

# Association analysis and functional marker development of *soluble starch synthase IIa (SSIa)* and gelatinization properties in Thai rice

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## Abstract

*Soluble starch synthase (SSIa)* plays an important role in amylopectin biosynthesis. The *SSIa* governs differences in amylopectin chain-length profile associated with gelatinization properties of starch among rice species. The association has been demonstrated in the *alk* gene on chromosome 6 controlling an alkali disintegration of rice grain. An induced mutant, BW1, different in gelatinization temperature compared to its high-nutritional-value wild type, Jao Hom Nin (JHN), was identified. The fine-structure of endosperm amylopectin, as well as the alkali digestibility of polished grains, was clearly different between the mutant and the wild-type. Sequence comparison of the entire *SSIa* between BW1 and JHN revealed the gain-of-function allele in this mutant. Three functional single nucleotide polymorphisms (SNPs) causing amino acid substitutions were observed at position 264, 1810 and 2340-41 from the starting point of *SSIa* translation. Different haplotypes classified according to these three SNPs for BW1 and JHN are G-G-GC and C-A-TT, respectively. The most informative SNP 2340-2341, as well as the SNP 2209 that has been reported as another important SNPs, were selected as targets for developing bi-PASA markers to classify rice varieties based on their starch gelatinization properties. These agarose-gel based markers were efficiently used as co-dominant markers that can detect different genotypes, homozygote and heterozygote, simultaneously. These markers will be useful in rice breeding program for selecting rice lines with appropriate cooking quality using marker-assisted selection.

## Media summary

Development of DNA markers underlying gelatinization temperature of rice flour is useful in rice breeding program for developing new rice cultivars that possess unique starch properties suitable for specific end uses.

## Keywords

DNA marker, alkali disintegration, gelatinization temperature, soluble starch synthase, amylopectin, rice starch

## Introduction

The eating quality of the cooked rice and the processing quality of rice starch are largely governed by the starch properties such as amylose content and gelatinization properties. At present, several genomic blocks affecting rice grain-starch properties have been identified. For example, the regulation of 5'-splicing of the leader intron of *Wx* gene by a single (G/T) SNP has made rice to contain either high or low amylose content (Ishiki et al. 1998). In addition to amylose content characteristic, gelatinization temperature (GT) is another theological indicator of cooking quality and processing characteristic of rice starch. Rice with low GT disintegrate completely, whereas rice with intermediate GT show only partial disintegration. Rice with high GT remains largely unaffected in the alkali solution. Umemoto et al. (1999) reported the possibility of the relationship between alkaline disintegration and amylopectin structure. Starch granules containing amylopectin enriched in shorter chains are more easily disintegrated in alkali solution than starch granules having amylopectin enriched in longer chains. Genetic studies

with the rice of japonica-type and indica-type revealed that the genes controlling the varietal differences in the alkali disintegration of starch granules, amylopectin chain-length, and gelatinization in urea solution were mapped at the same region on chromosome 6 (Umemoto et al. 2002). Recently, the *soluble starch synthase IIa (SSIIa)* gene has been reported as the key gene responsible for differences among rice varieties in terms of amylopectin chain-length distribution. The different alleles of the *SSIIa* plays a distinct role in the elongation of short chains within clusters (A+B1 chains) of amylopectin and the physico-chemical properties of their starch granules (Umemoto et al. 2002). The present study was conducted to determine the inheritance of the ASV and amylopectin structure. A natural mutant of a low-amylose content and its wild-type differing in amylopectin chain-length profiles and alkali disintegration in 1.7% KOH were investigated. Furthermore, the agarose-based allele specific markers were designed in order to determine rice in the term of alkali disintegration characteristic.

## Methods

*Plant materials:* A high nutritional value/ low amylose-content rice, Jao Hom Nin (JHN) and its natural mutant, BW1, were sequenced their entire *SSIIa* genes. Ninety-six F<sub>2</sub> progenies of crosses between JHN and BW1 were determined the association between the functional SNPs in the *SSIIa* and grain-disintegration characteristics. In addition, twenty-six landrace rice varieties picked from farmer's fields in several places of Thailand were used to evaluate the bi-PASA markers for the alkali digestibility.

*Measurement of Alkali Spreading Value (ASV):* Disintegration of starch granules in alkaline solution was analyzed by soaking ten milled grains in 15 ml of 1.7 % KOH at the room temperature for 23 hours. The degree of spreading was categorized using a seven-point score (7 = completely spread, and 1 = no reaction). Gelatinization temperature of each rice line was estimated based on alkali spreading value (ASV) of its seeds as followed: 1–3, high (74.5–80°C); 4–5, intermediate (70–74°C) and 6–7 low (<70°C).

*Sequencing strategy:* The full 5-kb fragment of rice *SSIIa*, the annotated gene P0525F01.23 of the clone AP003509, was retrieved from the Genbank. The overlapping PCR strategy was applied in order to sequence the whole gene. Nine primer pairs were designed using Primer 3 program. The PCR templates were sequenced from both directions by ABI 377 XL. The sequences of each fragment were assembled using Phred/Phrap/Consed programs and further compared using multiple-sequence-alignment program ClustalW.

*Bi-directional polymerase chain reaction amplification of specific alleles (bi-PASA):* Bi-directional PCR amplification of specific alleles (bi-PASA) markers utilizing four primers in a single PCR amplification was developed. Primers were designed based on the guidelines proposed by Liu et al.(1997). Two sets of bi-PASA primers were developed for genotyping of SNPs at positions 2209 and 2340-41 of the *SSIIa* gene. The PCR products were examined by electrophoresis on 1.5% agarose gel.

## Results

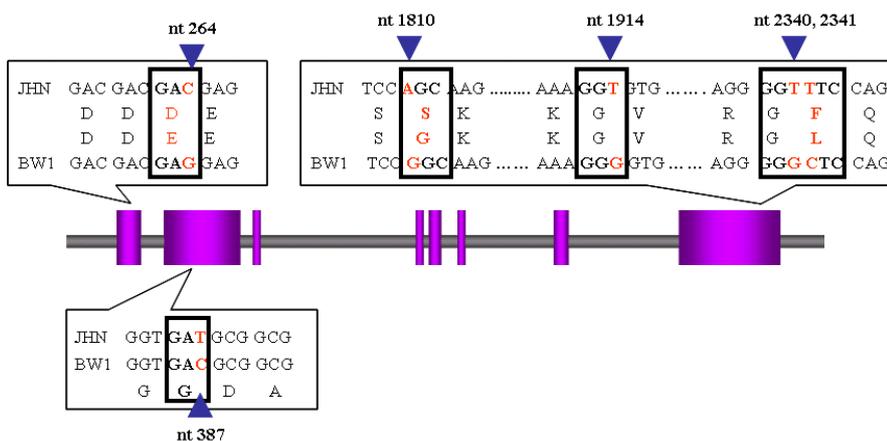
### *A mutant different in alkali digestibility*

Jao Hom Nin (JHN) is a non-photosensitive/purple-seed rice cultivar developed for nutrition purposes. BW1 is a natural mutant of JHN which has remarkable white grain-pericarp. Genome background investigation by SSR markers showed 75% similarity between JHN and BW1 (unpublished data). In addition this mutant contains the same nutritional value and amylose content level (12%) as those of JHN. However, a clear difference was observed in the degree of spreading of rice kernels in 1.7% KOH solution; JHN possessed intermediate alkaline digestibility, ASV = 5, while BW1 are resistant to alkali, ASV = 2. According to these observations, JHN and BW1 may contain different genetic blocks for the alkali digestibility. The further investigation of ASV in F<sub>3</sub> grains derived from 96 F<sub>2</sub> plants of these crosses revealed three types of F<sub>2</sub> plants which fits 1:2:1 ratio expected for a mendelian single gene ( $\chi^2 = 2.71$ , P = 0.05). Thus, it is possible that the difference in alkali digestibility between JHN and BW1 was caused from a mutation of a single gene. So far, the genetic control of GT has been reported to be governed by one major gene and modifier genes. Recently, the major gene regulating alkali disintegration was identified as *starch synthase II (SSIIa)* located on chromosome 6. The ASV dissimilarity between JHN and BW1 might reflect the variation in *SSIIa*. Furthermore, the chain length distribution analysis of amylopectin between JHN and BW1 was different (unpublished data). Therefore we hypothesized that the

differences in alkali digestibility and amylopectin fine structure of BW1, L-type, may reflect the gain-of-function of the *SSIIa* in this mutant.

### Sequence variations in *SSIIa*

Rice *SSIIa* comprises eight exons encoding 810 amino acids. Naturally, four functional SNPs causing amino acid substitutions have been observed at position 264, 1810, 2209 and 2340-41. In turn, four haplotypes were designated as followed: haplotype 1 (264C-1810G-2209G-2340G-2341C) haplotype 2 (G-G-G-GC), haplotype 3a (C-A-G-GC), haplotype 3b (C-A-G-TT) and haplotype 4 (C-A-A-GC) (Umemoto and Aoki 2005). In this study, the comparison of the coding sequences (CDS) between JHN and BW1 revealed six SNPs in the exon 1, 2 and 8 (Figure 1). Almost all SNPs resemble those found in previous reports except for the SNP at nucleotide 2209 that is not different between BW1 and JHN. JHN belongs to the haplotype 3b and BW1 belongs to haplotype 2. It has been stated that cultivars belong to haplotypes 1, 2, and 3a have higher amounts of granule-associated *SSIIa*, smaller amounts of amylopectin short chains ( $DP \leq 11$ ), higher gelatinization temperature, and less alkali spreading than cultivars with haplotype 3b and 4 (Umemoto and Aoki 2005). Based on *SSIIa* haplotypes, it is possible to conclude that the differences in alkali disintegration and amylopectin fine structure between JHN and its mutant BW1 were caused from sequence variations in their *SSIIa*.



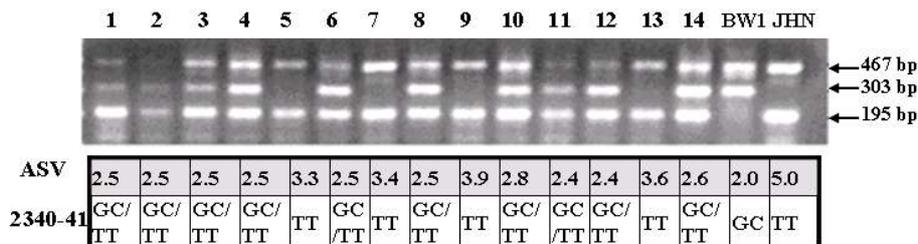
**Figure 1** SNP locations found in the exon 1 and exon 8 of the rice *SSIIa*. The SNP and the amino acid changes are shown in the square boxes. The nucleotide location of each SNP was also indicated.

Among the four functional SNPs, two SNPs at positions 2209 (G>A) and 2341 (C>T) were considered the most informative SNPs. These base substitutions caused amino acid changes, 737 (Val to Met) and 781 (Leu to Phe), respectively. The SNPs 2209 and 2341 each changed the enzyme activity and ability to bind to starch granules, and consequently, starch gelatinization properties (Umemoto and Aoki 2005). Replacements of Val-737 by Met or Leu-781 by Phe caused clear reduction of *SSIIa* activity. According to the analyses of starch-associated protein of different rice *SSIIa* haplotypes, combinations of SNPs 2209 and 2341 could be used to determine the *SSIIa* activity and gelatinization properties; 2209G-2341C is assigned for optimal *SSIIa* activity and high gelatinization temperature, while 2209G-2341T and 2209A-2341C are assigned for no *SSIIa* activity and low gelatinization temperature. These assignments of SNPs and gelatinization property, or alkali digestibility, are also valid for JHN and BW1. Since the base at the location 2209 of JHN and BW1 is the same (G allele), the combination G-GC is indicated the low alkali digestibility (ASV = 2) and the combination G-TT is for the high alkali spreading value (ASV = 5) (Figure 2).

### Functional markers for rice *SSIIa*

The alkali disintegration has been used as a phenotypic genetic marker, since the degree of disintegration correlates with the gelatinization temperature (GT) of rice flour. The estimation of alkali digestibility of rice grain may also be affected by the environment, especially the temperature during grain-filling. In the present study, the effective DNA markers were developed for determining the GT by utilizing the imformative SNPs found in the

*SSIIa*. The SNPs at the positions 2209 and 2341 were chosen to design PCR-based markers for alkali digestibility of rice grains. A PCR-based method recognizing different alleles (bi-PASA, bi-directional PCR amplification of specific alleles) of the rice *SSIIa* was developed. Two outer primers were designed to amplify a 467-bp fragment from exon 8 of the *SSIIa*, encompassing the SNP 2209 and 2340-41. Two sets of inner primers for the SNPs 2209 and 2340-41 were designed to amplify different sizes of amplified fragments specific to unique allele of each SNPs. Since the two adjacent SNPs at positions 2340 and 2341 are co-occurrence as TT or GC, both SNPs were co-genotyped and assigned as one SNP. Both homozygous and heterozygous genotypes for the SNPs 2209 and 2340-41 are efficiently identified on the basis of size differences of amplified fragments (Figure 2).



**Figure 2 Genotyping F<sub>2</sub> using the bi-PASA marker for the SNP 2340-41. The averages of alkali spreading score (ASV) from ten seeds of each rice line are shown under the DNA bands. The 467-bp allele-non specific fragments, 303-bp allele GC-specific and 195-bp allele TT-specific are indicated by arrows.**

A result of assaying the 96 F<sub>2</sub> plants revealed that 52 F<sub>2</sub> lines containing segregating seeds for alkali spreading have all heterozygous alleles at the SNP 2340-41, both 195-bp and 303-bp fragments were amplified. F<sub>2</sub> plants containing all seeds that were resistant to alkali digestion and all highly disintegrated seeds have the homozygous alleles for BW1 (303-bp fragment) and JHN (the 195-bp fragment), respectively. All F<sub>2</sub> plants have the same G-allele (the 325-bp fragment) at the SNP 2209 as the parental lines BW1 and JHN (data not shown). The two bi-PASA markers for the rice *SSIIa* were further validated by screening twenty-six cultivars of landrace Thai rice germplasms (unpublished data) and the result has clearly shown that these bi-PASA markers can be accurately used to identify the alkali digestibility of rice varieties.

## Conclusion

The present investigation has proven that the different in alkali digestibility and amylopectin fine structure between JHN and its mutant, BW1, were caused by sequence variation in *SSIIa*. This information facilitates the development of functional DNA markers for selecting rice lines with appropriate cooking quality. The SNPs 2209 and 2341 of the rice *SSIIa* were selected as targets for developing bi-PASA markers to classify rice varieties based on their starch gelatinization temperature. These agarose-based markers could be useful for rice breeding programs using marker-assisted selection in order to identify genotype based on their alkali spreading values.

## References

- Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K. 1998. A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5' splice site of the first intron. *Plant J.* 15: 133-8.
- Liu Q, Thorland EC, Heit JA and Sommer SS. 1997. Overlapping PCR for bidirectional PCR amplification of specific alleles: a rapid one-tub method for simultaneously differentiating homozygotes and heterozygotes. *Genome Res.* 7: 389-98.
- Umamoto T and Aoki N. 2005. Single-nucleotide polymorphism in rice *starch synthase IIa* that alter starch gelatinization and starch association of the enzyme. *Funct Plant Bio.* 32: 763-8.
- Umamoto T, Nakamura Y, Satoh H and Terashima K. 1999. Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during grain-filling. *Starch* 51: 58-62.
- Umamoto T, Yano M, Satoh H, Shomura A and Nakamura Y. 2002. Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theor Appl Genet.* 104: 1-8.