waxy. Rice varieties are divided into two sub-classes (waxy and non-waxy) based on the grain amylose content. Since waxy rice varieties absorb a little amount of water during cooking and are also very soft and sticky after cooking, they are less noticeable for consumers, but non- waxy rice varieties (19 to 21% amylose content), such as Tarom do not stick together, are separate after cooking and will remain soft for a long time. Therefore, these varieties are more considered by consumers. Rice contains two polymeric forms of glucose, including non-branched amylose and branched amylopectin. Amylose contains 16-30% starch that has a key role in the cooking and preparation of rice. Amylose synthesis is done by granol-bound starch synthase via the waxy gene, located on chromosome 6 (Smith *et al.,* 1997; Chen *et al.,* 2008). Numerous studies have

shown that the waxy gene is a major gene for the flavor and quality traits in rice (Sano *et al.,* 1985; Wang *et al.,* 1995; Larkin and Park 2003; Liu *et al.,* 2003; Lestari *et al.,* 2009; Yi *et al.,* 2009). The gene is responsible for the synthesis of amylose, which is a key factor in the process of cooking rice. According to the importance of this gene in rice quality, the evaluation of variation and identifying various forms of alleles in this locus is very important. Recognition of genetic differences between individuals and determination of their effect on individual's performance is the genetic basis in genomic research. SNPs are considered as the most common kind of genetic variation and also, the gene markers have been considered as a criterion for an individual's performance recognition. Genetic diversity involves many applications in biological fields such as plant breeding, phylogenetic studies, population genetics and evaluation of plant pathogenic factors. Nowadays, to evaluate the genetic variation and identification of polymorphism, different methods such as molecular markers are used (White and Potts 2006). Melting curve analysis technique is one of the most economical methods for genotyping of samples. Melting curve analysis is easier and less costly than the probe-based methods, and unlike SSCP-PCR, PBR, DNA sequencing, and HPLC, it is performed at a low cost and in a less time. As a closed-tube method, it has a less possibility of contamination of the PCR products. HRM method, in addition to the genotyping SNPs at the DNA level, has also other applications such as detection of mutations, species identification, DNA mapping, DNA methylation analysis, detection of internal tandem duplications, haplotype data analysis, DNA fingerprints and etc (Vaughn and Elenitoba-Johnson 2004). In the new high resolution melting method, all types of DNA polymorphisms such as SNP and deletion or insertion of nucleotides are detectable (White and Potts 2006). The aim of this study was to detect SNPs in the waxy gene, a closely-linked SSR marker to it and to evaluate the possible association between the genotyped marker loci with some quality traits in different rice varieties.

MATERIALS AND METHODS

DNA extraction

DNA was extracted from leaf samples (500 mg from each sample) from 68 different rice varieties (Table 1), provided by the research farm of Tabarestan Institute of Genetics and Biotechnology with the CTAB method (Doyle, 1987). In the present study, analysis of the waxy gene was performed targeting di-nucleotides CT-microsatellite in exon 1 and a G–T SNP at the 5′ splice site of the first intron. The quality and quantity of DNA samples were tested by spectrophotometry and PCR-products were tested by electrophoresis on a 2% agarose gel.

Detection of G-T polymorphism by melting curve analysis

Using a pair of specific primers (Forward: 5'- TCTGCTTCACTTCTCTGCTTGTGT -3' and Reverse: 5'-TTTCCAGCCCAACACCTTACAGA -3'), designed by VectorNTI9.0 software, a 187-bp fragment from intron 1 region of the waxy gene was amplified. In this study, the Rotor Gene 3000 instrument was used for melting curve analysis.

Detection of G-T polymorphism by PBR and PCR-SSCP analysis

Two methods of PCR-SSCP and PBR were used in order to confirm the genotyping results obtained from the melting curve analysis. For PBR, 7 µl of each PCR product was digested with 1 unit of *Acc*I endonuclease (Fermentas) in a final volume of 15 µl. The mixture was incubated for 13 h at 37 °C. The digested product was electrophoresed on 2% agarose gels and stained by the ethidium bromide solution. The SSCP-PCR analysis was carried out on a 14% polyacrylamide gel and silver nitrate staining.

Microsatellite analysis

To evaluate the SSR markers closely linked to the waxy gene, a part of the first exon of this gene was amplified by a pair of primers including F: 5′- CTTTGTCTATCTCAAGACAC-3′ and R: 5′- TTGCAGATGTTCTTCCTGATG-3′(Ayres 1997). PCR reaction was conducted in a 25 µl reaction mixture containing 100 ng of template DNA, 1× PCR buffer (CinnaGen, Iran), 1 mM $MgCl_2$, 200 mM of dNTPs, 1 mM of each primer and 1 unit of Taq DNA polymerase (CinnaGen, Iran). The PCR profile was set at 95 °C for 5 min, followed by 35 cycles of 94 °C for 60 s, 52 °C for 60 s, 72 °C for 60 s, and the final extension for 5 min at 72 °C. The PCR products were genotyped using a 8% polyacrylamide gel in 1× Tris-Borate EDTA buffer (TBE). The gel was run at 250 V for 14 h and stained with silver nitrate solution. The molecular size of the amplification products was estimated using a 50 bp ladder (Invitrogen). The gels were scored and different alleles were detected using Quantity One and Total Lab software.

Statistical analysis

The genotype frequency of the waxy gene was tested using the Popgene version 1.32 software for Hardy-Weinberg equilibrium (HWE). P-value ≥ 0.05 was considered a deviation from the equilibrium. The GLM

Table 1. Identification of waxy microsatellite and G–T SNP variations in 68 rice varieties.

Sample No.	Variety	SSR	G-T	Sample No.	Variety	SSR	G-T
1	BINAM219	CC	GG	36	PR111	AA	TT
2	SANGJO	FF	GG	37	IR06N213	AA	GG
3	HASANSARAII2	FF	GG	38	MTU1108	EE	GG
4	GHEREBO	BB	TT	39	PK82454	BH	GG
5	SEPIDROD63	DD	GG	40	HARDINATH1	FF	GG
6	AHLAMITAROM6	DD	GG	41	IR72	.	GG
7	CRIPTO49	EG	GG	42	OMCF6	CC	GG
8	BINAM280	CC	TT	43	IR10N105	CC	GG
9	MOSATAROM	HH	GG	44	BASMATI370	.	GG
10	IR58	CC	GG	45	MTU1081	CC	GG
11	RASHTI	DD	GG	46	CB05501	CC	GG
12	AMOL3	CC	GG	47	IR50	BB	GG
13	SADRI	CC	GG	48	NEAMAT	DD	GG
14	TAROMAMIRI	CC	GG	49	CO47	BB	GG
15	IR24	.	GG	50	AG7	BB	GG
16	RIBE	EE	TT	51	IR7858112322	AA	GG
17	DASHT	EE	GG	52	IR 64	BB	GG
18	SARDAK	CC	GG	53	SACG-7	AA	TT
19	AHLAMITAROM	DD	GG	54	WANXIAN 763	HH	TT
20	ROMA	BB	TT	55	HUA564	AA	TT
21	HASANSARAI1	FF	GG	56	HUA565	BB	GG
22	PSBRC2(IR328092633)	EG	GG	57	RC8	BB	GG
23	BP10620FBB815BB4	CC	TT	58	PSB RC18(IR51672)	BB	GG
24	CT145441M233MMMSR	DD	GG	59	SACG4	DD	GG
25	CB05219	EG	.	60	HEXI41	AA	TT
26	IR07A109	CC	GG	61	LUYIN46	DD	.
27	UPR184031116	FF	GG	62	CAU1	BB	GG
28	OM4900	CC	TT	63	PAU-3111-25-5-1-2	EE	GG
29	PAU201	BB	TT	64	MASUL1	.	GG
30	BR692631454	BB	GG	65	YUNJING 23	DD	GG
31	PANTDHAN19	BH	TT	66	PATNAGIRI 711	FF	.
32	LOCALCHECK (SPECIFYNAME)	DD	GG	67	CT17330	EE	GG
33	PR31132B11133	DD	GG	68	AAI R2	EE	.
34	B12743MR1823	BB	GG				
35	IR09A105	AA	GG				

procedure was carried out for the association study between polymorphisms, based on DNA markers and the traits including NTT, number of total tillers, FGN, number of filled grain/panicle; UFGN, number of unfilled grain/panicle; PL, panicle length; PH, plant height; GL, grain length; GW, grain width; LWR, grain length/width ratio; TGW, 1000-grain weight; Flw - Days to 50% flowering and PAccp - Phenotypic acceptability (1- excellent; 3- good; 5- fair; 7- poor; 9- unacceptable). All statistical analuses were considered significant with a level of $p \leq 0.05$. The differences between means were tested using Tukey's test and were considered to be significant at $P< 0.05$. All statistical analyses in our study were carried out with SAS 9.1.

RESULTS AND DISCUSSION

Genotyping by melting curve analysis

In this study, two genotypes (TT and GG) were observed in the waxy and non-waxy rice cultivars (Figure 1). It is noticed that the heterozygote genotypes (TG) were not observed in our study. Our results showed the HRM can easily discriminate the different genotypes. Recently, several studies have used the melting curve analysis for genotype determination and molecular diagnostics (Bullock *et al.*, 2002; Kwok and Chen 2003; Vaughn and Elenitoba-Johnson 2004; Yeh *et al*., 2004; Pont-Kingdon and Lyon 2005; Minucci *et al.,* 2010; Montgomery *et al.,* 2010; Ganopoulos *et al.*, 2011). In order to genotype five wheat varieties, the melting

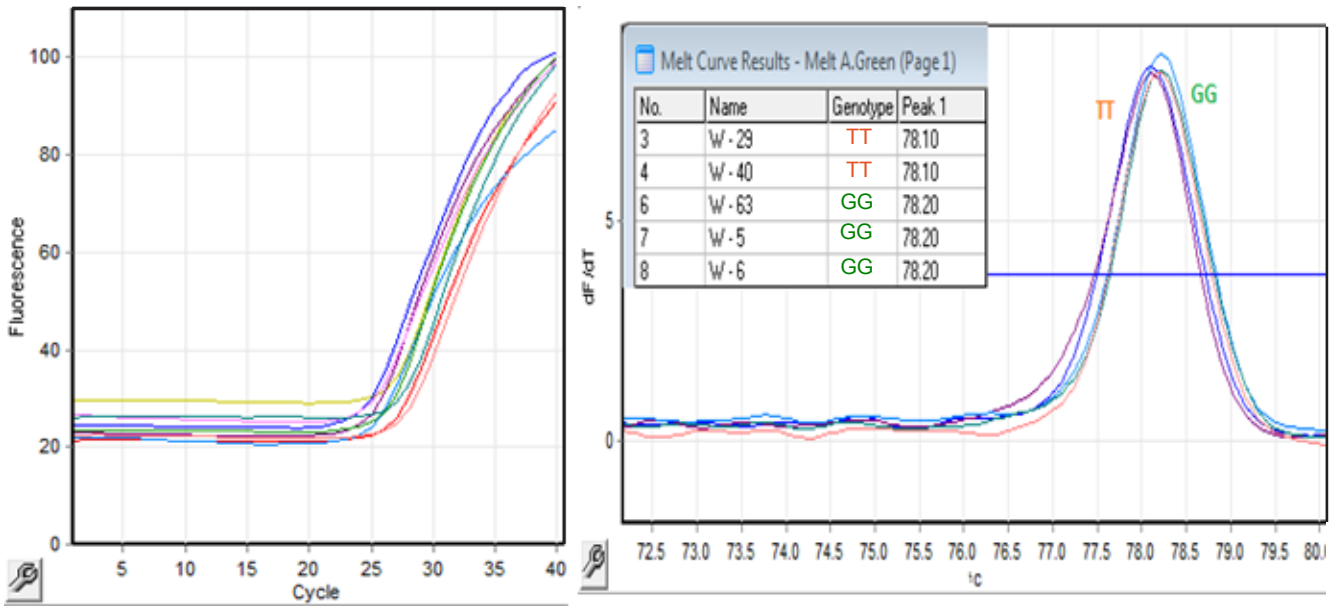

Figure 1. The amplification procedure and two genotypes of TT and GG detected by the melting curve analysis.

curve analysis was used and different genotypes were identified by this method (Shepherd and Henry 1998). Deylami *et al*. (2009) in a study for the evaluation of the SNP in *Plasmodium falciparum* resistant to chloroquin reported that using the melting curve analysis technique by Real-time PCR could potentially be a remarkable way to detect the single nucleotide polymorphisms (Deylami *et al.,* 2009). Also, Omrani and Ansari (2009) genotyped hepatitis C virus in the north west of Iran by melting curve analysis method and reported that the melting curve method could be used for routine HCV genotyping. In order to fingerprint three hybrid F1 rice cultivars and their parental lines, the high resolution melting curve analysis was used by Zhu *et al.* (2013). Their results suggested that the high resolution melting curve analysis could be used as potential, efficient and valuable methods for fingerprinting hybrid rice cultivars.Also high resolution melting curve analysis should be given priority compared with capillary electrophoresis for its high accuracy and high efficiency.

HRM can be used for SSR, InDel and SNP genotyping in rice. Comparing with PAGE, HRM has the advantages of high resolution, high through-put, simplicity and safety. It is a promising technology for molecular marker analysis in rice (Junliang *et al.,* 2011). Recently, several studies have shown that the high resolution melting curve analysis is an efficient method in plant research (Akiyama *et al.,* 2009; Tan *et al.,* 2013;Simko 2016). In general, according to our results and also previous results, HRM could be useful for genotyping and also for breeding programs in plants.

Genotyping by PBR and PCR-SSCP analysis

To confirm the detected SNP by melting curve analysis, two PBR and PCR-SSCP techniques were used. As expected, the obtained results of the PBR method and the PCR-SSCP band patterns showed both T and G alleles and TT and GG genotypes (Figure 2) with allelic and genotypic frequencies of 25 and 75 percent, respectively.

It was shown that a single nucleotide mutation can be involved in the mRNA processing and therefore, it could have a significant effect on the amylose content (Ayres *et al.,*1997). Several single nucleotide polymorphism are associated with the waxy gene such as CT repeat sequences in the 5'-UTR (Bligh *et al.,* 1995), single nucleotide polymorphism (T / G) at the splicing position in the first intron (Wang *et al.,* 1995), two SNPs in exon 6 and a 10 (Larkin and Park 2003) and 23-bp duplication in exon 2 (Wanchana *et al.,* 2003).

Microsatellite analysis

In this study, nine banding patterns were observed with SSR markers (Figure 3) and Jayamani *et al*. (2007) and Chen *et al*. (2008) reported nine SSR banding patterns in international rice cultivars. However, the size of the observed amplified fragments ranged between 107 to 127 bp in both mentioned

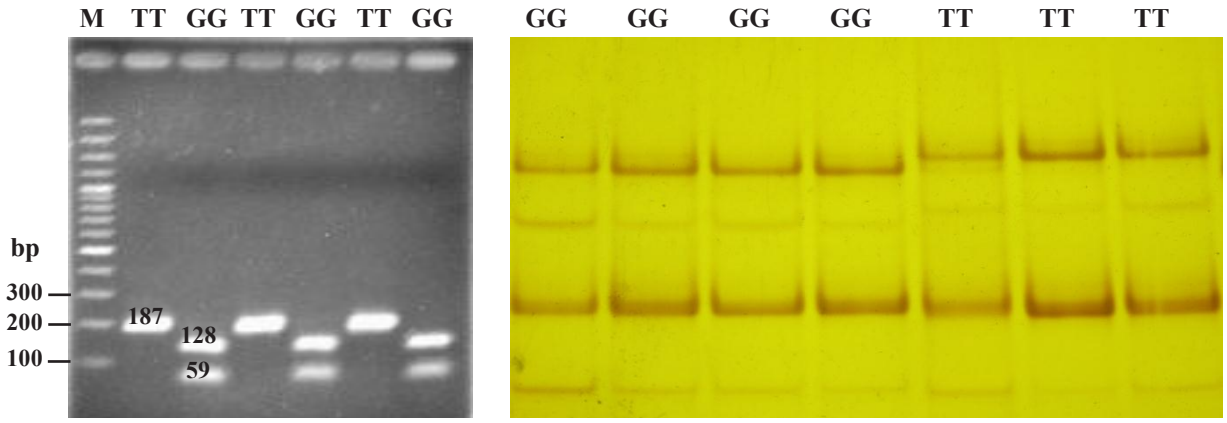

Figure 2. The samples of genotypes resulting from the waxy gene, using PBR and PCR-SSCP techniques.

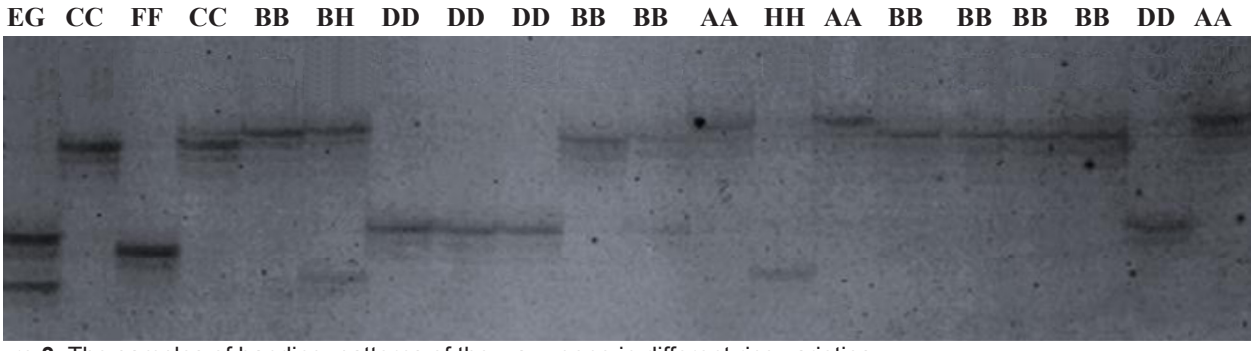

Figure 3. The samples of banding patterns of the waxy gene in different rice varieties.

studies which is consistent with our results.

In the present study, both homozygote and heterozygote genotypes were observed for microsatellite marker site in the waxy gene region. The frequency of homozygote (95%) was higher than heterozygote (5%) genotypes while in Jayamani *et al.* (2007) study, all genotypes were homozygote.

Statistical analysis

Chi-square test was used to determine whether the subjects met the Hardy-Weinberg equilibrium. We confirmed that none of the markers were compatible with the HWE ($p<0.01$) equilibrium. Furthermore, the association of different patterns in SSR marker with the GW, TGW and FGN traits was significant. However, we found no association between G-T polymorphisms in the waxy gene marker site and the mentioned traits.

Melting curve is a powerful technique for the detection of SNP. Since other techniques for genotyping suffer from high costs, time consuming, involve multiple steps and are expensive procedures, the melting curve analysis could be preferred. In other hand this technique has profound applications such as detection of mutations, discovering heterozygosity, DNA fingerprinting, species identification, evaluation of the ratio of somatic mutations, haplotype studies, estimation of the allelic frequency in populations and identifying the candidate genes, using low–cost dyes.

As the reports have shown, polymorphisms are useful to identify the alleles and variations between different cultivars and also, these polymorphisms are associated with high amylose content and thus could be effective on the rice quality. Wang *et al*. (1995) reported that a single nucleotide mutation in the splicing position at the first intron could be involved in mRNA processing, which subsequently was confirmed by Ayres *et al.* (1997) (cited in Wang *et al.,* 1995). Additionally, Ayres *et al*. (1997) have shown that 30 varieties with

low amylose content had AGTTATA sequences in the 5′splicing region in introns, while 59 varieties with intermediate and high amylose content had AGGTATA sequences. The researchers identified a G to T single nucleotide polymorphism that could explain79.7% of the variation in amylose content from non-waxy varieties. Hirano *et al.* (1998) showed that varieties containing AGGTATA sequences had Wx^a allele while varieties containing AGTTATA sequences had Wx^b allele (Hirano *et al.,* 1998). The difference between the two alleles is considered labile for the variation in the wx gene product levels in the endosperm and the difference in amylose content. Although, several studies have reported a significant association between this polymorphism and amylose content, however, these two alleles alone are not sufficient to explain all the observed variations in amylose content in rice varieties. In a study, on the waxy gene microsatellite and single-nucleotide polymorphisms for developing Japonica varieties with desired amylose levels in rice, it was reported that a high percentage of variation (86.5%) was justified by the polymorphisms present in the haplotypes. (Jayamani *et al.,* 2007). In addition, in another study it was shown that the shorter repeat alleles such as CT10 and CT11 and the sequence AGGTATA at the 5′-leader intron splice site in all studied varieties were associated with an apparent amylose content higher than 24%. Furthermore, researchers suggested that the varieties with desirable amylose levels can be improved quickly using both the Wx microsatellite and G-T SNP (Cheng *et al.,* 2012). Recently, the nucleotide diversity of the waxy gene in Chinese Micro core Rice Germplasm was evaluated. The results of the mentioned study showed 51 and 226 SNPs or insertions /deletions in the cultivated and wild rice varieties, respectively. The results showed that the diversity in the wild rice accessions was higher than the cultivated ones. Also, the association study between the polymorphisms of CTn microsatellite, G/T SNP, and 23-bp insertion in the waxy gene and amylose content in the cultivated rice revealed that the G/T allele and a 23-bp insertion had better association with amylose content than it had with the CTn alleles. (QIAO *et al.,* 2012).

CONCLUSION

Our findings indicate the presence of a high variation among different rice varieties in the waxy gene. This could be used as a useful marker to select for amylose content and qualify for rice breeding. On the other hand, recent advances in molecular genetics play an important role in the plant breeding and genetic programs. This is the first time that the technique of melting curve analysis was performed without the use of labeled probes. The results of our study showed that this technique can detect single nucleotide polymorphism in the waxy gene. The employment of this technique is suggested since traditional methods have some limitations and MCA technique possesses advantages over them. In general, the present results suggest that the mentioned markers could be used as molecular tools by the rice breeders to improve quality and desired amylose content in Iranian and other varieties of rice.

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