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Variation on Starch Properties and the Relationship to Single Nucleotide Polymorphism in SSIIa in Waxy Rice Collected from Central of Vietnam

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Twenty two waxy rice cultivars originated from Central Vietnam were used for studying the variation on starch properties and the relationship to single nucleotide polymorphism in the SSIIa gene. A wide range of alkali digestibility level among 22 waxy rice cultivars was observed and recorded as low, intermediate and high alkali digestibility groups. All 22 waxy rice cultivars has significantly higher proportion of fa (DP≦12) and markedly lower proportion of fb1 chains with DP from 12 to 24 whereas the little difference was observed in proportion fb2 (25≦DP≦36) and fb3 (DP≧37) between waxy cultivars tested and IR36. The nucleotide changes in three exons (exon 1, exon 2 and exon 8) were observed in 22 waxy rice cultivars. Of the six single nucleotide polymorphism (SNPs) in the coding region, two SNPs, C/T (at site 516 bp) in exon 2 and G/T (at site 3903) in exon 8, were silent substitution while other four caused amino acid replacement. The SNP at position 264 bp in exon 1, was found an G–to–C transition causing change of glutamic to aspartic while the SNP at 3,799 bp in exon 8, resulting in glycine to serine change. The SNP at 4,198 bp, causing methionine to valine. The SNP at 4,329–4330 bp was observed a GC–to–TT transition leading to change of glycine–leucine change to glycine–phenylalanine. The study suggested that the wide variation on alkali digestibility and amylopectin fine structure in waxy rice starch was caused by the nucleotide diversity of SSIIa gene besides of other starch synthase genes involving in amylopectin synthesis.

Key words: rice, starch properties, amylopectin, SSIIa, SNP

INTRODUCTION

Rice is main food for 17 countries in Asia and the Pacific, some countries in Africa and Latin America. Being consumed like staple food, rice provides 27% of dietary energy supply and 20% of dietary protein intake.

Quality of rice is determined by key components such as appearance, milling quality, cooking and eating quality and nutritional quality. Comprised up 90% of milled rice, endosperm starch properties play a critical role in eating and cooking quality of rice. The physicochemical properties for starch rice is affected by the the ratio of amylose to amylopectin and the structure of starch granule. Amylopectin constitutes up to 70–80% of rice starch and consists of α–1–4 linked glycosidic chains and branched with α–1–6 linked glucosidic chains. The variations of cluster fine structure are responsible for the variations on starch physicochemical properties between species (Nakamura *et al*., 2005). Some previous studies reported that there are relations between amylopectin fine structure and the disintegration of starch granule in alkali solution as well as gelatinization temperature in rice starch (Nishi *et al*., 2001; Umemoto *et al*., 2002). Starch granule containing amylopectin with longer A and B1 chains is predicted to be resistant to gelatinization and less soluble in alkali solution. Nakamura used ACR value (the ratio of the short chains of DP≦10 to the short and intermediate chains of 12≦DP≦24) for characterizing the structure of amylopectin of rice cultivars. He reported that the fine structure of amylopectin in endosperm of 129 rice cultivars can be divided into two types, L–type and S–type based on the ACR value(Nakamura *et al*., 2002). S–type amylopectin cultivars had low GT starches and L–type amylopectin cultivars had intermediate or high GT (Nakamura *et al*., 2006).

Two most important quality indicators, including amylose content and gelatinization temperature (GT) are used as determinant for cooking and eating quality of rice. Gelatinization property is measured by the degree of disintegration of rice starch granule in potassium hydroxide (Little *et al*., 1958). Because the susceptibility to alkali disintegration correlated inversely with gelatinization temperature of rice (Juliano *et al*., 1964), the alkali digestibility test is used to index gelatinization properties. One nature variation in rice, alkali disintegration, may also affect grain quality (Umemoto *et al*., 2004).

Using gene–mapping analysis, Umemoto reported that the starch synthase IIa (SSIIa) gene is located at the alk locus on chromosome 6 in the rice genome (Umemoto *et al*., 2002). Recently, the relationship between the single nucleotide polymorphism (SNP) of SSIIa gene and amylopectin structure and starch properties in rice was reported (Umemoto *et al*., 2002; Umemoto *et al*., 2004; Nakamura *et al*., 2005; Waters *et al*., 2006; Bao *et al*., 2006). The physicochemical properties in rice starch, which differ from variety to variety, are related

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to their structure. Therefore, characterization of starch properties and the relation to nucleotide diversity of starch genes in local rice is necessary to provide valuable information for the improvement rice quality program.

Central and Northern mountainous areas in Vietnam are considered to be the regions of richness diversity in rice genetic resources (Chang, 1976b; Okuno *et al*., 1996, Suu *et al*., 2012). In our previous study, ninety eight rice cultivars collected from Central Vietnam were used to investigate the variation on grain quality (Suu *et al*., 2012). In this study, twenty two waxy rice cultivars were chosen from above mentioned ninety eight rice cultivars for studying the variation on starch properties and relationship to single nucleotide polymorphism in SSIIa gene.

MATERIALS AND METHODS

Plant materials

Twenty two waxy rice cultivars collected from Central Vietnam were used in this study. Two rice cultivars including IR36 (*Indica*), TC65 (*Japonica*) were used as controls.

Evaluation of Alkali digestibility

The degree of alkali digestibility value was recorded after 24 hour soaking rice kernels in 1.3% KOH solution at 25°C. The alkali digestibility was scored by method of Little *et al*. (1958) and was classified as low (1–3), intermediate (4–5) and high (6–7).

Chain–length distribution analysis of amylopectin by capillary electrophoresis

Measurement of the amlopectin chain–length distribution of amylopectin was performed according to Nakamura method (Nakamura *et al*., 2002).

Three matured dry seed free from the embryo were crushed to fine powder. 20 mg of seed powder from each sample was put into the glass tube and suspended in 5 ml of methanol and boiled for 10 minutes. The homogenate was centrifuged at 2,500 rpm for 5 minutes and the precipitant was collected and washed with 5ml of 90% v/v methanol twice. After washing the precipitant was suspended in 285 μL of distilled water and 15 μL 5 N NaOH and boiled for 5 minutes and then cooled at room temperature. The cooling sample test tube was added with 9.6 μL of 100% CH_3COOH, 100 μL of 600 mM Na–acetate buffer (pH 4.4), 15 μL of 2% NaN_3 (Sodium azide) and 1089.6 μL of distilled water. The solution was stirred by small magnet and 3 μL of isoamylase enzyme were added and incubated at 37°C for 8 hours. Then, 3 μL of isoamylase enzyme were again added into the gelatinized polyglucan sample and incubated at 37°C, speed 2 for 7 hours. The hydrolysed samples in glass tubes was boiled for 20 min and then centrifuged at 14,000 rmp in 2 min. The supernatant were deionised by filtration over exchange resign using Bio–Rad AG 501–X8(D). The amount of supernatant was calculated by Park and Johnson's method. The supernatant was evaporated in a centrifugal vacuum for overnight. The dry sample was dissolved in 2 μL of 8–amino–1,3,6–pyrenesulfonic acid (APTS) and then added with 2 μL of 1 M sodium cyanoborohydride in tetrahydrofuran and incubated at 55°C for 90 min. After incubation, 46 μL distilled water was added into the sample and was stored at –30°C until use. 5 μL of sample was used for capillary column of P/ACETM MDQ (Beckman Coulter, Inc.).

Ecotilling SNPs analysis of SSIIa gene and sequencing

DNA extraction

Genomic DNAs were extracted from young leaf of 10 to 14 days seedling using the CTAB method (Doyle, 1991). DNA solution of all samples was adjusted to a concentration of 15 ng/lμL.

EcoTILLING

The EcoTILLING method was used to investigate SSIIa gene of Vietnamese rice germplasm according to Comai, Comai and Henikoff and Raghavan (Comai *et al*., 2004; Comai and Henikoff., 2006; Raghavan *et al*., 2007). Primers were designed by using the Primer3 software (http://frodo.wi.mit.edu/cgi–bin/primer3) with sequence information received from Genebank accession: AY423717.1. Heteroduplexes between wild type (two control cultivars: IR36–*indica* or TC65–*japonica*) and the DNA samples were formed by denaturing and reannealing PCR products. The PCRs were performed in 10 μL final volume using 0.4 U/reaction of Taq DNA polymerase (TaKaRa Ex TaqTM). The forward and reverse primers (primer 1 to primer 5) were used for EcoTLLING in Table 1. The PCR reaction was run at 94°C for 4 min; followed by 29 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 1 min and 30 s, and 149 cycles at 96°C for 10 min, 85°C for 20 s and final step at 25°C for 1 s. After PCR amplifying, the digestion was carried out at 37°C for 20 min by CEL I. The 10 μL of digested products was used for electrophoresis on 1.5% agarose gel in TAE buffer at 5 V/cm. The bands in addition to the full–length product indicated the presence of SNPs in the pool.

Table 1. List of primers used for Ecotilling and amplifying the SSIIa gene

Primer	Forward primer (5'–3')	Reverse primer (5'–3')
1	atccaccacgttcctcgtc	atcagcccagtcatcatcgt
2	atcgaccaggatgacgattc	tcctacatcctgggcttcc
3	ctgctggacaggtgtgtgtc	tcacaaggacagagcgagtg
4	aggagaaatgaggtcgcagt	cgtacagcttgaagtgatcca
5	tgtgatgctaaatggttcgtg	accattggtacttggccttg
6	atccaccacgttcctcgtc	atctaagcggctacgccata
7	gctcgagaggtgttctctgc	tttacgggctgtttgtttga
8	ggactctcggtgacttcctg	caaggacctcctcgtagagc

Sequencing analysis

Primer 6, primer 7 and primer 8 in the table 1 were used for amplifying the SSIIa gene. PCR amplification

for sequencing was performed using 1 μL of extracted DNA in a total volume of 10 μL containing 5 μL 2x FOD buffer, 0.3 μL of each primer, 1.2 μL Milli Q water and 0.2 μL KOD_FX. A total reaction of 29 cycles was programmed for 2 min at 94˚C, 15 s at 94˚C, 30 s at 61˚C, and 5 min at 68˚C. PCR products were purified using Microcon Centrifugal filter Devices. PCR products were directly sequenced from both strands with a BigDye Terminator Cycle Sequencing Kit using an ABI 3100–Avant Genetic Analyzer (Applied Biosystems). Data was analyzed by using http://clustalw.ddbj.nig.ac.jp.

RESULTS

Variation on alkali digestibility

Variation of alkali digestibility was showed in the Figure 1 and Table 2. IR36 was resistant in the alkali solution and scored as 1. In contract, Taichung 65 (TC65) was easily degraded in alkali solution with alkali score of 6. The wide range of alkali digestibility level among waxy rice cultivars was recorded from 2 to 6. Alkali digestion types were classified as low (score 2 to 3), intermediate (score 4 to 5) and high (score 6). Frequency of low, intermediate and high alkali digestibility among 22 waxy rice cultivars was 36%, 59% and 5%, respectively.

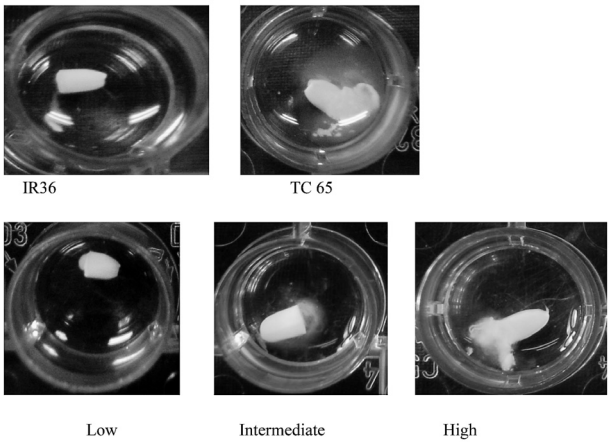

Fig. 1. Variation of alkali digestibility in waxy rice in 1.3% KOH. Alkali digestibility low (score 1,2,3); Alkali digestibility intermediate (score 4,5); Alkali digestibility high (score 6). Alkali digestibility of IR36 and TC65 were scored as 1 and 6, respectively.

Table 2. Variation of alkali digestibility in waxy rice

Alkali digestibility level	Number of cultivars	Frequency (%)
Low (score 2 to 3)	8	36
Intermediate (score 4 to 5)	13	59
High (score 6)	1	5
Total	22	100

Variation on amylopectin chain length distribution in waxy rice

According to the degree of polymerization, Hanashiro proposed the chain–length distribution of amylopectin as A chain with (DP≦12), B1 chain (12≦DP≦24), B2 chain (25≦DP≦36) and B3 chain (DP≧37) (Hanashiro *et al*., 1996).

In this study, the difference in distribution of amylopectin chain length fraction in waxy rice was showed in Figure 2–2 and Table 3. The proportion of amylopectin chain length fraction in TC65 was distinguished from that in IR36 by the higher proportion of fraction fa (A chain) and lower proportion of fb1 (B1 chain) (Figure 2–1). The proportion of fb2 (B2 chain) and fb3 (B3 chain) showed slight difference between two cultivars (TC65 and IR36). On the other hand, the result showed that starch granule from IR36 was resistant in alkali solution and scored as 1, while starch granule from TC 65 easily degraded in alkali solution and scored as 6.

Based on the ratio of total of DP≦10 per total of DP≦24 (ACR value), all 22 waxy rice cultivars had sort type (S type) of amylopectin with ACR values ranged from 2.52 to 2.81.

As shown in Table 3 and Figure 2–2, the amylopectin fine structure varied significantly in waxy rice cultivars in the ratios of fa chains and fb1 chain compared with that of IR36. All waxy rice cultivars has significantly higher proportion of fa (DP≦12) and markedly lower proportion of fb1 chains with DP from 12 to 24 whereas the little difference was observed in proportion fb2 (25≦DP≦36) and fb3 (DP≦37) between waxy cultivars tested and IR36.

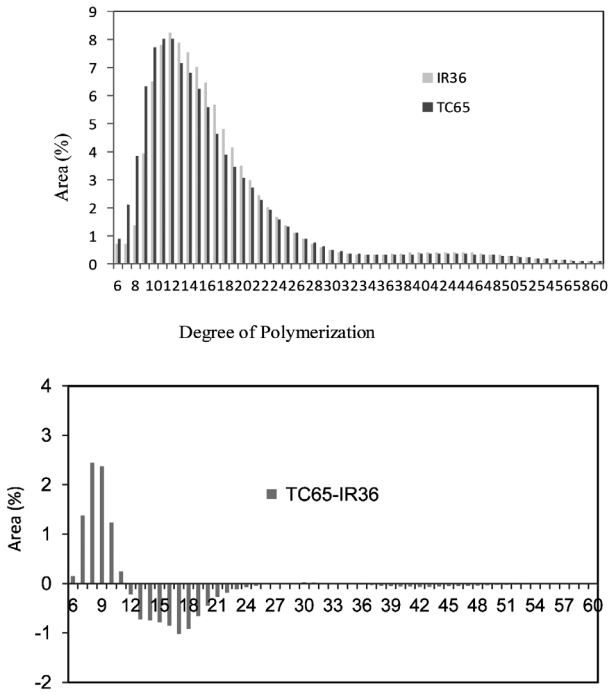

Fig. 2–1. Comparison of amylopectin chain–length distribution between IR36 and TC 65. IR36 shows the low alkali digestibility (score 1). TC 65 shows the high alkali digestibility (score 6).

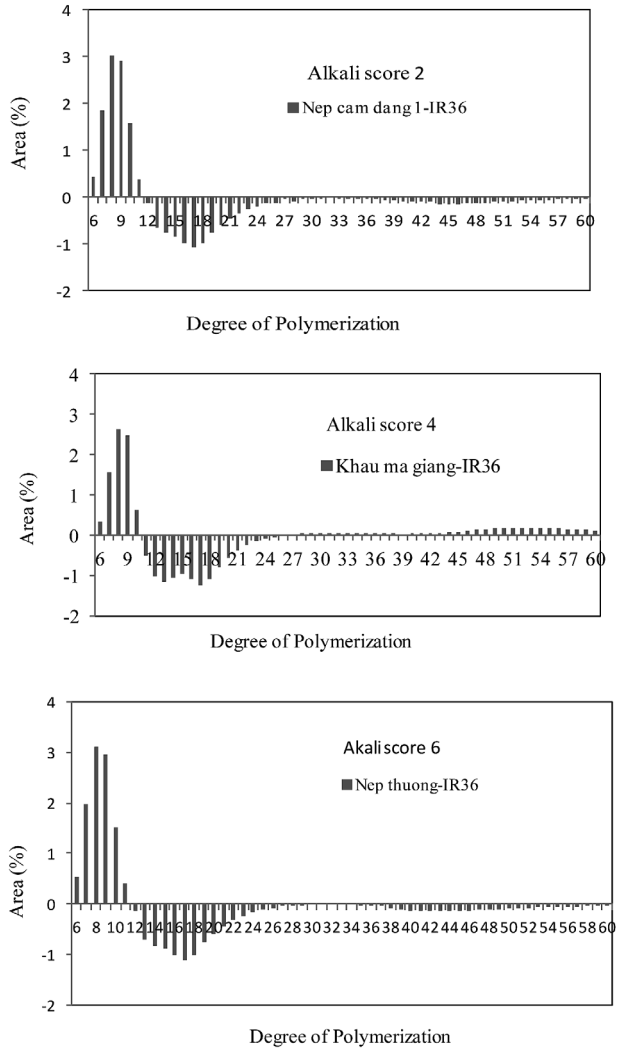

Fig. 2–2. Differences in chain–length distribution of amylopectin between waxy rice cultivars and IR36. IR36 shows the low alkali digestibility (score 1). Nep cam dang 1 : alkali score 2, Khau ma giang : alkali score 4; Nep thuong : alkali score 6.

Among low alkali digestibility waxy cultivars (alkali score 2 and alkali score 3), the slight difference in proportion of fa chain and the proportion of fb1, fb2 and fb3 chains was observed while the significant difference in proportion of fa chains was recorded among waxy cultivars with intermediate alkali digestibility (alkali score 4 and alkali score 5). The higher proportion of fb2 was found in two cultivars (Khau hin and Khau non). The amylopectin chain length fraction from Khau non and Khau ma giang showed the significantly higher proportion of fb3 as compared to that of IR36, TC65 and other waxy cultivars.

Relationship between alkali digestibility and amylopectin chain length distribution

Correlation between alkali digestibility and amylopectin chain length distribution was presented in Table 4. Alkali digestibility was weakly negative correlated with short chain fa while weakly positive with fb2 (25≦DP≦36) and fb3 (37≦DP≦60) chains.

Single nucleotide polymorphism (SNP) of SSIIa gene in waxy rice

As showed in the Table 5, the nucleotide changes in three exons (exon 1, exon 2 and exon 8) were observed. Of the six SNPs in the coding region, two SNPs, C/T (at site 516 bp) in exon 2 and G/T (at site 3903) in exon 8, were silent substitution while other four caused amino acid replacement. The SNP at position 264 bp in exon 1, was found an G–to–C transition causing change of glutamic to aspartic amino acid while the SNP at 3,799 bp in exon 8, causing change of glycine (encoded by GGC) to serine (encoded by AGC). Furthermore, the SNP at 4,198 bp, causing methionine (encoded by ATG) to valine (encoded by GTG). The SNP at 4,329–4330 bp, a GC–to–TT transition, was resulting in amino acid glycine–leucine (IR36 and TC65) to glycine–phenylalanine substitution. All 22 waxy rice cultivars had the same types of SNP in three exons.

DISSCUSSION

Waxy rice is a major type of cultivated rice with long–standing cultural importance in Asia (Olsen *et al.*, 2002). The wide variation on the physicochemical properties of waxy rice starch in local rice cultivars is important for broadening waxy rice cultivars for specific food usage.

In this study, a wide variation on alkali digestibility of starch in 22 waxy rice cultivars was observed (Table 2). The intermediate alkali digestibility was recorded commonly among tested waxy rice cultivars (59% of cultivars). Some previous studies reported that the variation on alkali digestibility of rice starch related to amylopectin structure (Umemoto *et al.*, 2004; Umemoto *et al.*, 2002; Nakamura *et al.*, 2002). Nakamura *et al.* (2002) reported that amylopectin file structure of Asian rice was classified into two groups L and S type based on ACR value. Starch of S type amylopectin rice was easier disintegrated in alkali solution than that of L type amylopectin. In this study, all 22 waxy rice cultivars had S type of amylopectin and most of them exhibited intermediate alkali digestibility. Indeed, the correlation between alkali digestibility and fa, fb2 and fb3 of amylopectin chain length was observed. However, this correlation was not strong. The length of A amylopectin chains plays a critical role in determining the physiochemical properties of starch (Umemoto *et al.*, 2004; Nakamura *et al.*, 2006; Inouchi *et al.*, 2005). In tropical non–waxy and waxy rice starch, the amylopectin chain ACR (ratio A chains/A+B1 chains) is the major factor that differentiates the alkali digestibility of *indica* and *japonica* rice varieties (Nakamura *et al.*, 2002). A wide variation on ACR value and the length of fa chain were observed among 22 waxy rice cultivars in the study. Thus, the differences in length of fa chains may contribute to the alkali digestibility differences among tested waxy rice cultivars.

The association of SNPs in rice SSIIa gene and starch properties was mentioned in some reports (Umemoto *et al.*, 2004; Nakamura *et al.*, 2005; Waters *et al.*, 2006;

Table 3. Distribution of amylopectin chain–length fraction in waxy rice cultivars

Cultivar name	Alkali score (1.3% KOH)	ΣDP≦10/ΣDP≦24 (ACR)	fa (DP≦12)	fb1(13≦DP≦24)	fb2 (25≦DP≦36)	fb3 (37≦DP≦60)
Te mun	2	0.252	38.892	48.548	7.193	5.367
Ngoo vai	2	0.257	38.797	48.761	7.258	5.184
Luot cay	2	0.263	39.536	48.639	7.159	4.666
Nep cam dang 1	2	0.263	39.432	48.389	6.934	5.245
Nep cam den	2	0.264	39.194	47.937	7.266	5.602
Lo cang	2	0.266	39.793	47.630	7.126	5.451
Ngoo nac	3	0.267	40.291	48.020	7.103	4.585
But veng	3	0.270	39.628	47.008	7.215	6.150
Khau cam panh	4	0.261	38.921	48.559	7.269	5.251
Lo xo	4	0.262	39.174	48.198	7.314	5.314
Nep ech dang 1	4	0.268	40.146	47.798	7.276	4.780
Nep vang som	4	0.268	39.771	47.838	6.934	5.457
Khau pan	4	0.271	40.464	48.586	6.677	4.273
Nep bo giua	4	0.276	40.681	47.906	6.999	4.414
Sang chu	4	0.281	41.110	47.183	7.085	4.621
Khau hin	5	0.250	34.446	50.032	8.613	6.910
Khau non	5	0.252	35.864	48.206	8.267	7.664
Khau ma giang	5	0.253	35.432	47.362	7.504	9.703
Nep man	5	0.266	40.011	48.558	6.967	4.464
Khau do don	5	0.269	40.394	47.706	6.984	4.915
Khau san	5	0.276	40.853	47.379	6.886	4.882
Nep thuong	6	0.266	39.695	48.192	7.143	4.969
TC 65	6	0.26	36.10	49.79	6.45	5.57
IR 36	1	0.17	29.97	53.98	6.11	3.80

Table 4. The correlation of alkali digestibility and amylopectin chain length distribution in waxy rice

	Alkali score	*ΣDP≦10/ΣDP≦24 (ACR)*	*fa (DP≦12)*	*fb1 (13≦DP≦24)*	*fb2 (25≦DP≦36)*	*fb3 (37≦DP≦60)*
Alkali score	1					
ΣDP≦10/ΣDP≦24 (ACR)	0.08610	1				
fa (DP≦12)	−0.20457	0.86000	1			
fb1 (13≦DP≦24)	−0.03073	−0.57166	−0.45454	1		
fb2 (25≦DP≦36)	0.23506	−0.69686	−0.88108	0.45821	1	
fb3 (37≦DP≦60)	0.22121	−0.65999	−0.85413	−0.05178	0.644413	1

Table 5. SNP analysis of SSIIa gene in waxy rice cultivars

Types	Exon 1	Exon 2	Exon 8				Alkali score (1.3%KOH)
	264	516	3799	3903	4198	4329–4330	
IR 36	G	C	G	G	G	GC	1
AY423717.1	G	C	G	G	G	GC	
TC65	C	T	A	T	A	GC	6
1 (22*)	C	T	A	T	G	TT	2–6

*: The number in parenthesis is the number of rice cultivars in this group

Bao *et al*., 2006). SSIIa plays a distinct role in the elongation of short chains within clusters (A+B1) of amylopectin (Umemoto *et al*., 2002). In our study, EcoTILLING and sequencing were used for detecting SNPs of SSIIa gene in 22 waxy rice cultivars. The combination of four SNPs C/A/G/TT found in exon 1 and exon 8 in SSIIa from 22 waxy rice cultivars was the same as SNPs of Kinmaze (Nakamura *et al*., 2005). However, alkali digestibility of 22 waxy rice cultivars was varied from low (36% of rice cultivars) to intermediate (59%) and high (5%).

Some other studies showed that the SNP at site 264 did not affect greatly to activity of SSIIa (Umemoto *et al*., 2005; Nakamura *et al*., 2005). On other hand, the combination of four SNPs in SSIIa of Nipponbare and Kinmaze caused clearly reduction of SSIIa activity. Consequently, the low gelatinization of starch and the high alkali digestibility score of two cultivars were found in these cultivars (Nakamura *et al*., 2005). Waters *et al*. (2006) found that SNPs in SSIIa gene of low gelatinization rice cultivars contained two haplotypes of SNP including A/G/TT and A/A/GC. In other study, the GC/TT at site 4329–4330 could differentiate rice with high or intermediate GT from low GT rice (Bao *et al*., 2006). Analysing SSIIa of 65 rice cultivars, Umemoto reported that rice cultivar, which had SNP A/G/TT (haplotype 3), can be grouped as susceptible to alkali solution (rice with higher short–chain ratio) and resistant to alkali solution (rice with fewer short chain) (Umemoto *et al*., 2004). As a whole, the natural variation on SSIIa affects the starch properties (Waters *et al*., 2006). The results of our study suggested that the wide variation on alkali digestibility and amylopectin fine structure in waxy rice starch was caused by the difference in activities of enzymes involving in amylopectin synthesis. Since the chain length distribution of amylopectin affects the rheological properties of cooked rice, the further genetic analysis should be focused on the diversity of amylopectin structure and the relationship with the physical behaviours of endosperm starch in Vietnamese local rice cultivars.

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