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ORIGINAL PAPER

A single nucleotide polymorphism in the *Waxy* gene explains a significant component of gel consistency

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Abstract Gel consistency (GC) is a standard assay used in rice improvement programmes to determine whether rice cultivars/breeding lines of high amylose content are soft or firm textured when cooked. In this study, we show that sequence variation in exon 10 of the *Waxy* (*Wx*) gene associates with GC using RILs derived from parents with high amylose content that differ in GC. The association was validated using a diverse set of traditional varieties, selected on the basis of amylose content, from the generation challenge programme. Structural investigations to explain how the mutation leads to differences in GC showed a strong association between GC and the proportion of amylose that leaches. It was shown that cooked rices of hard GC do not change in hardness over 24 h, whereas rices of soft GC retrograde significantly over 24 h. This leads to the conclusion that the mutation on exon 10 of the *Wx* gene affects the proportion of amylose bound to amylopectin and the proportion able to leach, and these structural differences alter the composition of the gel, which affects the amount of time the gel takes to reach a final hardness. The SNP described here completes the set of markers required to genotype for the current traits of cooking quality, but selecting the allele for soft texture has the negative result of also selecting for retrogradation potential.

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Introduction

Rice is the staple food of Asia. In recent years, consumer preferences have shifted towards better-quality rice, particularly towards varieties of good eating quality. Each country, or even province, requires rice to have a particular suite of quality traits. A common trait across most countries of Asia is soft texture of freshly cooked rice, and maintenance of that soft texture on storage (Champagne et al. 2010). The firmness of freshly cooked rice relates to the amount of amylose present in the grain, but the relationship does not hold within the high amylose category. Grain of high amylose can be either hard or soft when cooked (Cagampang et al. 1973; Juliano et al. 1964). In order to enable rice improvement programmes to select for soft-textured rices, the gel consistency test was developed as an indirect method for routine screening of cooked rice hardness for rices with amylose above 25% (Juliano 1985).

The gel consistency test measures the distance travelled, after 1 h, of a gel made from rice flour and KOH. For rices

with a hard texture, the gel is short, travelling only a small distance, but for those with soft texture, the gel is viscous and travels up to 10 cm within the hour (Cagampang et al. 1973). Different setting rates of the gel suggest different rheological properties. A gel made from rice flour will be a composite material of swollen granules and hydrated proteins bathed in a continuous phase of leached amylose and solubilised small amylopectin molecules (Biliaderis et al. 1986; Biliaderis and Zawistowski 1990; Cuevas et al. 2010b; Nguyen et al. 1998; Sodhi et al. 2010). The leached carbohydrates, which define the continuous phase, affect rheological properties (Itturiaga et al. 2010; Tsai and Lii 2000), such as flow and set properties of a gel, with different effects from different chain lengths (Biliaderis and Zawistowski 1990).

A number of studies, using different mapping populations, have located the gene for gel consistency at the *Wx* locus (Bao 2002; Bao et al. 2003; He et al. 2006; Lanceras et al. 2000; Li et al. 2003; Tang et al. 1991; Tian et al. 2005; Wang et al. 2007), which encodes the enzyme responsible for amylose synthesis. This finding is counter-intuitive since the *Wx* locus controls amylose content (Sano 1984), but gel consistency does not associate with amylose content. The implication is either that the *Wx* locus is not the major gene for gel consistency, or amylose content is not the only phenotype under its control.

Single nucleotide polymorphisms (SNPs) have been found at the splice site of exon 1 (Cai et al. 1998; Isshiki et al. 1998; Wang et al. 1995), exon 4 (Mikami et al. 2008), exon 6 (Chen et al. 2008a; Larkin and Park 2003; Mikami et al. 2008) and exon 10 (Chen et al. 2008b; Larkin and Park 2003; Mikami et al. 2008) of the *Wx* gene. Different haplotypes of the *Wx* gene, based on the SNPs at exon 1, 4 and 6, determine the different classes of amylose (Chen et al. 2008b; Mikami et al. 2008). However, both states of the SNP on exon 10 are found in the high amylose class, and this SNP associates with the firming of the gel as the temperature cools from 95 to 50°C in a rapid visco analyser (RVA) (Chen et al. 2008b). This translates to the part of the amylograph curve that was first associated with gel consistency (Cagampang et al. 1973), leading to the hypothesis that the SNP on exon 10 associates with gel consistency.

Using a population derived from parents of high amylose, developed for identifying genes of gel consistency, as well as a diverse set of traditional varieties with high amylose content, the objectives of this work were to determine (1) whether the SNP on exon 10 of the *Wx* gene associates with gel consistency, (2) differences in features of amylose, other than content, that could explain gel consistency and (3) associations between gel consistency, amylose properties, SNP status, and texture of freshly cooked and retrograded rice.

Materials and methods

Material

A population (F_6) of recombinant inbred lines (RILs) derived from a cross between IR8 and IR5 was developed at the International Rice Research Institute (IRRI). Both parents carry the Wx^a allele and have 29% amylose (Cuevas et al. 2010a), but the parents differ significantly in gel consistency, offering an opportunity to find the genetic basis of gel consistency without the confounding effect of different amylose contents. Gel consistency of IR8 in the wet and dry seasons of 2006–2009 was 34 ± 2 mm and for IR5 from the same eight trials was 97 ± 3 mm. The RILs were grown in two seasons at IRRI's experimental farm during the wet season of 2008 (427 F_5 RILs) and dry season of 2009 (316 F_6 RILs). Fewer lines were grown in 2009 because a tungro infestation in the 2008 wet season led to the loss of 111 lines from the population.

In order to test any associations found in the RILs, a set of diverse traditional varieties was also grown and tested. The set consisted of a subset of the Generation Challenge Programme (GCP), which are diverse traditional varieties (*Oryza sativa* L.) selected by the TT Chang Genetic Resources Centre at IRRI to represent all sub-populations of *O. sativa* (Supplementary Table S1). The GCP set was grown in the dry season of 2008; the subset used in the present study (163) was selected on the basis of amylose content (>25%). All harvested seeds were stored and dried to required moisture (12–14%). All samples were dehulled (Satake Engineering Co. Ltd, Japan), and then polished using a commercial paint shaker adapted to polish small samples of rice (Skandex SO400, Fast and Fluid Management, Netherlands). Samples of polished rice were ground to pass a 0.5 mm screen using a Cyclotec Mill (UDY Corp, USA).

In order to determine trends for gel consistency in material being considered as new varieties, gel consistency values were retrieved from IRRI's quality evaluation database for late-generation material evaluated in the quality evaluation program between 2005 and 2009.

Gel consistency

Flour (100 mg) from all samples was weighed in duplicate into 13 mm × 100 mm tubes and 200 µL of ethyl alcohol (95%), containing 0.025% thymol blue, was added to each tube as well as 2 mL 1N KOH. Tubes were mixed using a Vortex Genie mixer (Scientific Industries, Inc., NY, USA), then placed in a vigorously boiling water bath for 8 min. The tubes were then removed from the water bath, held at room temperature for 5 min, and then cooled in an ice water bath for 15 min. Following this, tubes were laid

horizontally on a light box on top of graphing paper. After 1 h, the distance that the gel migrated in the tube was measured.

Genotyping at exon 10

Thirty-day-old seedlings of the diverse set of 163 traditional varieties and the F_5 and F_6 progeny of the cross derived from IR8 and IR5 were grown in the glasshouse at IRRI before transplanting. Leaf tissue was harvested before transplanting and then frozen with liquid nitrogen and kept at −80°C for DNA extraction. DNA was extracted from the leaf tissue of individual plants exactly as previously described (Cuevas et al. 2010a).

In order to genotype the SNP on exon 10 of the *Wx* gene, primers were designed and PCR was conducted to amplify a region of exon 10 consisting of 745 bp, and then a restriction enzyme was used. The PCR reactions contained 10 μL reaction mixture with 2 μL of template DNA, 5 μL 2× Biomix (Bioline, MA, USA), 0.2 μL 10 mM (forward primer: 5′-GCATCACCGGCATCGTC-3′), 0.2 μL of 10 mM (reverse primer: 5′-GCTCCGGCCATGATGA-GATG-3′) and 2.6 μL sterile molecular biology grade water. Using a thermocycler GStorm Thermal Cycler (model GS1, Gene Technologies Ltd, Essex, UK), the PCR cycle was set at 94°C for 1 min, followed by 35 cycles of 94°C denaturation for 30 s, annealing 67°C for 45 s, 72°C elongation for 30 s and were stored at 4°C. The products were detected using 2% agarose gel in 1× Tris–Borate EDTA buffer (TBE). The gel was run at 100 V for 35 min and was stained with SYBR Safe (Invitrogen, Carslsbad, CA, USA) solution. The gels were observed under UV transilluminator (DR 195 M, Clare Chemicals, Dolores, CO, USA) and photographed using Alpha Imager software (Alpha Innotech Corp, CA, USA). The molecular size of the amplification products was estimated using a 1-kb ladder (Invitrogen, Carlsbard, CA, USA). In order to determine the C → T polymorphism, 5 mL of each PCR product was digested with 0.2 μL of *Apa*I restriction enzyme (Takara, Shiga, Japan) in a total volume of 10 μL. This restriction enzyme recognises the sequence GGGCCC and cleaves at the second C leading to two products of 424 and 321 bp. In the case of the polymorphism, the second C becomes a T, so the enzyme does not have a recognition site, and the 745-bp PCR product remains intact (Fig. 1). The mixtures were incubated for 1 h at 37°C. The digested products were electrophoresed for 45 min at 100 V on 2% agarose gels in 1× TBE buffer with ladder standard.

Texture profiling

A subset of the population, 21 with C and 21 with T at exon 10, was chosen for all subsequent analyses. Polished grains

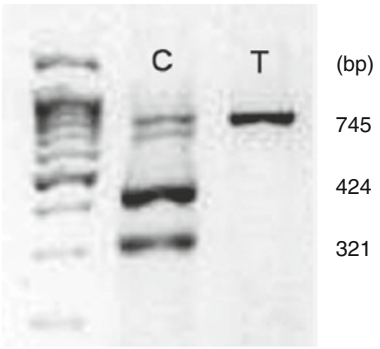

Fig. 1 PCR amplification products of the two alleles of the *Wx* gene as defined by the SNP at exon 10 using IR5 (C) and IR8 (T). Primer sequences are indicated in the Sec."Materials and methods"

of each sample (25 grains) were soaked in excess water (20 mL) for 30 min. The tubes, each containing 25 grains, were then cooked in a boiling water bath for 15 min, and then cooled in an ice water bath for 2 min before the water was decanted. The grains were then placed on absorbent paper to drain any excess water. The cooking preparation was replicated five times. For each replicate, three grains were analysed for hardness and stickiness using a TA.XT-Plus Texture Analyser with a cylindrical probe attachment (diameter = 35 mm, Stable Micro Systems Ltd., Surrey, UK). Strain was set at 90% and the test speed at 0.5 mm s^{-1}. Texture was measured twice per replicate, immediately after cooking and after 24 h of storage at 4°C, with rewarming to room temperature.

Quantifying HWS and HWI starch

The hot-water-soluble (HWS) and -insoluble (HWI) fractions of starch were collected from flour of 21 progeny with C and 21 with T at exon 10 of the *Wx* gene. Starch in the fractions was debranched and separated by size exclusion chromatography (SEC). Separation, debranching and SEC analysis was carried out, in duplicate, exactly as previously described (Cuevas et al. 2010b; Ward et al. 2006).

Statistics

Analysis of variance (ANOVA) was performed with *R* statistical software (2.11.0). Fisher's Least Significant Difference (LSD) was used to compare means at $p < 0.05$.

Results

Association between gel consistency and SNP

Figure 2 shows the distribution of the values obtained for gel consistency for the F_6 population grown in the dry

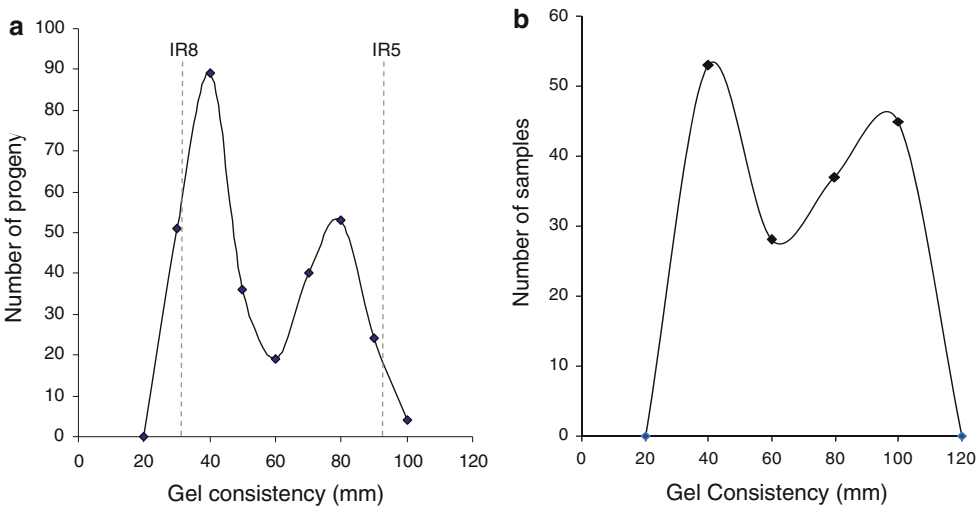

Fig. 2 Distribution of gel consistency values of (a) progeny of the F_6 population derived from an IR8/IR5 cross and grown in the dry season of 2009 at IRRI and (b) the diverse set of traditional varieties with amylose >25%. All the progeny/lines with C at exon 10 of the *Wx* gene had gel consistency >60 mm, and those with a T ranged from 20 to 60 mm

Table 1 Association between the SNP on exon 10 of the *Waxy* gene and gel consistency in a diverse set of 163 traditional varieties with amylose content >25%, and in the RILs grown in 2008 and in 2009

	Gel consistency (mm)		
	Diverse set	F_5 population 2008	F_6 population 2009
IR8	35.0	35.0	30.0
IR5	100.0	100.0	92.0
C exon 10	82.7[a]	90.6[a]	75.2[b]
T exon 10	40.1[b]	34.6[c]	33.2[d]
$LSD_{0.05}$	2.3	1.2	

In a column, numbers followed by the same letter are not significantly different at 5% LSD

season of 2009 (a) and for the diverse set of traditional varieties of 25% amylose and above (b). The distribution for both is bimodal, showing two broad peaks that intersect at a gel consistency of about 60 mm. The average for each group is about 40 and 80–85 mm, respectively, and the range for both modes is about 40 mm.

The population and the diverse set were genotyped at the SNP site on exon 10 using a restriction enzyme. All progeny with C at the site in exon 10 were of gel consistency >60 mm and those with a T at that site showed a gel consistency <60 mm and the correlation was highly significant (Table 1). When the association found in the RILs population was tested using a set of 163 diverse traditional varieties, all with amylose content above 25%, the same result at the same level of significance was found (Table 1). In both the population and the diverse material, 61 lines and 33 varieties showed gel consistency values that are traditionally defined as intermediate—between 40 and 60 mm. All of these lines/varieties had T at the SNP site (Tables 1, S1).

Figure 3 shows the proportion of late generation material undergoing final selection before consideration for

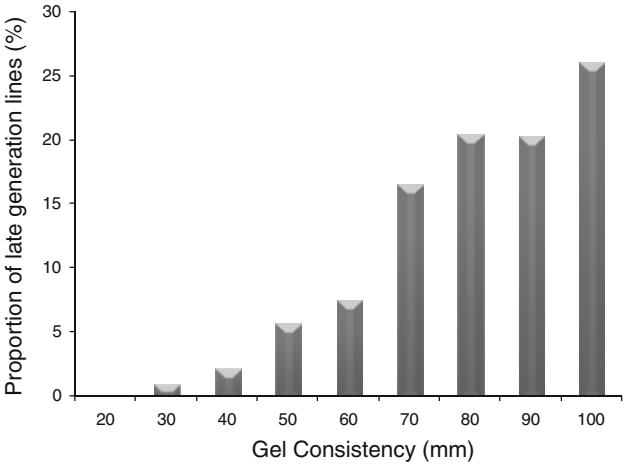

Fig. 3 Frequency histogram of 3,300 late generation breeding lines from replicated yield trials at IRRI from 2005 to 2009 showing the proportion that fall into each 10 mm division of gel consistency values

varietal release each year at each 10 mm interval of the scale used to measure gel consistency. Over the past 5 years, 84% of lines that are progressed to late generation trials have gel consistency greater than 60 mm (Fig. 3).

Association between gel consistency and texture

Texture profiling was carried out to test the association between firmness of cooked rice and the SNP. Table 2 shows that IR5 and progeny with C at exon 10 are significantly softer when freshly cooked than IR8 and progeny with T at exon 10. However, Table 2 shows that when the same set of rices are tested 24 h later, after storage at 4°C, those that were soft became much harder, showing significant retrogradation, but those that were initially firm changed little over storage.

Table 2 Association between the hardness of freshly cooked and stored rice grains of the parents and a subset of the RILs from the 2009 season

	Hardness (g)	
	Freshly cooked	Stored 24 h
IR8	2,880.5	2,904.0
IR5	1,968.9	2,803.0
C exon 10	2,344.4[d]	3,177.2[a]
T exon 10	2,789.4[c]	2,962.3[b]

Values with the same letter are not significantly different ($LSD_{0.05} = 53.5$)

There were significant differences between the SNP and the proportion of soluble and insoluble amylose and amylopectin (Table 3) for both seasons. For samples with C, a higher proportion of starch became soluble in hot water than samples with a T at exon 10. The amount of soluble material is highly significantly associated with gel consistency, and the amount of insoluble starch, by corollary, is also highly significantly associated with gel consistency (Table 3).

Discussion

Utility of the SNP in rice improvement programmes

Gel consistency is one of three indirect indicators of cooking quality tested in rice improvement programmes. Three classes of gel consistency have traditionally been measured, with an intermediate class defined as a gel travelling 40–60 mm during the assay. However, the present study clearly shows a bimodal distribution, indicating a major effect on gel consistency from biallelic variation at a single locus. The mapping population is derived from parents that differ in gel consistency, so a bimodal distribution in gel consistency values of the progeny (Fig. 2a) is not an unexpected result. However, finding a similar distribution in a set of diverse traditional varieties (Fig. 2b) strongly supports the hypothesis that a single locus has a large effect on gel consistency. Both the progeny and the diverse set indicate that allelic variation defined by the SNP on exon 10 of the *Wx* gene separates high amylose varieties into two classes of gel consistency.

Table 3 Association between the proportion of soluble and insoluble starch fractions and the SNP genotype using the RILs grown in two seasons

Proportion of HWS and HWI (%)								
	HWSA		HWSAP		HWIA		HWIAP	
Year	2008	2009	2008	2009	2008	2009	2008	2009
IR8	64.3	70.1	9.3	9.0	35.7	29.9	90.7	91.0
IR5	65.0	68.7	14.5	19.7	35.0	31.3	85.5	80.3
T exon10	66.8[c]	65.7[d]	11.6[c]	9.5[d]	33.2[a]	34.3[a]	88.4[b]	90.5[a]
C exon 10	72.9[a]	71.4[b]	13.6[b]	15.8[a]	27.1[d]	28.6[c]	86.4[c]	84.2[d]
$LSD_{0.05}$	0.9		0.8		0.9		0.8	

For each fraction, numbers followed by the same letter are not significantly different at 5% LSD

HWSA Hot-water-soluble amylose, *HWSAP* Hot-water-soluble amylopectin, *HWIA* Hot-water-insoluble amylose, *HWIAP* Hot-water-insoluble amylopectin

All progeny of the mapping population and the diverse traditional varieties with gel consistency <60 mm had T at the SNP site on exon 10 of the *Wx* gene and all those with gel consistency >60 mm had C at that SNP site (Table 1). The data in the present study clearly suggest that the SNP on exon 10 of the *Wx* gene, from C → T, is a significant factor influencing gel consistency (Table 1) and is consistent with previous mapping studies that have associated the *Wx* gene with gel consistency. The range of each mode in Fig. 2, spanning 40 mm, suggests that other genes, in combination with each SNP status, lead to differences in gel consistency within each of the modes. Moreover, the first mode in Fig. 2a, b includes the 61 progeny and 33 traditional varieties with gel consistency of 40–60 mm; this is traditionally defined as the intermediate class (Cagampang et al. 1973). These all have T at exon 10, so they associate genetically with those progeny/varieties of hard gel, with gel consistency <40 mm. While this SNP associates with gel consistency above and below 60 mm, it does not identify an intermediate class.

Data accumulated over 5 years in IRRI's quality evaluation program indicates that rice improvement programmes are selecting for soft gel consistency, which means gel consistency >60 mm (Fig. 3), in order to meet market requirements for soft texture immediately after cooking (Juliano et al. 1964). Breeding lines in late generation trials are those that offer potential as new varieties. With so few late generation lines showing gel consistency <60 mm (Fig. 3), it is likely that the SNP on exon 10 of the *Wx* gene, which separates gel consistency values at about 60 mm, will be useful to rice improvement programmes, and that breeders working with high amylose material will select those carrying the allele with C to ensure gel consistency >60 mm. However, selecting for C at this locus selects not only for soft texture immediately after cooking, but data in the present paper suggest that the SNP also selects for retrogradation of stored rice, which is an undesirable property of rice.

The first three quality traits measured in rice improvement programmes are amylose content, gelatinisation temperature and gel consistency (Juliano 1985). Molecular markers have already been developed and validated for

both amylose content and gelatinisation temperature (Chen et al. 2008a; Cuevas et al. 2010a). The present study completes the set of markers for early generation cooking indices, by adding a useful marker for soft gel consistency, with the caveat of retrogradation potential, and a low-cost restriction assay for measuring it. Now, early generation testing for cooking quality can be assayed with a single DNA extraction and a number of SNP markers.

Association between texture, retrogradation and the SNP

Texture of freshly cooked and stored rice associates significantly with the SNP on exon 10 (Table 2). Further, all those progeny of the population that are soft when freshly cooked, with C at exon 10, are significantly firmer on storage, whereas those with T at exon 10 are firm when freshly cooked and do not change a lot upon storage (Table 2). Retrogradation is a trait that is not desired by any rice consumer. The association between retrogradation potential and this SNP suggests that the SNP affects, and perhaps predicts, retrogradation. It is important that rice breeding programmes are aware that by selecting for soft freshly cooked rice they are likely to be selecting also for retrogradation. However, this association was found using progeny of a population and it needs to be verified in other genetic backgrounds. Nevertheless, it offers potential insights into the biological basis of texture in freshly cooked and stored grains of rice.

The samples with a C at exon 10 gave soft, viscous gels, softer cooked rice, but high retrogradation, and those with T gave a short, firm gel, firm texture of freshly cooked rice and little change to texture over storage (Tables 1, 2, 3). As rice cooks, two phases form within the grain. The first is a continuous phase that contains solubilised starch molecules connected by weak covalent bonds, and the second is a dispersed phase of hydrated starch granules (Nguyen et al. 1998). A higher concentration of starch polymers in the continuous phase is associated with a viscous gel that flows (Debet and Gidley 2007; Gidley 1989). Insoluble amylose chains, covalently linked to amylopectin (Aoki et al. 2006), have been shown to reinforce hydrated starch granules and increase the rigidity of the dispersed phase (Sodhi et al. 2010), leading to a firm gel that fractures but does not flow (Debet and Gidley 2007). Table 3 shows that the C on exon 10 is significantly associated with a larger proportion of soluble starch polymers in freshly cooked rice, and a T on exon 10 is associated with a larger proportion of insoluble amylose.

Amylose in the continuous phase does not contribute to firmness while it remains soluble in that phase (Genovese and Rao 2003), perhaps explaining the softer texture of the freshly cooked rice from these samples (Table 2). However, the amylose in the continuous phase will eventually undergo aggregation and gelation (Gidley 1989), forming an interconnected gel embedding the swollen granules (Biliaderis and Zawistowski 1990; Ellis and Ring 1985). Gelation is dependent on the length of the polymers, with long chains >1,100 degrees of polymerisation (DP) undergoing gelation and shorter chains precipitating (Gidley and Bulpin 1989). Analysis of the amylose chains in the hot water-soluble fraction shows that the average chain length is greater than DP 1,100 (Ward et al. 2006), so soluble amylose in the continuous phase is likely to undergo gelation rather than precipitation. Aggregation and gelation of long chains in solution occurs over a number of hours (Clark et al. 1989), so is not captured in a 60-min gel consistency test. Presumably in the 24 h after cooking, the amylose polymers formed an aggregated and cross-linked gel, leading to the significant increase in hardness of the grains (Table 2). The higher proportion of insoluble starch in the samples with T at exon 10 (Table 3), leading to a more rigid dispersed phase, possibly explains the harder gel consistency of those samples, and the small change in firmness after 24 h (Table 2), since a gel with those properties sets rapidly and does not continue to flow (Debet and Gidley 2007).

The data in the present study show that the SNP on exon 10 of the *Wx* gene explains a significant component of differences in gel consistency, firmness of freshly cooked rice and retrogradation. The *Wx* gene encodes granule-bound starch synthase I (GBSSI), which elongates amylose chains (Sano 1984). This SNP associates with differences in the proportion of amylose chains that are free to leach into the continuous phase and those that are covalently linked to amylopectin, which has significant implications for the properties of the gel, and therefore the texture of the cooked rice. This raises interesting questions about how the SNP alters the location and nature of the amylose chains to give such fundamentally different phenotypes. Exon 10 of the *Wx* gene encodes part of the glycosyl transferase domain of the gene for the transfer of ADP or UDP linked sugars (Shapter et al. 2009). The SNP leads to a change from a non-polar (Pro) to a polar (Ser) amino acid residue in the glycosyl transfer region of the encoded enzyme, and this might alter the way the enzyme elongates amylose or binds to the starch granule, leading to the large differences in texture of the cooked and stored rice. The present study suggests that the SNP on exon 10 of the *Wx* gene predicts soft gel consistency, and along with that, soft texture of freshly cooked rice, and perhaps high retrogradation potential of rices with high amylose.

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