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Influence of single-nucleotide polymorphisms in the gene encoding granule-bound starch synthase I on amylose content in Vietnamese rice cultivars

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Amylose content is one of the most important factors influencing the physical and chemical properties of starch in rice. Analysis of 352 Vietnamese rice cultivars revealed a wide range of variation in apparent amylose content and the expression level of granule-bound starch synthase. On the basis of single-nucleotide polymorphisms (SNP) at the splicing donor site of the first intron and in the coding region of the granule-bound starch synthase I gene, *Waxy* gene, alleles can be classified into seven groups that reflect differences in apparent amylose content. The very low and low apparent amylose content levels were tightly associated with a G to T in the first intron whereas intermediate and high amylose was associated with a T genotype at SNP in exon 10. The correlation between the combination of T genotype at SNP in the first intron, C in exon 6, or C in exon 10 was predominant among low amylose rice varieties. Our analysis confirmed the existence of Wx^{op} allele in Vietnamese rice germplasm. The results of this study suggest that the low amylose properties of Vietnamese local rice germplasm are attributable to spontaneous mutations at exons, and not at the splicing donor site.

Key Words: amylose, granule-bound starch synthase I, rice germplasm, single-nucleotide polymorphism, *Waxy* gene.

Introduction

Starch consists of two kinds of glucan polymers, amylose and amylopectin. Amylose is predominantly a linear molecule of α-1,4-linked D-glucose, although some of the molecules are slightly branched by α-1,6-glucosidic linkages (Takeda and Hizukuri 1987). Amylopectin is composed of highly branched α-1,4-polyglucans, which are short, linear α-1,4-glucan chains that are regularly branched by α-1,6-glucosidic linkages (Takeda *et al.* 1987). The physicochemical properties of rice starch are affected by the ratio of amylose to amylopectin and their molecular structures (Nakamura *et al.* 2006).

Amylose is synthesized by the granule-bound starch synthase (GBSS), whereas amylopectin is synthesized by the concerted action of four classes of enzymes: ADP-glucose pyrophosphorylase, starch synthase, branching enzyme, and debranching enzyme (Denyer *et al.* 2001, Hannah and James 2008). Granule-bound starch synthase I (GBSSI) in rice is encoded by the *Waxy* (*Wx*) gene (Hirano and Sano 1991, Hirose and Terao 2004, Okagaki 1992). In rice cultivars, there are three functional alleles at the *Waxy* locus: Wx^a, Wx^b, and Wx^{op}. Wx^a and Wx^b are found mainly in Indica and Japonica rice, respectively. The expression level of Wx^a and Wx^b are associated with amylose content (Sano 1984, Sano *et al.* 1985, 1986). Expression level of mRNA and accumulation of waxy protein in Wx^a cultivars is 10-fold higher than that of Wx^b cultivars (Isshiki *et al.* 1998). The *Waxy opaque* (Wx^{op}) allele is the modified form of the Wx^a gene and endosperms with the Wx^{op} allele have 10% amylose content (Mikami *et al.* 1999, 2008).

The association between amylose content (AC) and single-nucleotide polymorphisms (SNPs) in the rice *Wx* gene has been described at the splicing donor sites of the first intron (Isshiki *et al.* 1998, Sano *et al.* 1985), exon 4 (Larkin and Park 1999, Mikami *et al.* 1999, 2008), exon 6 (Cai *et al.* 1998, Larkin and Park 2003, Mikami *et al.* 2008, Wang *et al.* 1995), and exon 10 (Cai *et al.* 1998, Hirano *et al.* 1996, Mikami *et al.* 2008, Wang *et al.* 1995). The cytosine and thymidine (CT_n) dinucleotide repeats in the 5′-untranslated region (UTR) of the *Wx* gene were reported to be a factor associated with AC (Ayres *et al.* 1997, Bergman *et al.* 2001, Bligh *et al.* 1995). However, the relationship between these polymorphisms and amylose contents is not clear.

Subsequent studies demonstrated that the SNP at the splicing donor site of the first intron reduces the efficiency of GBSS prior to processing of mRNA and causes the low levels of the mature *Waxy* transcript, GBSS, and apparent amylose content (AAC) (Cai *et al.* 1998, Hirano *et al.* 1996, 1998, Larkin and Park 1999, 2003, Wang *et al.* 1995). Moreover, recent reports show a tight correlation between SNP in the first intron, coding regions, and AAC (Chen *et*

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al. 2008a, 2008b, Liu *et al.* 2009, Dobo *et al.* 2010). Two SNPs in exons 6 and 10, the A/C and C/T polymorphisms, respectively, resulted in nonsynonymous amino acid changes (Chen *et al.* 2008a, 2008b, Dobo *et al.* 2010). The combination of two SNPs in the *Waxy* gene, including a single G/T polymorphism at the splicing donor site of the first intron and an SNP in exon 6, can effectively differentiate all three classes of low, intermediate, and high AAC (Chen *et al.* 2008a). The combination of three SNPs (the single G/T polymorphism at the splicing donor site of the first intron, A/C in exon 6, and C/T in exon 10) was associated with AAC among US rice varieties and in European rice germplasm (Chen *et al.* 2008b, Dobo *et al.* 2010).

Vietnamese rice cultivars display a wide range of variation in amylose content (Suu *et al.* 2012). According to Nakagahra *et al.* (1986), the center of diversity for amylose content is the hilly area of southeast Asia. Indeed, wide variation in apparent amylose content has been reported in rice germplasm from many Asian countries, including Myanmar, northeastern India, China, and Vietnam (Aung *et al.* 2002, Heu 1986, Jahan *et al.* 2002, Nakagahra *et al.* 1986, Suu *et al.* 2012). Vietnam is one of the centers of diversity of cultivated rice, as shown in previous studies that confirmed the great diversity of Vietnamese rice landraces (Fukuoka *et al.* 2003, Suu *et al.* 2012).

In this report, we describe the variation in AAC, GBSS levels, and the SNP of the GBSSI gene in the germplasm of Vietnamese rice cultivars. We also examine the association between the variation in AAC and the SNP distribution at the splicing donor site of the first intron or in the coding region of the GBSSI gene.

Materials and Methods

Plant materials

Of the 352 Vietnamese local rice cultivars examined in this study, 98 cultivars were previously examined for AAC by Suu *et al.* (2012). Three rice cultivars, 'Taichung65' (Japonica), 'IR36' (Indica), and 'EM21' (*waxy* mutant) were used as a control. Out of 352 rice cultivars, 87 non waxy rice cultivars were used for EcoTILLING SNP analysis. For sequencing analysis of the GBSSI gene, 23 were chosen from 87 non waxy rice cultivars. All Vietnamese local rice cultivars are preserved in National seed genebank of Plant Resources Center, Vietnam.

Estimation of the AAC

Apparent amylose content was estimated by using the DU 7500 Spectrophotometer (Beckman), following Satoh *et al.* (1990). A single seed was cut into two pieces, gelatinized by treatment with 2 ml of 1 N NaOH, and kept at room temperature for 24 h. Next, 4 ml of 1 N CH_3COOH and 4 ml of distilled water were added and the solution was homogenized. A 0.8-ml volume of this solution was transferred into a small tube, to which 0.2 ml of 0.2% (w/v) I2, 2% (w/v) KI, and 4 ml of distilled water were added. The AAC was determined by measuring the blue value at 680 nm and λ-max.

Western blotting

To each 20 mg of seed powder, 700 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffers was added. The material was vortexed for 3 h at room temperature and then centrifuged at 20°C, 6,000–7,000 rpm for 10 min. SDS-PAGE was performed using 10% SDS polyacrylamide gel. A polyvinylidene difluoride (PVDF) membrane was used to blot the band of proteins, and the membrane was incubated with the primary antibody (rabbit anti-wheat GBSS waxy protein). Immunoreactive proteins were detected by incubation with the secondary antibody (goat anti-rabbit IgG H + L). An equal amount of the enhanced chemiluminescence substrate (GE Healthcare) was mixed, and the membrane was incubated for 1 min in this mixture before use. The x-ray film was developed, washed, and fixed.

DNA extraction

A set of 87 nonwaxy rice cultivars was selected to represent a wide range of AACs from 352 rice cultivars. Total genomic DNAs were extracted from young leaves of 10–14-day-old seedlings using the hexadecyl trimethylammonium bromide (CTAB) method (Doyle 1991). DNAs from all samples were adjusted to a concentration of 15 ng/µl.

EcoTILLING SNP analysis

We used the EcoTILLING method to investigate the *GBSSI* gene in germplasm from Vietnamese rice cultivars, following the method of Comai *et al.* (2004) and Till *et al.* (2006). The Primer3 software was used to design primers for the *Wx* gene (http://frodo.wi.mit.edu/cgi-bin/primer3). *GBSSI* (AccNo Os06t0133000-01 of the 'Nipponbare' *Wx* genome sequence), which was acquired from the DNA Data Bank of Japan (DDBJ), was used to design the primer.

Heteroduplexes between the wild-type (one of the control cultivars, 'IR36' - Indica or 'Taichung65' - Japonica) and the DNA samples were formed by denaturation followed by reanneaning of PCR products. PCR was performed in a 10-µl final volume by using 0.4 U/reaction of Taq DNA polymerase (TaKaRa Ex Taq™). The forward and reverse primers were used for PCR amplification (Table 1). PCR was performed as follows: one cycle at 94°C for 4 min; followed by 29 cycles at 94°C for 30 s, 61°C for 30 s, and 72°C for 1 min 30 s. The subsequent denaturation and reannealing steps followed the method described by Suzuki *et al.* (2008). The digestion was carried out at 37°C for 20 min by using a crude celery juice extract (CEL I enzyme) (Anai *et al.* 2008). The CEL I reaction was stopped by 10 mM EDTA (final conc.). Then, 10 µl of digested product was resolved on 1.5% agarose gel in TAE buffer, run at 5 V/cm. The gel was stained with ethidium bromide and visualized under a UV transilluminator.

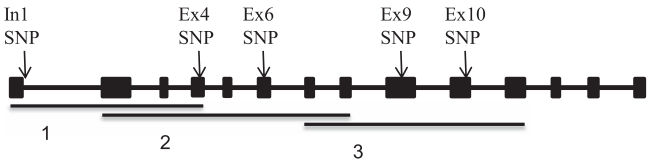

Fig. 1. Schematic representation of *GBSSI* gene structure and the position of single-nucleotide polymorphism in *GBSSI* gene. The boxes and the lines between boxes indicate the exon and the intron, respectively. Lines under the gene and number from 1 to 3 indicate the region amplified by PCR and the primer set used for amplification, respectively.

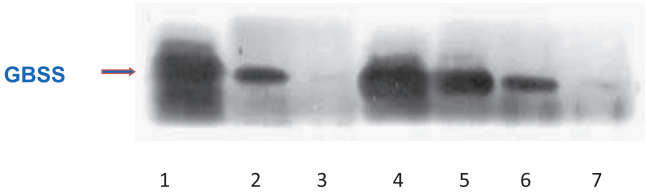

Fig. 2. The expression level of granule-bound starch synthase I by Western blotting analysis in Vietnamese rice cultivars. Lane 1 to lane 3: Control cultivars: Lane 1: 'IR36'; Lane 2: 'Taichung65'; Lane 3: 'EM21'; Lane 4 to lane 7: The expression level of granule-bound starch synthase I of local rice cultivars were classified into four types (high, intermediate, low and absent) as compared with that of 'IR36'. Lane 4: 'Accession number 2479: Te Moc chau'; Lane 5: 'Accession number 2217: Me noll'; Lane 6: 'Accession number 2596: Khau se tao'; Lane 7: 'Accession number 2468: Khau pe'.

Sequencing analysis

We constructed a PCR primer set 1 including a forward primer (5′-CCATTCCTTCAGTTCTTTGTCT-3′) and a reverse primer (5′-CACTGACCTGGCAAAGAAGG-3′), which amplified the fragment containing the first exon–intron junction of the *Waxy* gene. To amplify the fragment containing exons 4–10, we designed the following PCR primer sets: primer set 2, containing the forward primer (5′-TAGCCGAGTTGGTCAAAGGA-3′) and reserve primer (5′-AAGCACAGGCTGGAGAAAT-3′), and primer set 3, containing the forward primer (5′-TCGCATTGGATG GATGTGTA) and reserve primer (5′-GCATAAAACAAA AATGGCATGG-3′) (Fig. 1). PCR amplification was performed using KOD_FX (TOYOBO). PCR was performed as follows: one cycle at 94°C for 2 min, followed by 29 cycles of 94°C for 15 s, 61°C for 30 s, and 68°C for 5 min. PCR products were purified using Microcon Centrifugal Filter Devices (GE Healthcare). Purified PCR products were directly sequenced from both strands using 11 primers (Table 1) with a BigDye Terminator Cycle Sequencing Kit using an ABI 3130*xl* Genetic Analyzer (Applied Biosystems). Data were analyzed using ClustalW (http://clustalw.ddbj.nig.ac.jp).

Results

Variation in GBSS levels and AAC

Amylose is synthesized by GBSSI protein. Three control cultivars, 'IR36,' 'Taichung65,' and 'EM21,' showed high, low, and absent GBSS levels, respectively, according to the intensity of 60 kDa bands by Western blot analysis (Fig. 2). Based on the amount of GBSS protein, 352 rice cultivars were divided into four classes, high in 122 cultivars (34.6%), intermediate in 13 cultivars (3.7%), low in 52 cultivars (14.8%), and absent in 165 cultivars (46.9%) (Figs. 2, 3). Intermediate between 'IR36' and 'Taichung65' was defined as intermediate (lane 5 in Fig. 2)

The AAC of Vietnamese rice cultivars varied from 0 to 32% (Fig. 3). Based on varied range of AAC, rice cultivars can be grouped into five groups: glutinous (0–6%), very low (6–12%), low (12–20%), intermediate (20–25%), and high (25–32%) (Suu *et al.* 2012) (Table 2 and Fig. 3). Nearly half (46.9%) of the cultivars belonged to the glutinous group (0–6%), while the other 53.1% belonged to nonglutinous groups (>6%). The nonglutinous group was divided into four groups on the basis of AAC: very low (3.4% of cultivars), low (15.1%), intermediate (12.2%), and high (22.4%).

The relationship between GBSS level and AAC in Vietnamese rice cultivars is shown in Fig. 3. Cultivars with different GBSS levels showed continuous variation in AAC. All the cultivars with no GBSS exhibited the glutinous phenotype (waxy endosperm), and the AAC of the varieties was distributed from 0% to 6%. In the nonglutinous group, the varieties with low, intermediate, and high levels of GBSS varied in AAC from 8% to 26%, from 14% to 26%, and from 18% to 32%, respectively (Fig. 3).

Table 1. List of primers used for EcoTILLING and sequencing

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
1	CCATTCCTTCAGTTCTTTGTCT	CCTTTGACCAACTCCGGCTAC
2	TAGCCGAGTTGGTCAAAGGA	TGGATTGGGGATTAGAATTTGA
3	TGCAGAGATCTTCCACAGCA	CTCCTAGCCACAACG
4	TCGCATTGGATGGATGGATGTGTA	CACTGACCTGGCAAAGAAGG
5	TTCAGGTTTGGGGAAAGACC	TGTTGTGGATGCAGAAAGCA
6	TGCACACTGCATTCTGTTCA	GTCGTACTTGGCGGTGATGT
7	ACATCACCGCCAAGTACGAC	GGTCTTCCGGCTAACTCCAC
8	TCAGAACAAATTCAGTGGCAAA	AAGCACAGGCTGGAGAAAT
9	ATTTCTCCAGCCCTGTGCTT	TTGACCGTTCGTCTTGTTCA
10	TGAACAAGACGAACGGTCAA	GCATAAAACAAAAATGGCATGG
11	TCTTATCGGACCCTGAATTTATGT	TCCTGAGTCAAACTACTGCTCCTT

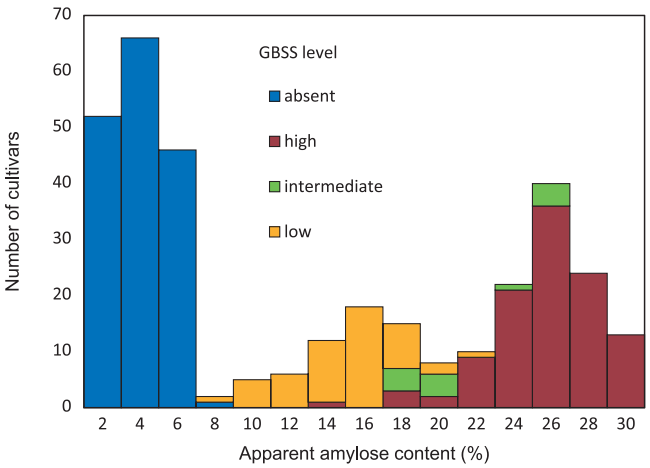

Fig. 3. The relationship between apparent amylose content and the expression level of granule-bound starch synthase in Vietnamese rice cultivars.

Table 2. Variation in apparent amylose content (%) in Vietnamese rice cultivars

Apparent amylose content (%)	Amylose class	Number of cultivars	Frequency (%)
0–6	Glutinous	165	46.9
7–12	Very low	12	3.4
12–20	Low	53	15.1
20–25	Intermediate	43	12.2
25–32	High	79	22.4

SNPs of the GBSS1 gene

The SNPs in *GBSSI* gene of 23 nonglutinous rice cultivars were determined by the EcoTILLING and the sequencing of the genomic DNA. The G/T polymorphism at the splicing donor site of the first intron and four SNPs in *GBSSI* gene were found in the 23 rice cultivars (Table 3). Based on the combination of the G/T polymorphism and SNPs in exon 4 (Ex4), exon 6 (Ex6), exon 9 (Ex9), and exon 10 (Ex10), the cultivars were classified into seven types.

The 23 cultivars were divided into two groups based on the G/T polymorphism at the splicing donor site of the first intron. In the first group ('IR36' type), containing 12 cultivars, the splicing donor site of the first intron was AGGTA TA. The AAC of this group ranged from 13 to 30. In the second group ('Taichung65' type), containing 11 cultivars, the splicing donor site of the first intron was AGTTATA. The AAC of the second group varied from 9 to 20.

A sequence analysis of 23 rice cultivars indicated seven allelic patterns: Wx^{g1} (G-A-A-C-T), Wx^{g2} (G-A-A-C-C), Wx^{g3} (G-G-A-C-C), Wx^{g4} (G-A-C-T-C), Wx^{t1} (T-A-A-T-C), Wx^{t2} (T-A-A-C-C), and Wx^{t3} (T-A-C-T-C) (Table 3). The first letter in the allelic pattern corresponds to the G/T at the splicing donor site of the first intron, the second letter to the A/G polymorphism in Ex4, the third letter to the A/C polymorphism in Ex6, the fourth letter to the C/T polymorphism in Ex9, and the fifth letter to the C/T polymorphism in Ex10. The Wx^{g1} pattern (G-A-A-C-T - 'IR36' type) was found in five cultivars with intermediate and high AAC, whereas the Wx^{g4} pattern (G-A-C-T-C) was found in two cultivars with low AAC. The Wx^{g3} pattern (G-G-A-C-C) allele was associated with the low AAC class, while the Wx^{g2} pattern (G-A-A-C-C) was associated with both low and high AAC. As the cultivars in Wx^{g2} contained both low and high levels of GBSS, Wx^{g2} cultivars were divided into subgroups, $Wx^{g2\text{-}1}$ and $Wx^{g2\text{-}2}$ (Fig. 4). The Wx^{t1} pattern (T-A-A-T-C - 'Taichung65' type) was associated with low AAC. The Wx^{t2} pattern (T-A-A-C-C) was found in two low AAC cultivars. The Wx^{t3} pattern (T-A-C-T-C) was found in eight cultivars with low AAC.

We found four SNPs on four exons (Ex4, Ex6, Ex9, Ex10) of the *GBSSI* gene. The SNP at the position of 3,013 bp from ATG starting site was a synonymous C to T substitution (Ex9). On the other hand, A to C substitution at 2,016 bp (Ex4), A to C substitution at 2,385 bp (Ex6), and C to T substitution at 3,377 bp (Ex10) were responsible for

Table 3. *Waxy* gene haplotypes and geographical distribution of *Wx* alleles in Vietnamese local rice cultivars

Wx alleles	G/T in first intron	Exon 4	Amino acid	Exon 6	Amino acid	Exon 9	Amino acid	Exon 10	Amino acid	GBSS level	Apparent amylose content (%)	Geographical distribution
TC65	T	GAC	Asp	TAT	Tyr	CCT	Pro	CCT	Pro	Low	20	
IR36	G	GAC	Asp	TAT	Tyr	CCC	Pro	TCT	Ser	High	23	
Wx^{g1} (5*)	G	GAC	Asp	TAT	Tyr	CCC	Pro	TCT	Ser	High	20–32	Northwest, Northeast
Wx^{g2} (2)	G	GAC	Asp	TAT	Tyr	CCC	Pro	CCT	Pro	High, low	13–26	Northeast
Wx^{g3} (3)	G	GGC	Gly	TAT	Tyr	CCC	Pro	CCT	Pro	High	13–18	Northwest
Wx^{g4} (2)	G	GAC	Asp	TCT	Ser	CCT	Pro	CCT	Pro	High	17–20	Northwest, South central coast
Wx^{t1} (1)	T	GAC	Asp	TAT	Tyr	CCT	Pro	CCT	Pro	Low	17	Northwest
Wx^{t2} (2)	T	GAC	Asp	TAT	Tyr	CCC	Pro	CCT	Pro	Low	15–16	Northeast, Central highland
Wx^{t3} (8)	T	GAC	Asp	TCT	Ser	CCT	Pro	CCT	Pro	Low	9–16	Northwest, Northeast, Central highland

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

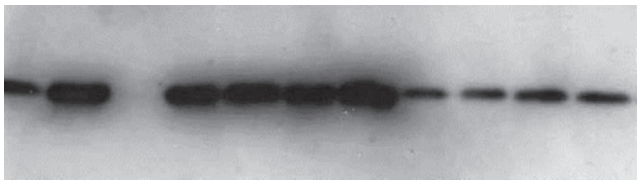

Fig. 4. The expression level of granule-bound starch synthase in Vietnamese rice cultivars by Western blotting analysis, grouped according to the single-nucleotide polymorphism at the 5′ splicing donor site of the first intron and at the coding region of the granule-bound starch synthase I gene.

non-synonymous changes from Asp to Gly, from Tyr to Ser, and from Pro to Ser, respectively (Table 3).

Waxy gene haplotypes and geographical distribution

Table 3 showed the geographical distribution of *Waxy* allele haplotypes. Northwest regions is richness diversity of *Waxy* allele with the distribution of all *Waxy* haplotypes were found except Wx^{g2}. Most of Wx^{t2} and Wx^{t3} cultivars with low AAC were detected in Northeast and Central highland of Vietnam. The Wx^{g3} and Wx^{t1} were distributed only in Northwest, Wx^{g1} was found in Northeast regions.

Discussion

One of the useful tools for identifying the GBSS allele and explaining variations in AC is the CT dinucleotide repeats in Ex1 (Ayres *et al.* 1997, Bergman *et al.* 2001, Bligh *et al.* 1995). For example, the CT dinucleotide repeats can explain 75.6% of the variation in AAC in international rice germplasm (Chen *et al.* 2008a) and 81.2% of the variation in AAC in European rice germplasm (Dobo *et al.* 2010), However, recent studies indicate that the combination of an SNP in the splicing donor site of the first intron, Ex6, or Ex10 can more effectively distinguish all three classes of apparent amylose than the CT dinucleotide repeats can (Chen *et al.* 2008a, Dobo *et al.* 2010). The present study identified a diversity of nucleotides in the *GBSSI* gene sequence among 23 rice landrace cultivars. As the SNP C-to-T change in Ex9 was a silent mutation, we present here our finding of an association between the AAC and the combination of an SNP in the first intron and an SNP in Ex4, Ex6, or Ex10 of the *GBSSI* gene in Vietnamese rice germplasm.

The G/T SNP at the splicing donor site of the first intron was observed in several of the Vietnamese rice cultivars. The very low and low AAC levels were tightly associated with the T SNP in the first intron in the rice cultivars we investigated. This finding was consistent with previous studies that showed that the non waxy cultivars, which carried T in the first intron, decreased levels of AAC (Bligh *et al.* 1998, Cai *et al.* 1998, Hirano *et al.* 1998, Isshiki *et al.* 1998, Olsen and Purugganan 2002, Wang *et al.* 1995, Yamanaka *et al.* 2004). Thus, T SNP in the first intron may be used to discriminate between the low AAC and the intermediate to high AAC classes (Ayres *et al.* 1997, Hirano *et al.* 1998, Isshiki *et al.* 1998, Larkin and Park 1999, Liu *et al.* 2009). Those studies assumed that the T SNP in the first intron was attributable to control the processing and steady levels of GBSS mRNA and the subsequent low amylose in these cultivars.

In the present study, three classes of low, intermediate and high AAC were found in cultivars with the presence of G in the first intron. This finding contrasted with the conclusion of some other studies (e.g., Ayres *et al.* 1997, Dobo *et al.* 2010, Larkin and Park 1999). Those studies concluded that intermediate or high AAC of cultivars from US and European rice germplasms was associated with G genotype.

The association between the combination of SNPs in the first intron, Ex6, or Ex10 and low amylose content in US, European, Asian rice germplasms was reported (Chen *et al.* 2008b, Dobo *et al.* 2010). However, whereas most rice varieties from Europe had the combination of TAC, the combination of TCC was predominant in US rice germplasm. In the present study, more cultivars had the TCC combination (Wx^{t3}) than the TAC combination (Wx^{t1} and Wx^{t2}), suggesting that the Wx^{t3} allele was predominant among low amylose rice varieties from Vietnam.

Regarding the relationship between the combination of three SNPs in In1, Ex6 and Ex10 and AAC, we found that the Wx^{t1} (TAC), Wx^{t2} (TAC) and Wx^{t3} (TCC) were associated with low AAC. This finding supposed that the change of A to C in Ex6 and T to C in Ex10 may affect the enzyme activity, reducing the amylose content. Our results suggest that the combination between three SNPs in the first intron, Ex6, or Ex10 (TAC and TCC) may be used as a tool to distinguish low amylose content from other classes of amylose content (intermediate and high). In Chen's study and Dobo's study, Waxy-H (G in In1, A in Ex6, C in Ex10) had intermediate or high ACC, whereas in this study, Wx^{g2} and Wx^{g3} (Waxy-H) had both low and high AAC. Thus, this finding contrasts with the results of previous studies (Chen *et al.* 2008b, Dobo *et al.* 2010). It is interesting to note that in the present study, all Wx^{g1} cultivars (Waxy-HH) having the combination of three SNPs (G in In1, A in Ex6 and T in Ex10) showed intermediate or high AAC. Our finding supports the previous studies that the combination of three SNPs in In1, Ex6 and Ex10 (GAT) could be associated with intermediate or high amylose (Chen *et al.* 2008b, Dobo *et al.* 2010, Mikami *et al.* 2008).

In the present study, we also found that ranges in AAC of Wx^{t2} and Wg^{t3}, which differ only by Exon 6 and 9, are also overlapping with each other (Table 3), suggesting the influence of other genes or genetic background. Similar observation was recorded in Wx^{g2} and Wx^{g4}. Ranges in AAC of Wx^{g2} and Wx^{g4}, which differ only by Ex6, are overlapping with each other (Table 3), suggesting the influence of other genes or genetic background.

In our study of 23 rice cultivars, most of the cultivars with low amylose had the C SNP in Ex10, whereas all the cultivars with intermediate and high amylose had the T SNP

in Ex10. The results suggested that the low amylose content observed in Vietnamese local rice germplasm might be attributable to the SNP in Ex10, not by the SNP at the splicing donor site of the first intron.

Recently, Teng *et al.* (2012) demonstrated that the Ex10 SNP was the cause of the AAC phenotypic diversity between the high amylose sub-class I (24.36–25.20%) and high amylose sub-class II (25.81–26.19%) lines, and used this SNP to subdivide the Wx^a allele into two subgroups demonstrating high AAC. The lines with serine residue from the amino acid substitution had higher GBSS activity, suggesting that the *Wx* protein might be phosphorylated. This SNP was responsible for amino acid change to Ser, which might play an important role in regulating GBSS activity through phosphorylation. Rice pasting viscosity determined by Rapid Visco Analyzer (RVA), is used to estimate cooking and eating quality characteristic. The association of SNP in *Waxy* gene with viscosity characteristics was reported in some studies (Chen *et al.* 2008b, Larkin *et al.* 2003, Larkin and Park 2003). Larkin *et al.* (2003) reported that the *Waxy* locus has significant effects on viscosity characteristics such as peak viscosity, hot paste viscosity, cool paste viscosity, breakdown and setback viscosity. Chen *et al.* (2008b) demonstrated that the SNP in Ex10 associated with RVA profiles differ between the two high AAC types. Recently, Traore *et al.* (2011) reported that the *Waxy* Ex10 SNP marker was associated with most RVA profiles. Furthermore, Tran *et al.* (2011) also found that the SNP on Ex10 of the *Wx* gene explained a significant component of the differences in gel consistency. The results from previous studies suggested that the SNP in Ex10 can be used in molecular breeding program focused on quality improvement. In the present study, we assume that this polymorphism results in the substitution of the polar amino acid serine for the nonpolar proline in Ex10, which might lead to a change in GBSS activity and thus reduce amylose content. Our results suggested that the T genotype at SNP in Ex10 could be used as a marker for discriminating the high amylose class. The one exception to this rule was the Wx^{g2} group, which included both low and high AAC levels. The expression of GBSS in this group suggested that other factors might contribute to the variation in AAC among these rice varieties.

Our research confirmed that the combination of the G/T SNP in the first intron and SNPs in Ex4, Ex6, Ex9, and Ex10 were related to the AAC. The Wx^{g3} cultivars had opaque endosperm types and low AAC. Two SNPs, G in the first intron and G in Ex4, were observed in Wx^{op} cultivars, and the C SNP in Ex10 was also found in Wx^{g3} cultivars. Thus, the Wx^{g3} in this study was similar to the Wx^{op} and Wx^{hp} types reported by other studies (Liu *et al.* 2009, Mikami *et al.* 1999, 2008). Mikami *et al.* (1999) reported that the level of gene product bound to starch granules was markedly reduced in the Wx^{op} line, resulting in a lower amylose content. Apparently the combination of SNPs at the splicing donor site of the first intron, Ex4, and Ex10 affects the binding of GBSS to starch granules, causes a low activity level in GBSS, and results in low AC of these cultivars.

In nonwaxy rice, the presence of all amylose classes in Vietnamese rice germplasm support the fact that the Southeast Asian countries is the center of genetic diversity for amylose content in rice (Aung *et al.* 2002, Fukuoka *et al.* 2003, Heu 1986, Liu *et al.* 2009, Nakagahra *et al.* 1986, Suu *et al.* 2012). Those results imply that the local rice resources in Vietnam are rich in spontaneous mutations that play a vital role in rice quality breeding programs.

The findings of this research will contribute to our understanding of the molecular mechanisms of amylose biosynthesis that are controlled by naturally occurring alleles at the *Wx* locus in rice.

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