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 Wxop 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 amyllopectin. 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-glycosidic 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-glycosidic linkages (Takeda et al. 1987). The physico-chemical properties of rice starch are affected by the ratio of amylose to amyllopectin and their molecular structures (Nakamura et al. 2006).

Amylose is synthesized by the granule-bound starch synthase (GBSS), whereas amyllopectin 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: Wxa, Wxb, and Wxop. Wxa and Wxb are found mainly in Indica and Japonica rice, respectively. The expression level of Wxa and Wxb are associated with amylose content (Sano 1984, Sano et al. 1985, 1986). Expression level of mRNA and accumulation of waxy protein in Wxa cultivars is 10-fold higher than that of Wxb cultivars (Isshiki et al. 1998). The Waxy opaque (Wxop) allele is the modified form of the Wxa gene and endosperms with the Wxop 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 (CTn) 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
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 study, 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 CH₃COOH 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 Os060133000-01 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 reannealing of PCR products. PCR was performed in a 10-μl final volume by using 0.4 U/reaction of Taq DNA polymerase (TakaRa Ex TaqTM). 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 1 enzyme) (Anai et al. 2008). The CEL 1 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.
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 \textit{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 forward primer (5’-TCGCATTGGATGGATGTGTA) and reserve primer (5’-GCATAAAACAAAAATGGCATGG-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 3130xl 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      | CCAACgAGtgGCTTGCTTgTcT | CcTgTTGACcAAtcCGGcTaC  |
| 2      | TCAGgGAGTTgGCTGATAAGG  | TggATTTGGGGAGTTAGATTg  |
| 3      | TgCAGgAGATGTCGACA  | CtcTGACGcAAcGcG  |
| 4      | TcGCATGgGATTGgATGgATGgTA | CACgTGACGTGcAAgGAG  |
| 5      | TcCAGggTTGgGcAAAGGcC | TGGTTgGTtgGcGAAgAGc  |
| 6      | TgCaCAGGACTgCggTgTcC  | GTCgTACgTTGcGcGATG  |
| 7      | AcATcAGGcGcaAGGAc   | GGTcGGCTCgCgAATcCc  |
| 8      | TcAGgACACgAAATCAGGgGAA  | AAGGCAGcGgGcAAgAcAT  |
| 9      | ATtCtCACgGCCGTgTcT  | TtgACGgTTGgTCgtgTgc  |
| 10     | TgAAGAgGcGAAcGgGg  | GcTAAacAAcAAATGgGAgc  |
| 11     | TcTtACGGGACCCTGaATTTAgT  | TcCtGAGTGcAaACTcGTCgCt  |
SNPs of the GBSSI 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 AGGTGTA. 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 AGTTTATA. The AAC of the second group varied from 9 to 20.

A sequence analysis of 23 rice cultivars indicated seven allelic patterns: \(W_x^{\text{ex}4}\) (G-A-A-A-T-C), \(W_x^{\text{ex}2}\) (G-A-A-C-C), \(W_x^{\text{ex}3}\) (G-G-A-C-C), \(W_x^{\text{ex}1}\) (T-A-A-T-C), \(W_x^{\text{ex}2}\) (T-A-A-C-C), and \(W_x^{\text{ex}3}\) (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 \(W_x^{\text{ex}1}\) pattern (G-A-A-C-T- ‘IR36’ type) was found in five cultivars with intermediate and high AAC, whereas the \(W_x^{\text{ex}2}\) pattern (G-A-A-C-T-C) was found in two cultivars with low AAC. The \(W_x^{\text{ex}3}\) pattern (G-G-A-C-C) allele was associated with the low AAC class, while the \(W_x^{\text{ex}4}\) pattern (G-A-A-C-C) was associated with both low and high AAC. As the cultivars in \(W_x^{\text{ex}2}\) contained both low and high levels of GBSS, \(W_x^{\text{ex}3}\) cultivars were divided into subgroups, \(W_x^{\text{ex}2,1}\) and \(W_x^{\text{ex}2,2}\) (Fig. 4). The \(W_x^{\text{ex}1}\) pattern (T-A-A-T-C- ‘Taichung65’ type) was associated with low AAC. The \(W_x^{\text{ex}2}\) pattern (T-A-A-C-C) was found in two low AAC cultivars. The \(W_x^{\text{ex}3}\) 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

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**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          |

**Table 3. Waxy gene haplotypes and geographical distribution of \(W_x\) alleles in Vietnamese local rice cultivars**

| \(W_x\) 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        | CTC    | Pro        | Low        | 20                          | Northwest, Northeast      |
| IR36            | G                   | GAC    | Asp        | TAT    | Tyr        | CCG    | Pro        | TCT    | Ser        | High       | 23                          |                          |
| \(W_x^{\text{ex}1}\) (5*) | G                   | GAC    | Asp        | TAT    | Tyr        | CCG    | Pro        | TCT    | Ser        | High       | 20–32                       |                          |
| \(W_x^{\text{ex}2}\) (2) | G                   | GAC    | Asp        | TAT    | Tyr        | CCG    | Pro        | CTC    | Pro        | High, low | 13–26                       | Northeast                   |
| \(W_x^{\text{ex}3}\) (3) | G                   | GCC    | Gly        | TAT    | Tyr        | CCG    | Pro        | CTC    | Pro        | High       | 13–18                       | Northwest                  |
| \(W_x^{\text{ex}4}\) (2) | G                   | GAC    | Asp        | TCT    | Ser        | CCT    | Pro        | CTC    | Pro        | High       | 17–20                       | Northwest, South central coast |
| \(W_x^{\text{ex}1}\) (1) | T                   | GAC    | Asp        | TAT    | Tyr        | CCT    | Pro        | CTC    | Pro        | Low        | 17                         | Northeast                  |
| \(W_x^{\text{ex}2}\) (2) | T                   | GAC    | Asp        | TAT    | Tyr        | CCG    | Pro        | CTC    | Pro        | Low        | 15–16                       | Northeast, Central highland |
| \(W_x^{\text{ex}3}\) (8) | T                   | GAC    | Asp        | TCT    | Ser        | CCT    | Pro        | CTC    | Pro        | Low        | 9–16                        | Northwest, Northeast, Central highland |

*: The number in parenthesis is the number of rice cultivars in this group.
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 content 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 ($W_x^{c3}$) than the TAC combination ($W_x^{cl}$ and $W_x^{c2}$), suggesting that the $W_x^{c1}$ 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 $W_x^{cl}$ (TAC), $W_x^{c2}$ (TAC) and $W_x^{c3}$ (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, $W_x^{c2}$ and $W_x^{c3}$ (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 $W_x^{cl}$ 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 $W_x^{c2}$ and $W_x^{c3}$, 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 $W_x^{c2}$ and $W_x^{c4}$. Ranges in AAC of $W_x^{c2}$ and $W_x^{c4}$, 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 $W_x^{op}$ allele into two subgroups demonstrating high AAC. The lines with serine residue from the amino acid substitution had higher GBSS activity, suggesting that the $W_x$ 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 non-polar 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 $W_x^{op}$ 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 $W_x^{g3}$ cultivars had opaque endosperm types and low AAC. Two SNPs, G in the first intron and G in Ex4, were observed in $W_x^{op}$ cultivars, and the C SNP in Ex10 was also found in $W_x^{g3}$ cultivars. Thus, the $W_x^{g3}$ in this study was similar to the $W_x^{op}$ and $W_x^{op}$ 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 $W_x^{op}$ 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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