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SNP in starch biosynthesis genes associated with nutritional and functional properties of rice

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Starch is a major component of human diets. The relative contribution of variation in the genes of starch biosynthesis to the nutritional and functional properties of the rice was evaluated in a rice breeding population. Sequencing 18 genes involved in starch synthesis in a population of 233 rice breeding lines discovered 66 functional SNPs in exonic regions. Five genes, AGPS2b, Isoamylase1, SPHOL, SSIIb and SSIVb showed no polymorphism. Association analysis found 31 of the SNP were associated with differences in pasting and cooking quality properties of the rice lines. Two genes appear to be the major loci controlling traits under human selection in rice, GBSSI (*waxy* gene) and SSIIa. GBSSI influenced amylose content and retrogradation. Other genes contributing to retrogradation were GPT1, SSI, BEI and SSIIIa. SSIIa explained much of the variation in cooking characteristics. Other genes had relatively small effects.

Rice is a major human food composed largely of starch. Starch properties determine the key functional properties of rice such as cooking temperature and influence human health through its contribution to the glycemic index and levels of resistant starch. The incomplete digestion-absorption of resistant starch in the small intestine leads to non-digestible starch fractions with physiological functions similar to dietary fibre with significant beneficial impacts[1].

Retrogradation describes the hardening of cooked starch after cooling due to re-crystallization of gelatinized starch components during storage[2]. It is believed there is a significant correlation between the tendency of any one starch sample to retrograde and its levels of resistant starch. Hence, in this study the term retrograded-resistant rice starch is used. Assessment of the *in vivo* digestion and structural features of maize, bean and potato flake high amylose resistant-retrograded starch in the ileal contents of four human populations found resistant starch consisted mainly of retrograded amylose with degree of polymerization of approximately 35 glucose units and a melting temperature of 150°C[3]. Pea, maize, wheat, and potato retrograded amylose are highly resistant to amylolysis and digestibility[4]. Factors other than amylose content which may have a direct or indirect influence on the rate of starch retrogradation, firmness and resilience of rice starch after cooking are protein and lipid contents[5].

High-amylose rice cultivars usually have more resistant starch (RS) and lower estimated glycemic index (EGS), suggesting highly-retrograded cooked rice cultivars tend to a reduction of hydrolysis index (HI) and glycemic index (GI)[6]. Conversely, starch of low-amylose rices, which have higher HI, are more quickly hydrolysed than intermediate and high-amylose rice (high HI)[6,7]. Characteristics of high amylose rice cultivars are normally determined by RVA (Rapid Visco Analysis) which are described by parameters such as peak viscosity (PKV), hot paste viscosity (HPV) and cool paste viscosity (CPV).

Seven starch synthesis enzyme classes have been defined, including ADP-glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSS), starch synthase (SS), branching enzyme (BE), debranching enzyme (DBE), starch phosphorylase (PHO) and glucose 6-phosphate translocator (GPT). These genes/enzymes contribute directly or indirectly to the production of starch granules.

The link between natural variation in particular starch synthesis genes and starch properties is well established in some cases. GBSSI (*waxy* gene) is primarily responsible for the synthesis of linear chains of glucose molecules

found in amylose is the most well characterised cereal grain starch synthesis enzyme. A number of SNP in the rice *waxy* gene, at the intron/exon 1 junction site, exon 6 and exon 10, impact starch quality[8–10] by effecting amylose content. The gene encoding starch synthase IIa (SSIIa), *alk,* is exclusively expressed in the rice endosperm and has been extensively studied in the context of its effect on cooking quality and starch texture[11,12]. Two SNPs within exon 8, [A/G] and [GC/TT] are significantly associated with rice alkali disintegration and eating quality and starch gelatinisation temperature (GT)[13].

More recently, Yan et al. (2010) analysed the association of 17 starch synthesis genes with RVA profile parameters in a collection of 118 glutinous rice accessions using 43 gene-specific molecular markers. They found 10 of 17 starch-related genes have an impact on rapid visco analyzer (RVA) profile parameters. The association analysis revealed pullulanase plays a dominant role in control of PKV, HPV, CPV, breakdown viscosity (BDV), peak time (PKT), and pasting temperature (PT) in glutinous rice. Nine other starch genes had a minor impact on only a few RVA profile parameters. However, RVA parameters such as starch paste viscosity and other starch quality traits may be controlled by a complex genetic system involving many starch-related genes[14].

Many induced mutations that have been studied[15] result in loss of function or drastically alter starch biosynthesis resulting in poor yielding rice. These studies are useful in understanding the biochemical function of enzymes in starch biosynthesis. However, because the rice varieties are not viable as crop plants these mutants are not directly relevant to rice improvement or the understanding of human selection during domestication that involves more subtle selection for mutants that do not impact adversely on productivity. In this study we have worked with material within a rice breeding program to explore the diversity that is available in the domesticated genepool. With the exception of GBSSI and SSIIa, most studies of the molecular basis of starch synthesis have focused on comparison of gene-deficient mutants[15] rather than analysis of allelic diversity, perhaps in part due to a lack of high-throughput technologies to discover and analyse new variants in diverse populations. Single nucleotide polymorphisms (SNP) are the most abundant type of genetic variation found within all species and many important plant traits and human diseases are attributed to these sequence variations[16]. Identifying SNP and associating them with grain starch quality advances our understanding of the starch biosynthesis pathway and highlights ways to improve crops that are higher yielding and of better quality, directly impacting food security and human nutrition and health.

Massively parallel sequencing (MPS) technology is a high-throughput platform for genetic analysis based on ultra deep DNA sequencing[17]. Kharabian-Masouleh et al. (2011) discovered more than 501 SNPs and 113 In/dels in 17 starch synthesis genes in an Australian rice breeding population using a combination of a target-pooled long range PCR and MPS. By combining MPS with high throughput genotyping technologies such as multiplexed-MALDI-TOF (Sequenom), rapid polymorphism discovery followed by association analysis is now possible[18].

In this study we investigated the role of 18 starch-related genes and their SNPs by assessing their contribution to variation in starch properties in a rice breeding population. We report a novel SNP in Glucose-6-Phosphate Translocator 1 (GPT1) gene which is associated with amylose content and retrogradation rate of resistant starch and establish an explicit-coherent gene by gene approach to unveil association of 18 starch-related genes and their SNP polymorphisms with rice starch physiochemical properties.

Results

Assays for 66 SNPs were designed and 233 individuals genotyped. The identification of genes (Figure 1) and code and coordinate of all SNPs studied appears in Table 1. SNP IDs starting with TBG or TBU

Gene

ADP glucose pyrophosphorylase (AGPase)

Granule bound starch synthases (GBSSI and GBSSII)

Starch synthases (SSI, SSIIa, SSIIb, SSIIIa, SSIIIb, SSIVa, SSIVb)

Branching enzymes (BEI, BEIIa, BEIIb)

Debranching enzymes (ISA1, ISA2, Pullulanase)

Starch phosphorylase (SPHOL)

Glucose 6-phosphate translocator (GPT1)

Figure 1 | Genes associated with variation in rice starch properties in a population of 233 Australian rice breeding lines. The genes in red are those most correlated with starch properties, those in green do not explain variation in starch properties while genes in black have low to medium effects on rice starch quality.

were extracted from databases and the remainder, mainly starting with GA, were reported by Kharabian-Masouleh et al. (2011). No functional polymorphisms were found in this population in AGPS2b, SPHOL, SSIIb, SSIVb, ISA1 suggesting these genes have no effect on the phenotypes investigated in this population. A gene by gene approach was applied to find associations between individual genes and physiochemical and quality-related properties of rice grain.

GBSSI (Granule bound starch synthase I). There was a strong correlation between the G/T SNP at the exon1/intron1 boundary and the RVA curve characteristics of PKV and BDV (Table 1 and Table 2). The highest *F*-value in this experiment was for this SNP and retrogradation rate (Martin test) (*F*-value=223.29) and amylose content (*F*-value=121.52). The R^2 value for retrogradation and amylose content were 0.66 and 0.51, respectively. The second SNP in GBSSI associated with grain properties was the C/T SNP at co-ordinate 3486 (exon 10) which creates a P→S substitution and has a significant association with trough and final viscosity (FV), set back, retrogradation (Martin test) and amylose content. The R^2 value for retrogradation and amylose content were 0.39 and 0.16, respectively.

The exon 6 SNP also revealed some significant association according to *p*-values≤ 0.01 but did not show any remarkable *F* and R^2 values (which suggest it has little control on critical pasting properties). In combination, the results suggest this gene is responsible for determining a significant proportion of the variation in retrograded-resistant rice starch.

GBSSII (Granule bound starch synthase II). GBSSII synthesises amylose and is found exclusively bound to starch granules in green tissues. During pre-heading, about 1–3 days after flowering, this gene/enzyme is expressed in leaf, leaf sheaths, culm, and pericarp tissue at a low level[19]. The synthesised amylose is subsequently consumed by the plant or mobilised to the endosperm[20]. One non-synonymous SNP (nsSNP) found at position 1638 of this gene was tested for association with starch physiochemical traits (Table 1). Only one association with PT with R^2 value of 0.20 was observed for this SNP, although some minor association also calculated with GT and Peak time (Table 2).

SSI. Only one ′T/C′ nsSNP at position 5153 of this gene showed minor associations with FV, SB and Martin test (MT), with R^2 values of 0.16, 0.11, 0.16, respectively (Table 1).

Table 1 | Name and characteristics of SNPs genotyped in 18 rice starch-related genes in a population of 233 Australian rice breeding lines

No	Gene	SNP ID*	Coordinates on gDNA	Expected SNP	SNP Assayed†	Association with Physiochemical traits	Status
1	AGPS2b	TBGU388647	233	G/T	G/G	N/A	No polymorphism
2	AGPS2b	TBGI050742	1507	T/C	T/T	N/A	No polymorphism
3	SPHOL	TBGU168031	2501	G/T	G/G	N/A	No polymorphism
4	SPHOL	TBGU168032	2920	C/T	C/C	N/A	No polymorphism
5	SPHOL	TBGU168027	1001	C/A	C/C	N/A	No polymorphism
6	SPHOL	TBGU168024	176	G/T	G/G	N/A	No polymorphism
7	SPHOL	TBGU168039	5514	G/T	G/G	N/A	No polymorphism
8	GBSSI	WAXYEXIN1	246	T/G	T/G	P1,BD,FV,SB,MT,AC,PN	Highly associated
9	GBSSI	WAXYEX6	2494	A/C	A/C	SB,BD,MT,AC	Highly associated
10	GBSSI	WAXYEX10	3486	C/T	C/T	T1,FV,SB,MT,AC,PN	Highly associated
11	GBSSII	GBSSII_GA_1638	1638	G/A	G/A	PT, GT	Low-Medium association
12	SSI	TBGU272768	5153	T/C	T/C	FV,SB,MT	Low-Medium association
13	SSIIa	SSIIa_GA_Ref631	631	G/T	G/T	N/A	No association
14	SSIIa	ALKSSIIA4	4827–4828	GC/TT	GC/TT	BDV,SB,PKT,PT,GT,CHK	Highly associated
15	SSIIb	TBGU116115	3416	A/G	A/A	N/A	No polymorphism
16	SSIIb	TBGU116120	3948	G/C	G/G	N/A	No polymorphism
17	SSIIb	TBGU116121	3979	T/C	T/T	N/A	No polymorphism
18	SSIIb	TBGU116109	330	G/A	A/A	N/A	No polymorphism
19	SSIIb	TBGU116119	3946	C/T	C/C	N/A	No polymorphism
20	SSIIb	TBGU116116	3487	T/G	T/T	N/A	No polymorphism
21	SSIIIa	GA_Ref1058	1058	T/A	T/A	PT,MT,	Low-Medium association
22	SSIIIa	GA_Ref1680	1680	G/A	G/A	SB,PT,MT,AC,PN,GT	Low associated
23	SSIIIa	GA_Ref3136	3136	G/A	G/A	N/A	No association
24	SSIIIa	GA_Ref3391	3391	T/A	T/A	N/A	No association
25	SSIIIa	GA_Ref3559	3559	T/A	T/A	CHK	Low association
26	SSIIIa	GA_Ref4384	4384	G/A	G/A	N/A	No association
27	SSIIIa	GA_Ref1379	1379	A/C	A/C	FV,SB,PT,MT,AC,PN	Low-Medium association
28	SSIIIa	GA_Ref1708	1708	G/A	G/A	MT,AC,PN,GT	Low-Medium association
29	SSIIIa	GA_Ref3274	3274	G/A	G/A	N/A	No association
30	SSIIIa	GA_Ref6242	6242	T/C	T/C	N/A	No association
31	SSIIIa	GA_Ref1457	1457	A/C	A/C	N/A	No association
32	SSIIIa	GA_Ref1615	1615	C/T	C/T	N/A	No association
33	SSIIIa	GA_Ref1834	1834	C/T	C/T	N/A	No association
34	SSIIIa	GA_Ref2758	2758	G/A	G/A	N/A	No association
35	SSIIIa	GA_Ref1722ER	1722	G/A	G/A	FV,SB,PT,MT,AC,PN,GT	Low-Medium association
36	SSIIIa	GA_Ref2488	2488	C/T	C/T	N/A	No association
37	SSIIIa	GA_Ref3073	3073	G/A	G/A	N/A	No association
38	SSIIIa	GA_Ref1357	1357	G/A	G/A	MT	No association
39	SSIIIa	GA_Ref2080	2080	C/T	C/T	N/A	No association
40	SSIIIa	GA_Ref3481	3481	G/A	G/A	N/A	No association
41	SSIIIa	GA_Ref5466	5466	G/A	G/A	FV,SB,PT,MT,AC,PN,	Low-Medium association
42	SSIIIa	GA_Ref10761	10761	C/T	C/T	PT	Low association
43	SSIIIb	GA_Ref1315	1315	T/C	T/C	PT	Medium association
44	SSIIIb	GA_Ref4543	4543	C/A	C/A	PT	Medium association
45	SSIIIb	GA_Ref5451	5451	T/C	T/C	PT	Medium association
46	SSIIIb	GA_Ref7232	3232	T/G	T/G	PT	Medium-High association
47	SSIIIb	GA_Ref7255ER	7255	C/A	C/A	PKV	Medium association
48	SSIIIb	GA_Ref7437	7437	A/C	A/C	PT	Low-Medium association
49	SSIVa	GA_Ref4048	4048	C/T	C/T	PT,GT	Low-Medium association
50	SSIVa	GA_Ref7160	7160	A/G	A/G	PKT,PT,AC,PN,GT	Low-Medium association
51	SSIVa	GA_Ref7506	7506	A/T	A/T	PT,GT	Low-Medium association
52	SSIVa	GA_Ref7823	7823	T/C	T/C	PT,GT	Low-Medium association
53	SSIVa	GA_Ref8383	8383	C/A	C/A	PT,GT	Medium association
54	SSIVb	TBGU260749	5090	G/C	G/G	N/A	No polymorphism
55	SSIVb	TBGU260765	9525	G/A	G/G	N/A	No polymorphism
56	BEI	GA_Ref1558	1558	C/T	C/T	PV,BDV,FV,SB,PT,MT,AC,PN	Low-Medium association
57	BEIIa	GA_Ref3266	3266	T/G	T/G	N/A	No association
58	BEIIb	GA_Ref9035	9035	C/T	C/T	N/A	No association
59	BEIIb	GA_Ref10068	10068	C/A	C/A	N/A	No association
60	ISA1	TBGU362347	1748	G/A	G/G	N/A	No polymorphism
61	ISA1	TBGU362346	1746	C/G	C/C	N/A	No polymorphism
62	ISA2	Iso2_GA_Ref960	960	T/C	T/C	BDV, PT, CHK	Low association
63	ISA2	Iso2_GA_Ref1712	1712	C/A	C/A	BDV, PT, CHK	Low association
64	Pullulanase	TBGU185983	1938	G/A	G/A	PT, GT	Low association
65	Pullulanase	TBGU185989	2380	T/C	T/C	CHK	Low association
66	GPT1	GPT1_GA_Ref_1188	1188	T/C	T/C	AC, MT,BD,FV,SB	Highly associated

*SNP identification can be found from Kharabian-Masouleh et al., 2011 (starting with GA code) or OryzaSNP MSU database (http://oryzasnp.plantbiology.msu.edu/) starting with TBG or TBU codes.
†Homozygosity of SNP calls mean no polymorphism in the corresponding allele.
MT=Martin test (retrogradation), PN=Predicted Nitrogen, CHK=Chalkiness (%).

Table 2 | Association of 18 rice starch-related genes with rice starch physico-chemical traits in a population of 233 Australian rice breeding lines

Gene		Trait	Locus/SNP	F-test	p-adjusted value	R^2_Marker
AGPS2b	Section 1	No functional polymorphism found in this gene		-	-	-
SPHOL	Section 2	No functional polymorphism found in this gene		-	-	-
GBSSI	Section 3	Peak1	WAXYEXIN1	34.346	9.99E-04	0.23
		Trough1	WAXYEX10	36.9498	9.99E-04	0.1384
		Breakdown	WAXYEXIN1	35.1893	9.99E-04	0.2343
		Breakdown	WAXYEX10	18.9223	9.99E-04	0.076
		Final Viscosity	WAXYEXIN1	15.0534	9.99E-04	0.1157
		Final Viscosity	WAXYEX10	106.068	9.99E-04	0.3156
		Setback	WAXYEXIN1	76.2739	9.99E-04	0.3988
		Setback	WAXYEX10	59.8068	9.99E-04	0.2064
		Martin_N	WAXYEXIN1	223.294	9.99E-04	0.6601
		Martin_N	WAXYEX10	147.783	9.99E-04	0.3912
		Martin_N	WAXYEX6	16.8014	9.99E-04	0.0681
		AC_percent	WAXYEXIN1	121.53	9.99E-04	0.5138
		AC_percent	WAXYEX10	44.0661	9.99E-04	0.1608
		AC_percent	WAXYEX6	16.2252	9.99E-04	0.0659
		predicted_N	WAXYEXIN1	121.543	9.99E-04	0.5138
		predicted_N	WAXYEX10	43.967	9.99E-04	0.1605
		predicted_N	WAXYEX6	16.3841	9.99E-04	0.0665
GBSSII	Section 4	Past_temp	GBSSII_GA_Ref1638	27.8519	9.99E-04	0.2028
		GT	GBSSII_GA_Ref1638	9.7254	9.99E-04	0.0938
SSI	Section 5	Trough1	SSI_TBGU272768_5153	14.2713	9.99E-04	0.0592
		FinalVisc	SSI_TBGU272768_5153	43.6138	9.99E-04	0.1612
		Setback	SSI_TBGU272768_5153	28.8805	9.99E-04	0.1129
		Martin_N	SSI_TBGU272768_5153	45.7145	9.99E-04	0.1676
		AC_percent	SSI_TBGU272768_5153	20.5891	9.99E-04	0.0832
		predicted_N	SSI_TBGU272768_5153	20.4244	9.99E-04	0.0825
SSIIa	Section 6	Breakdown	ALKSSIIA4	22.4536	9.99E-04	0.1682
		PeakTime	ALKSSIIA4	53.0867	9.99E-04	0.3235
		Past_temp	ALKSSIIA4	199.652	9.99E-04	0.6427
		GT	ALKSSIIA4	32.806	9.99E-04	0.2547
		Chalk%	ALKSSIIA4	8.9273	9.99E-04	0.0744
SSIIb	Section 7	No functional polymorphism found in this gene		-	-	-
SSIIIa	Section 8	FinalVisc	SSIIIa_GA_Ref1379	9.0413	9.99E-04	0.0753
		FinalVisc	SSIIIa_GA_Ref1722ER	8.8028	9.99E-04	0.0723
		FinalVisc	SSIIIa_GA_Ref5466	8.9423	9.99E-04	0.0736
		Setback	SSIIIa_GA_Ref1680	7.8821	9.99E-04	0.0655
		Setback	SSIIIa_GA_Ref1379	11.6269	9.99E-04	0.0948
		Setback	SSIIIa_GA_Ref1722ER	10.1037	9.99E-04	0.0821
		Setback	SSIIIa_GA_Ref5466	9.0543	9.99E-04	0.0745
		Past_temp	SSIIIa_GA_Ref1058	8.7158	9.99E-04	0.0722
		Past_temp	SSIIIa_GA_Ref1680	7.4574	9.99E-04	0.0622
		Past_temp	SSIIIa_GA_Ref1379	7.9273	9.99E-04	0.0667
		Past_temp	SSIIIa_GA_Ref1722ER	9.3315	9.99E-04	0.0763
		Past_temp	SSIIIa_GA_Ref10761	7.2026	9.99E-04	0.062
		Past_temp	SSIIIa_GA_Ref5466	8.756	9.99E-04	0.0722
		Martin_N	SSIIIa_GA_Ref1058	13.2478	9.99E-04	0.1058
		Martin_N	SSIIIa_GA_Ref1680	20.8545	9.99E-04	0.1564
		Martin_N	SSIIIa_GA_Ref1379	27.7893	9.99E-04	0.2002
		Martin_N	SSIIIa_GA_Ref1708	16.5211	9.99E-04	0.1301
		Martin_N	SSIIIa_GA_Ref1722ER	20.6652	9.99E-04	0.1546
		Martin_N	SSIIIa_GA_Ref1357	7.6136	9.99E-04	0.0639
		Martin_N	SSIIIa_GA_Ref5466	20.4182	9.99E-04	0.1536
		AC_percent	SSIIIa_GA_Ref1680	10.3167	9.99E-04	0.084
		AC_percent	SSIIIa_GA_Ref1379	14.2201	9.99E-04	0.1136
		AC_percent	SSIIIa_GA_Ref1708	9.3351	9.99E-04	0.0779
		AC_percent	SSIIIa_GA_Ref1722ER	10.6866	9.99E-04	0.0864
		AC_percent	SSIIIa_GA_Ref5466	11.1556	9.99E-04	0.0902
		predicted_N	SSIIIa_GA_Ref1680	10.2716	9.99E-04	0.0837
		predicted_N	SSIIIa_GA_Ref1379	14.2099	9.99E-04	0.1135
		predicted_N	SSIIIa_GA_Ref1708	9.3091	9.99E-04	0.0777
		predicted_N	SSIIIa_GA_Ref1722ER	10.6615	9.99E-04	0.0862
		predicted_N	SSIIIa_GA_Ref5466	11.1281	9.99E-04	0.09
		GT	SSIIIa_GA_Ref1680	10.0271	9.99E-04	0.0946
		GT	SSIIIa_GA_Ref1708	30.2791	9.99E-04	0.2436
		GT	SSIIIa_GA_Ref1722ER	15.2535	9.99E-04	0.1365
		Chalk%	SSIIIa_GA_Ref3559	8.9878	9.99E-04	0.0821
SSIIIb	Section 9	Peak Viscosity	SSIIIb_GA_Ref7255ER	7.7442	9.99E-04	0.0666

Table 2 | Cont

Gene		Trait	Locus/SNP	F-test	p-adjusted value	R^2_Marker
		Past_temp	SSIIIb_GA_Ref4543	21.3553	9.99E-04	0.2251
		Past_temp	SSIIIb_GA_Ref5451	23.0673	9.99E-04	0.176
		Past_temp	SSIIIb_GA_Ref1315	25.0653	9.99E-04	0.1849
		Past_temp	SSIIIb_GA_Ref7232	41.4018	9.99E-04	0.3151
		Past_temp	SSIIIb_GA_Ref7255ER	29.1937	9.99E-04	0.212
		Past_temp	SSIIIb_GA_Ref7437	21.0809	9.99E-04	0.1572
SSIVa	Section 10	PeakTime	SSIva_GA_Ref7160	10.7899	9.99E-04	0.0875
		Past_temp	SSIva_GA_Ref4048	27.6864	9.99E-04	0.1989
		Past_temp	SSIva_GA_Ref7160	39.5053	9.99E-04	0.2599
		Past_temp	SSIva_GA_Ref7823	30.2856	9.99E-04	0.2159
		Past_temp	SSIva_GA_Ref8383	30.8007	9.99E-04	0.2227
		Past_temp	SSIva_GA_Ref7506	29.3874	9.99E-04	0.205
		AC_percent	SSIva_GA_Ref7160	9.1222	9.99E-04	0.075
		predicted_N	SSIva_GA_Ref7160	9.077	9.99E-04	0.0747
		GT	SSIva_GA_Ref4048	8.5371	9.99E-04	0.0825
		GT	SSIva_GA_Ref7160	19.7873	9.99E-04	0.1709
		GT	SSIva_GA_Ref7823	10.209	9.99E-04	0.098
		GT	SSIva_GA_Ref8383	10.4426	9.99E-04	0.1014
		GT	SSIva_GA_Ref7506	10.6137	9.99E-04	0.0982
SSIVb	Section 11	No polymorphism detected in this gene				
BEI	Section 12	Peak Viscosity	BEI_GA_Ref1558	9.5546	9.99E-04	0.0796
		Breakdown	BEI_GA_Ref1558	11.2003	9.99E-04	0.092
		FinalViscosity	BEI_GA_Ref1558	13.0129	9.99E-04	0.1054
		Setback Viscosity	BEI_GA_Ref1558	32.1812	9.99E-04	0.2255
		Past_temp	BEI_GA_Ref1558	8.4131	9.99E-04	0.0708
		Martin_N	BEI_GA_Ref1558	34.5608	9.99E-04	0.2383
		AC_percent	BEI_GA_Ref1558	38.8652	9.99E-04	0.2602
		predicted_N	BEI_GA_Ref1558	39.1031	9.99E-04	0.2614
BEIIa	Section 13	No significant association was observed with starch traits	-	-	-	
BEIIb	Section 14	No significant association was observed with starch traits	-	-	-	
Iso1	Section 15	No polymorphism detected in this gene	-	-	-	
Iso2	Section 16	Breakdown	Iso2_GA_Ref1712	8.2378	9.99E-04	0.0768
		Breakdown	Iso2_GA_Ref960	9.0028	9.99E-04	0.076
		Past_temp	Iso2_GA_Ref1712	7.8355	9.99E-04	0.0733
		Past_temp	Iso2_GA_Ref960	7.8341	9.99E-04	0.0668
		Chalk%	Iso2_GA_Ref1712	7.2855	9.99E-04	0.0685
		Chalk%	Is02_GA_Ref960	8.2391	9.99E-04	0.07
Pullulanase	Section 17	Past_temp	Pullu_TBGU185983_1938	23.5989	9.99E-04	0.1747
		GT	Pullu_TBGU185983_1938	19.1496	9.99E-04	0.167
		Chalk%	Pullu_TBGU185989_2380	7.5266	9.99E-04	0.0666
GPT1	Section 18	Peak Viscosity	GPT1_GA_Ref_1188	21.1979	9.99E-04	0.092
		Break down	GPT1_GA_Ref_1188	37.1798	9.99E-04	0.148
		Final viscosity	GPT1_GA_Ref_1188	31.1074	9.99E-04	0.126
		Set back viscosity	GPT1_GA_Ref_1188	83.2826	9.99E-04	0.282
		Martin_N	GPT1_GA_Ref_1188	292.143	9.99E-04	0.577
		AC_percent	GPT1_GA_Ref_1188	123.047	9.99E-04	0.365
		Predicted N	GPT1_GA_Ref_1188	122.543	9.99E-04	0.364

SSIIa. Highly significant associations were found between SNP of SSIIa and PT, peak time (PKT), GT and breakdown viscosity. The highest *F*-test value of 199.65 was observed for the [GC/TT] SNP at position 4827–4828 of SSIIa and PT. This SNP is associated with PT, PKT and BDV with R^2 values of 0.642, 0.323 and 0.168, respectively. This SNP has one of the strongest associations among the physiochemical properties studied in this rice population (R^2=0.642). The G/T SNP at position 631 showed no singnificant association with any traits.

SSIIIa. The highest polymorphism was observed in this gene with 22 SNPs in the coding region causing amino acid changes. Polymorphism in this gene showed association with a FV, SB, PT, MT, AC, predicted N, GT and chalkiness. However, most revealed very low R^2 values of less than 0.1, indicating that although they are associated, they do not have a highly significant effect on physiochemical properties (Table 2). The highest R^2 values for GT, MT and AC were 0.243, 0.200, and 0.113, respectively (Table 2).

SSIIIb. The main effect of SSIIb was observed on PT. Associations were found between ´T/G´ and ´C/A´ SNPs at positions 7232 and 4543 with R^2 values of 0.315 and 0.225, respectively. These relatively high R^2 values suggest SNPs in the coding regions of this gene

influence PT, although a minor association was found with peak viscosity (PKV). These SNPs at positions 207 and 756 alter the corresponding amino acids Lys→Asn and Ser→Ile, respectively. This gene is a major gene contributing to PT as some other SNPs also exhibited significant associations with PT (Table 2).

SSIVa. Five SNPs were examined in this gene (Table 3), of which four showed significant association with PT (Table 2). There was a relatively high R^2 of 0.259 for the functional ´A/G´ SNP at position 7160, which influences PT. In addition, four other SNPs, with R^2 values ranging from 0.198–0.222, also have an influence on PT. A large portion of phenotypic variation of PT in this rice population seems to be explained by SNP in SSIVa. Some minor associations were observed with GT, PKT, AC and predicted nitrogen (PN). SSIIIb and SSIVa in combination contribute to PT in this rice population.

SSIVb. No polymorphism was detected in this gene. Therefore, it could be concluded that there is no association between this gene and the studied traits in this population.

BEI. Only one C/T SNP at position 1558 of this gene was discovered[21]. Nine out of 13 studied physiochemical traits were associated with this SNP at a medium level with the highest R^2 values observed for AC, MT, SB and FV. The relatively high R^2 values of 0.260 and 0.238 for AC and MT respectively suggests this gene has a prominent effect on amylose content and retrogradation. Minor associations were also found between this SNP and PV, BDV and FV (Table 2).

BEIIa. BEIIa is a leaf expressed gene involved in amylopectin synthesis. The ´T/G´ SNP at position 3266 displayed no significant association, confirming BEIIa as a green tissue-specific gene with no impact on grain starch properties (Table 2).

BEIIb. BEIIb is known as *amylose extender* (*ae*) in maize and other cereals (Yun and Matheson, 1993). Two SNPs in this gene were examined (Table 2) but no significant association was found with grain starch properties in this population (Table 1).

ISA1 (Isoamylase 1). No polymorphism was detected in ISA1 in this population.

ISA2 (Isoamylase 2). Two SNPs were assessed in this gene and all R^2 values were less than 0.1, indicating a very low association with breakdown viscosity and chalkiness traits (Table 2).

Pullulanase. A recent association study between pullulanase and RVA profile parameters in glutinous rice has shown strong relations of this gene with PKV, HPV, BDV, PKT[14]. In this study only weak associations with the two assayed SNPs in pullulanase, PT, GT and CHK with R^2 values of 0.174, 0.167 and 0.066, respectively were found (Table 2).

GPT1 (Glucose-6-Phosphate Translocator). For the first time we report that the *GPT1* gene, early in the biochemical pathway of starch synthesis, encoding the glucose-6-phosphate translocator enzyme, has a major association with resistant starch production in rice. A ´T/C´ SNP at position 1188 of the *GPT1* gene, alters Leu24 to Phe, and is highly associated with resistant-retrograded starch and amylose content (Table 2). The ´T´ and ´C´ alleles produce high and low levels of retrograded starch, respectively. An association study of 233 genotypes demonstrated a highly significant correlation (R^2) of 0.577 and 0.365 (P=0.00099) between this SNP and retrogradation degree and apparent amylose content, respectively (Table 2).

Table 3 | Range of phenotypic values (variation) for measured physiochemical properties in 233 Australian rice genotypes

Traits	Range
Peak 1	2168–3669
Trough 1	1312–2372
Breakdown Viscosity	667–1913
Final Viscosity	2560–4386
Set Back	−658–+1203
Peak Time	5.7–6.3
Pasting Temperature	65.65–78.40
Martin Test (N)	0.405–3.612
Amylose content (%)	14.10–28.85
Predicted N (N)	0.31–1.82
Gelatinization Temperature (°C)	62.00–82.98
Chalkiness (%)	0.709–44.55

Discussion

GBSSI and SSIIa are major genes involved in many grain quality properties such as amylose content and gelatinization temperature (Figure 1 and Figure 2). Highly significant associations were found between GBSSI and retrogradation and amylose content although this gene showed more significant relations with properties such as BDV, SB and FV. A number of authors have already reported the importance of this enzyme in determining the starch physiochemical properties in rice and other cereals. SNPs at the intron/exon 1 junction site, exon 6 and 10 in rice GBSSI (*waxy* gene) have the most significant impact on amylose content and by extension, starch quality[8–10]. This study confirms the ´T/G´ SNP at the intron1/exon1 junction site has a major influence on a number of physiochemical properties.

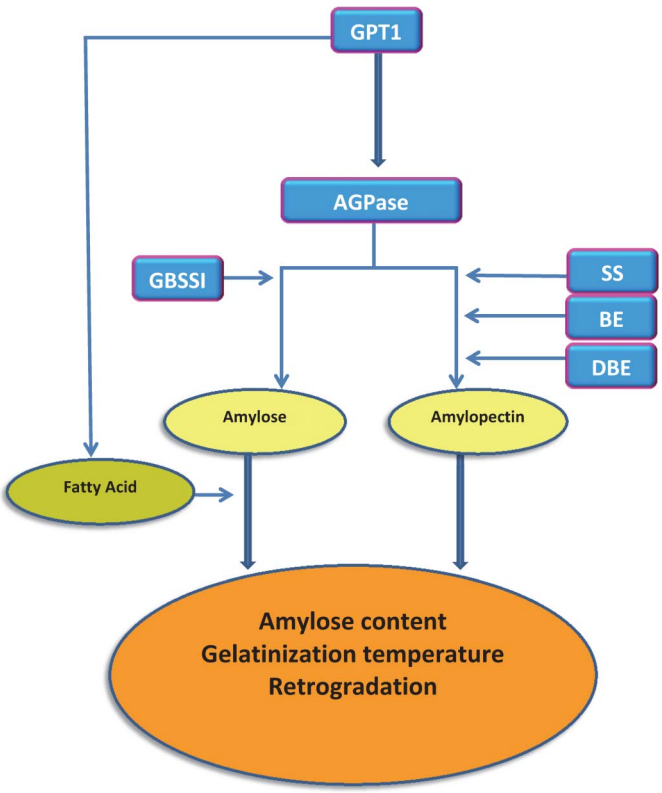

Figure 2 | Simplified pathway of starch synthesis in rice and interaction with starch properties.

SSIIa had a high association with pasting temperature, gelatinization temperature and peak time. The effect of this gene on cooking quality and starch texture has been extensively studied by many authors[11,12]. Umemoto and Aoki, (2005) found alkali disintegration and eating quality of rice starch were explained by two SNP, [A/G] and [GC/TT], within the exon 8 of *alk* locus. These SNPs also have significantly associated with starch GT[13]. Two SNPs at positions 631 and 4827–4828 (*ALKSSIIA4*) respectively were tested for association (Table 1 and 2). The effect of the [GC/TT] SNP on alkali disintegration and rice starch eating quality has already been explained by many authors[22,23]. Highly significant associations were found between SSIIa SNPs and important physiochemical properties such as PT, PKT, GT and BDV. Melting of starch crystalline regions is measured by pasting and gelatinisation temperature and peak time signifies the end of the melting process. The highest *F*-test value of 199.65 was observed for ALKSSIIA4 [GC/TT] SNP and PT. This SNP clearly controls PT, PKT and BDV with R^2 values of 0.642, 0.323 and 0.168, respectively. This SNP has one of the strongest associations among the physiochemical properties of rice studied in this population (R^2=0.642). The G/T SNP at position 631 showed no significant association with any traits.

SSIVa is one of the least well characterized starch genes in rice. This study showed a significant influence of this gene on PT and GT. In total, five SNPs were examined in this gene (Table 1), of which four SNPs showed significant association with PT (Table 2).

Six genes, GBSSII, SSI, SSIIIa, SSIIIb, SSIVa and BE, had low to medium effects on variation in starch traits. SNPs in these genes had association with a number of characters with low to medium R^2 values. The effect of these genes on starch traits have been studied at the gene level[20,15,24,11]. Here for the first time, SSIIIb and SSIVa have been identified as PT-associated (pasting temperature-associated) at a relatively medium to high level.

SSI transcript level has been measured at different seed developmental stages. A high expression level was reported at 1–3 days after flowering (DAF), peaking at 5 DAF, and then remaining almost constant during starch synthesis in the endosperm. This suggests that SSI is a major SS form in cereals[25]. Only one nsSNP in SSI, ´T/C´ at position 5153, in this gene showed minor associations with FV, SB and MT, with R^2 values of 0.16, 0.11, 0.16, respectively (Table 2).

Pullulanase had low associations with PT, GT and CHK in the population studied here. In contrast, a recent association study in glutinous rice has shown strong relationships between pullulanase and RVA profile parameters, PKV, HPT, BDV and PKT[11]. The differing observations are most likely due to the structure of each population. Minor genes are very population-specific and the analysis of Yan et al. (2010) was undertaken within a glutinous population composed of rice varieties which have very low amylose content and this would have revealed the role of pullulanse in this genetic background.

Seven genes of 18 did not contribute to starch physiochemical properties in this population. No polymorphisms were detected in five genes, AGPS2b, SPHOL, SSIIb, SSIVb and ISA1 while BEIIa and BEIIb displayed polymorphism but these were not associated with any physiochemical properties measured in this study. In contrast, other studies have suggested some of these genes are important in determining rice starch physiochemical properties and quality. For example, Kawagoe et al. 2005 found the AGPS2b subunit plays an important role in starch granule synthesis and is associated with rice *shrunken* mutants[26]. SPHOL is reported to be involved in starch degradation and biosynthesis by phosphorylation of some starch-related enzymes and proteins such as starch branching enzymes (SBEs) and starch synthase (SSIIa)[27]. Almost all of these studies have been based on mutants totally deficient in enzyme activity[28] which abolish the gene function and therefore have a significant effect on the content of soluble sugars, structure and appearance of starch granules and endosperm quality in rice and other species.

SSIIb and BEIIa are mostly expressed in green tissues and theoretically do not have major impact on grain quality traits[29]. In this study we confirm that SNPs in green-tissue related genes have no or very small effects on grain starch properties. No significant association was found between the two BEIIb SNPs and quality traits in this population. The differing results can be attributed to the different structure of each population, in each population each gene has a particular impact which is determined by the presence of the range of alleles present at other starch biosynthesis loci.

This study found BEIIb (*amylose extender*) and ISA1 had no association with any of the physiochemical properties of rice starch measured despite previous reports that these genes in several cereal species impact starch properties[30,31]. We examined two SNP in this gene (Table 1) but no significant association was found with starch properties. Previous biochemical analysis of rice (*Oryza sativa*) *amylose-extender* (*ae*) mutants revealed the influence of this gene on gelatinization properties through the structural alteration of amylopectin by reducing short chains and degree of polymerization[32]. However, these studies focused on mutant populations where a large segment of the gene has been deleted. Therefore, the results of those experiments are not comparable with our variation study at SNP level. Antisense inhibition of rice ISA1 has altered the structure of endosperm amylopectin and the starch physiochemical properties[33]. The ISA genes also contribute to the degree of setback on glutinous rice cultivars[14].

Philpot et al. (2006)[5] reported removal of lipid increased the rate of retrogradation and the firmness of gels significantly in rice. Analysis of *O. sativa* cultivar Koshihikari grown in Japan and Australia found individuals grown in Japan had a lower retrogradation rate, despite the fact that flour from both origins contained 18% amylose. Removal of the lipids from these samples resulted in retrogradation rates which were not significantly different. The amount of amylose complexed with lipids affects starch retrogradation[34] and so it was suggested this phenomenon can be attributed to the amount of lipid complexed with long amylose chains, the higher concentration of lipid linked to long amylose chains explained the lower retrogradation in the Japanese grown rice. GPT1 is required for transportation of reduced carbon into plastids which is ultimately utilised for both lipid and amylose synthesis (Figure 2). It has been suggested amylose content is correlated with lipid content[35] and it is thought lipids play a structural role as a core scaffold in holding together the helical architecture of amylose. GTP1 is involved in determining plastid fatty acid concentration[36] and this may influence the formation of lipid-amylose complexes. GPT1 is associated with amylose content and retrogradation rate in this set of germplasm. In addition to its impact on amylose content, GPT1 may also affect retrogradation rate by influencing lipid content in rice grain.

This study has found the genes which have an impact upon starch traits within the Australian rice breeding program display relatively low levels of diversity. This set of genes is bounded by two sets of genes, one which has no diversity and another which has high levels of diversity. Australian rice breeders are managing a small number of genes and alleles which have an impact on starch quality in order to achieve desirable starch quality within the breeding program. Construction of new quality classes may require access to a wider range of alleles, genes and germplasm.

Methods

Plant materials. Plant material was supplied by Department of Primary Industries NSW, Yanco Agricultural Institute, Australia. A population of 233 temperate (*japonica*-type) F_6 rice breeding lines was selected from pedigree rows . Selection had taken place on capacity of lines to flower and set seed and morphological traits of plant height, grain size and shape. No selection had taken place for grain starch quality traits.

Physiochemical properties. Physiochemical traits measured were apparent amylose content (AC), gelatinization temperature (GT) quantified according to standard differential scanning calorimetry methods (DSC)[37]. Percent grain chalk was estimated by a FOSS Cervitec according to the manufacturer's instruction. Retrogradation rate (Martin test[5]) was estimated by measuring the force in Newtons (N) required to push a probe into a gel derived from flour samples post viscosity measurements and stored overnight at 20°C (Lloyd texture analyser TAPlus, Hemisphere United Kingdom). Peak viscosity (PKV), trough viscosity (TV), final Viscosity (FV), breakdown viscosity (BDV), setback (SB), peak time (PKT) and pasting temperature (PT) were measured by a Rapid Visco Analyser to evaluate rheological properties of starch structure (Perten RVA 4500, Segeltorp, Sweden) according to the manufacturer's instructions. The range of values observed for these traits is shown in Table 3 and a list of all data in supplementary materials (Supplementary data 1).

Designation of starch-synthesis genes. The available literature was used to identify the most likely candidate genes associated with rice starch quality[19,23,24,38]. The general entries of nucleotide sequences (gDNA) and full-length cDNAs of important gene classes which were presumed to be involved in starch biosynthesis were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/) and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/cgi-bin/putative_function_search.pl) databases and then re-sequenced using integrated long range PCR in combination with massively parallel sequencing (Illumina) to find novel SNPs/Indels in the studied population[21]. A consensus sequence alignment was generated for each candidate gene to design the amplification primers.

Candidate genes/enzymes for SNP genotyping. Eighteen genes representing seven groups of enzymes, namely ADP-glucose pyrophosphorylase (AGPase), granule bound starch synthases (GBSSI and GBSSII), starch synthases (SSI, SSIIa, SSIIb, SSIIIa, SSIIIb, SSIVa, SSIVb), branching enzymes (BEI, BEIIa, BEIIb), debranching enzyme (ISA1, ISA2, Pullullanase), starch phosphorylase (SPHOL) and glucose-6-phosphate- translocator (GPT1) were selected for SNP genotyping (Figure 1).

SNP dataset. SNP data were primarily retrieved through SNP discovery within the population of 233 rice breeding lines[21]. The functional polymorphisms discovered within the studied population were then compared to SNPs available within the OryzaSNP MSU database (http://oryzasnp.plantbiology.msu.edu/) and extra SNPs harvested to minimise the possibility non-synonymous SNP (nsSNP) were missed. In total, 65 nsSNPs were chosen for genotyping, of which 48 were polymorphic or existing in the population (Table 1). The remaining 17 SNP which were not polymorphic in this population were mainly retrieved from data bases.

Primer design and SNP genotyping. Multiplexed assays were designed by Sequenom MassARRAY Assay design 3.1 software to cover all available SNPs. The optimal amplicon size containing the polymorphic site was set to 80–120 bp. A 10-mer tag (5-ACGTTGGATG-3) was added to the 5′end of each amplification primer to avoid confusion in the mass spectrum and to improve PCR performance[18] (Supplementary data 2).

Capture PCR protocol, primer extension and mass spectrometry. The steps of PCR capture, primer extension, resin cleanup and mass spectrometry were undertaken according to the manufacturer's instructions (Sequenom MassARRAY).

Association analysis. Assays were constructed for 110 polymorphisms defining each of the alleles of 18 genes controlling starch quality traits and retrogradation. SNP data of genotyped polymorphic alleles (Supplementary data 3) along with phenotypic data were analysed by TASSEL v2.1[39] software to find SNP associated with physiochemical properties. A gene by gene approach was employed to understand association of individual gene/SNP with each trait.

Statistical analysis. Genotypic and phenotypic files were prepared according to Bradbury et al. (2007) and then imported to TASSEL v2.1. The general linear model (GLM) was used for alignment of data with 1000 permutations. Critical statistics such as *F*-test, *p*-value, adjusted *p*-value and R^2 were calculated to measure associations. P-values ≤ 0.01 were considered to have a significant effect on each trait. After identifying significantly contributing SNP, *F*-test values were used for comparison, larger *F* values were interpreted as exhibiting a higher association between SNP and its corresponding trait. Finally, R^2 is the portion of total variation explained by the full model[39].

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Author contributions

RH, DW and RR designed the project. A K-M and RW performed the experiments. A K-M, DW and RH wrote the paper. All authors commented on the manuscript.

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