1 **Association mapping of starch physicochemical properties with starch synthesis**
2 **related gene markers in nonwaxy rice (*Oryza sativa* L.)**

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Abstract

Starch physicochemical properties strongly influence eating and cooking quality of rice. The CAPS, dCAPS and InDel markers for 13 starch synthesis related genes (SSRGs) were developed, and together with markers developed before, there are 35 markers tagged for 23 SSRGs, with each gene tagged with at least one marker. These and 108 other markers were used for association mapping for 20 starch physicochemical property parameters. A total of 64 main-effect loci or QTLs were detected. In addition, 56 and 62 loci were identified under the *Wx* and *SSIIa* background, respectively. *Wx* was a major main-effect QTL for AAC, pasting viscosity, gel texture, and retrogradation property ($P < 0.0001$). *SSIIa* was a major main-effect QTL for pasting temperature, thermal and retrogradation properties ($P < 0.0001$), but it was a minor main-effect QTL for some pasting viscosity parameters, such as BD, CS, Stab and SBratio. Four other SSRGs, *SSIIa*, *BE1*, *SSIIc* and *GBSSII* were detected for AAC under *Wx* background. *Wx* was detected for Tc and ΔHg under the *SSIIa* background. *PUL* was detected for HD as main-effect QTL and under *SSIIa* background. *AGPL2* and *ISA1* were detected respectively for ΔHg and retrogradation as main-effect QTL as well as under both *Wx* and *SSIIa* backgrounds. This study suggested that retrogradation properties were mainly controlled by *Wx*, *SSIIa* and *ISA1* with the relative effects in the order of *SSIIa* > *Wx* > *ISA1*. These results have direct applications to quality breeding programs.

Keywords: Rice; eating quality; amylose; gelatinization temperature; RVA pasting viscosity; association mapping; QTL

Abbreviations used:

AAC, apparent amylose content; ADH, gel adhesiveness; ASV, alkali spreading value; BD, breakdown viscosity; BE, starch branching enzyme; CAPS, cleaved amplified polymorphic sequences; dCAPS, derived CAPS; COH, gel cohesiveness; CPV, cold paste viscosity; CS, consistency; DBE, debranching enzyme; DSC, differential

scanning calorimetry; GBSS, granule-bound starch synthase; GT, gelatinization temperature; HD, gel hardness; HPV, hot paste viscosity; ISA, isoamylase; PT, pasting temperature; PV, peak viscosity; PUL, pullulanase; QTL: quantitative trait locus; R%, retrogradation percentage; RVA, rapid visco analyser; SB, setback viscosity; SBratio, setback ratio. Stab: stability; SS, starch synthase; SSRG, starch synthesis related genes; T_o，onset temperature; T_p, peak temperature; T_c, conclusion temperature; ΔHg, enthalpy of gelatinization; ΔHr, enthalpy of retrogration; $\Delta T_{1/2}$, width at half peak height.

Introduction

Rice serves as a staple food for about half of the world's people. New varieties with high yield, high quality and high resistance to biotic and abiotic stresses are bred and released continuously in order to meet the ever-increasing demand for more food as a consequence of human population growth coupled with the decrease in arable land. Improvement of rice quality is among the most important goals in current breeding programs, especially its eating and cooking quality as most rice is consumed as cooked rice. Starch is the major component of rice grain, the content and fine structure of its two constituents, amylose and amylopectin, determine rice eating and cooking quality. Biochemically, four classes of enzymes are involved in starch biosynthesis, i.e. ADP-Glucose pyrophosphorylase (*AGPase*), starch synthase (*SS*), starch branching enzymes (*BEs*), and starch de-branching enzymes (*DBEs*) (Nakamura, 2002; James et al. 2003; Hannah et al. 2008). AGPase converts ADP-glucose to glucose-1-phosphate in rice kernel, provides substrate for starch synthase. Granule bound starch synthase-I enzyme (*GBSS1*) is a primary enzyme responsible for amylose production in rice endosperm while other *SS* (soluble starch synthase), *BEs* and *DBEs* work together but with distinct roles to synthesize amylopectin. Many of these enzymes have multiple isoforms. The *AGPase* consists of four large (*AGPL1-4*) and two small (*AGPS1, AGPS2*) subunits (Ohdan et al. 2005; Lee et al. 2007). There are a total of 10 isoforms for starch synthase enzymes: *GBSS (I, II), SSI, SSII (SSIIa, SSIIb, SSIIc), SSIII (SSIIIa and SSIIIb),* and *SSIV (SSIVa and SSIVb)* (Hirose and Terao 2004; Tetlow et al. 2004; Ohdan et al. 2005; Zhang et al. 2011). Rice *BE* has three isoforms: *BEI, BEII (BEIIa, BEIIb)* (Nakamura 2002; Ohdan et al. 2005). Two types of *DBE*: isoamylase and pullulanase are both found involved in amylopectin biosynthesis in rice endosperm (Nakamura et al. 1996; Kubo et al. 1999; Fujita et al. 2003; Wong et al. 2003; Ohdan et al. 2005).

Genetic studies indicated that the starch physicochemical properties, such as AAC, gelatinization temperature, gel consistency, RVA pasting viscosity, gel texture, DSC

84   thermal property, and retrogradation, might be controlled by one or a few genes with

85   major effects (He et al. 1999; Bao et al. 2000; Bao et al. 2004a; Wan et al. 2004; Fan

86   et al. 2005). Linkage mapping of the quantitative trait locus (QTL) for AAC and

87   pasting viscosity (Bao et al. 1999; He et al. 1999; Bao et al. 2000, 2003; Larkin et al.

88   2003; Septiningsih et al. 2003; Aluko et al. 2004; Wan et al. 2004; Fan et al. 2005;

89   Wang et al. 2007; Lapitan et al. 2009; Traore et al. 2011) shows that they are largely

90   controlled by the *Wx* locus on chromosome 6. Since *GBSSI* is responsible for amylose

91   synthesis, *GBSS1* alleles correlated with variation in AAC of rice grain is not

92   surprising. Linkage mapping studies have identified only one major QTL, i.e. the

93   alkali degeneration (*alk*) locus on chromosome 6, as having major responsibility for

94   different gelatinization temperatures in diverse rice germplasm (He et al. 1999; Aluko

95   et al. 2004; Bao et al. 2004b; Fan et al. 2005; Tian et al. 2005; Wang et al. 2007;

96   Lapitan et al. 2009). Map-based cloning of the *alk* locus reveals that it encodes SSIIa,

97   which is the major gene responsible for GT (Gao et al. 2003). QTL mapping shows

98   that the amylopectin chain length distribution is also controlled by the *SSIIa* locus

99   (Umemoto et al. 2002). The function of *SSIIa* is to elongate the short A and B1 chains

100   with degree of polymerization (DP) < 10 to form long B1 chains of amylopectin

101   (Nakamura et al. 2005). Although the functions of several genes in determining the

102   rice eating and cooking qualities have been gradually clarified, such as *Wx* and *SSIIa*,

103   many of others still remain unknown even though their functions in starch

104   biosynthesis have been revealed.

105

106   In addition to *Wx* and *SSIIa*, the contribution of other genes to rice eating and cooking

107   quality is derived from analysis of allele variations and their association with the

108   quality parameters. Association analysis is a popular method to test the relationship

109   between specific sequence polymorphisms in candidate genes and phenotypic

110   variations (Thornsberry et al. 2001; Gupta et al. 2005). Molecular markers specific to

111   a simple sequence repeat polymorphism with respect to (CT)n repeats and to a single

112   nucleotide polymorphism of *Wx* gene have been successfully designed to distinguish

113   rice varieties with low amylose content from varieties with intermediate or high

amylose content (Ayres et al. 1997). Likewise, a marker specific to a SNP of *SSIIa* can differentiate varieties with low gelatinization temperature from those with intermediate or high gelatinization temperature (Bao et al. 2006b). By sequencing starch biosynthesis related genes, more and more allele variations have been revealed either in coding regions or un-translated regions of genes. For association analysis, previous studies (when SNP data were not available) focused more on the allele variations occurred in un-translated regions (Bao et al. 2006a), but recent studies have focused on the SNP of coding regions (Tian et al. 2009; Kharabian-Masouleh et al. 2012; Teng et al. 2013). Since starch biosynthesis is a complex network of many isoforms, both types of allele variations should be incorporated into a systematic association analysis. Furthermore, recently others genes have also been reported to affect the production of amylose or amylopectin. For instance, a SNP of *glucose-6-phosphate translocator 1* gene has been reported as highly associated with amylose content and retrogradation properties (Kharabian-Masouleh et al. 2012).

Previous studies often focused on three parameters affecting eating and cooking quality, apparent amylose content (AAC), gelatinization temperature (GT) and gel consistency. However, starch physicochemical properties consist of many parameters such as pasting, textural, thermal and retrogradation properties; and few genetic studies of other physicochemical properties have been conducted. Previously, we have established an association mapping panel consisting of 416 rice accessions (Jin et al. 2010) and genotyped the markers tagged for *Wx*, *SS1*, *BE1*, *BEIIb* (Bao et al. 2006a), *SSIIa* (Bao et a. 2006b), and *AGPase* (Bao et al. 2012). In this study, we aim to develop more markers for tagging other starch synthesis related genes (SSRGs), and to investigate the associations between SSRG markers and starch physicochemical properties. The results from this study will enhance our understanding of the genetic control of starch physicochemical properties, and provide markers for carrying out molecular breeding to improve rice grain quality.

Materials and Methods

Rice materials and physicochemical properties

Of the 416 rice accessions developed for association mapping (Jin et al. 2010), 379 accessions are nonwaxy rice with measured AAC, RVA pasting viscosity parameters and gel texture properties (Bao et al. 2006c), and 205 accessions with measured thermal and retrogradation properties (Bao et al. 2007). In brief, RVA pasting profile was determined using a Rapid Visco Analyser (RVA, Model 3-D, Newport Scientific, Warriewood, Australia) with the parameters including peak viscosity (PV), hot paste viscosity (HPV), cool paste viscosity (CPV) and their derivative parameters breakdown (BD, = PV-HPV), setback (SB, = CPV-PV), consistency (CS, =CPV-HPV), stability (Stab, =HPV/PV), and setback ratio (SBratio, =CPV/HPV), and pasting temperature (PT). The viscosities were measured in Rapid Visco Units (RVU). Gel texture properties including hardness (HD, *g*), adhesiveness (ADH, *g.s*) and cohesiveness (COH) were measured on a TA-XT2*i* Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) equipped with the Texture Expert software program (Version 5.16). Thermal properties were analyzed using a DSC 2920 thermal analyser (TA Instruments, Newcastle, DE, USA) and the parameters included onset (T_o), peak (T_p), and conclusion (T_c) temperature, width at half peak height ($\Delta T_{1/2}$) and enthalpy (ΔHg) of gelatinization. The retrogradation properties were measured with the same sample after measurement of the thermal properties, stored, and rescanned with DSC. The enthalpy (ΔHr) of the retrograded starch was used to calculate the percentage of retrogradation (R%) as (ΔHr)/ (ΔHg) ×100.

DNA isolation

Fresh leaf tissue was harvested at the flowering stage from plants grown in the field. DNA was extracted following a CTAB procedure (Doyle 1991).

Development of CAPS, dCAPS, and InDel markers and genotyping

Development of the cleaved amplified polymorphic sequences (CAPS) and derived

173 CAPS (dCAPS) follow the methods of Konieczny and Ausubel (1993) and Neff et al.

174 (1998).

175

176 The primers for PCR were synthesized by the Shanghai Shenggong BioTech Co. Ltd.

177 (Table 1). The PCR was carried out in a total volume of 20 μL containing 10 mM

178 Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM $MgCl_2$, 0.1 mM dNTPs,

179 200 nM primers, 1 unit of *Taq* polymerase, and 30 ng of genomic DNA. All

180 amplifications were performed on a PTC-100 thermal cycler (MJ Research, Inc.)

181 under the following conditions: 5 min at 95ºC; 35 cycles of 1 min at 95 ºC, 45 s at 55

182 ºC and 1 min at 72 ºC; and a final extension step at 72 ºC for 10 min.

183

184 Amplified PCR products were digested using suitable restriction endonucleases in a

185 total volume of 20 μL according to the manufacturer's instructions (Table 1). The

186 digests were resolved by electrophoresis in 1.5–2.0% agarose gel and visualized using

187 a VersaDoc imaging system (Bio-Rad) after staining with ethidium bromide.

188

189 **Statistical analysis**

190 The polymorphism information content (PIC) values were determined using

191 PowerMarker version 3.25 (Liu and Muse 2005). Nei's genetic distance (Nei et al.

192 1983) was calculated and used for unweighted pair-group method with arithmetic

193 means (UPGMA) analysis as implemented in PowerMarker, with the UPGMA tree

194 viewed using MEGA 4.0 (Tamura et al. 2007).

195

196 Analysis of variance (ANOVA) and principal component analysis were performed

197 using the SAS System for Windows version 8 (SAS Institute Inc., Cary, NC, USA).

198 Duncan's multiple range test was conducted for comparison of means at $P < 0.05$.

199 Cluster analysis of the starch properties parameters were performed in SPSS Statistics

200   20 (Windows) using Ward's method based on squared Euclidean distance.

201

202   **Association mapping**

203   The population structure (Q) was determined using the STRUCTURE program

204   (Pritchard et al. 2000), with 100 simple sequence repeat (SSR) markers (Jin et al.

205   2010), and the kinship coefficients (K) between accessions were estimated using the

206   SPAGeDi program (v. 1.2g) (Hardy and Vekemans, 2002) with the same set of SSR

207   markers (Shao et al. 2011). Association analysis between marker alleles and different

208   starch physicochemical properties was performed with TASSEL Version 2.1 software,

209   taking the gross level population structure (Q) and kinship (K) into account (Yu et al.

210   2006; Bradbury et al. 2007). The *P*-value determining whether a marker is associated

211   with a trait was set at *P*<0.01.

212

213   **Results**

214

215   **Marker development for starch biosynthesizing genes and their genotypes**

216   In addition to available SNP, InDel, and SSR markers tagged for *Wx*, *SSI*, *BEI*, *SSIIa*,

217   and all *AGP* genes (two small subunits and four large subunits) by Bao et al. (2002,

218   2006a, 2006b, 2012), we further developed CAPS, dCAPS and InDel markers for

219   tagging other starch biosynthesis related genes, i.e. *GBSSII, SSIIc, SSIIb, SSIIIa,*

220   *SSIIIb, SSIVa, SSIVb, BEIIa, BEIIb, ISA1-3* and *PUL* (Table 1) in this study. Most of

221   SNPs tagged were derived from the study of Tian et al. (2009) who reported many

222   functional SNPs for starch genes. All 13 new gene-specific markers (Table 1)

223   produced two or more alleles each, with the polymorphic information content (PIC)

224   ranging from 0.005 (InDel marker *SSIVb*) to 0.500 (marker *ISA2*). A deletion of 23bp

225   in *SSIVb* was reported by Tian et al. (2009) in rice Suyunuo; we developed the

226   primers to genotype this InDel in our rice accessions, and also found only Suyunuo

227   had this deletion. Due to its low PIC, we adapted another CAPS marker from Tian et

228   al. (2010) and Yan et al. (2011) to genotype this gene and found this marker had the

229   PIC value of 0.381 among all rice accessions.

230

In total, there are 35 markers tagged for 23 starch synthesis related genes (SSRGs), with each gene tagged with at least one marker. The UPGMA tree made with these 35 SSRG markers showed that all the 416 rice could be assigned into two large groups, representing *indica* and *japonica* subspecies, respectively (Supplementary Fig. 1), with rice BP459, BP464, BP465, BP470, BP474, BP476, BP487 and BP532 loosely affiliated with the *japonica* group. These same accessions were also assigned to the *japonica* group based on 100 SSRs, but with a much smaller membership probability (Jin et al. 2010). The waxy rice could also be classified into *indica* and *japonica* groups using the SSRGs markers (Xu et al. 2013). These results clearly indicated that SSRGs have diverged between the two subspecies during domestication and/or improvement.

The association mapping panel has been genotyped with 100 SSRs before with a few additional SSRs and other gene markers, such as *Gnla, fgr, Ra,* and *Rc* (Shao et al. 2011). In total, there are 143 markers that can be used for association mapping. Fig. 1 shows the position of each marker in the chromosome of rice genome in physical distance.

Phenotypes of starch properties of nonwaxy rice

The starch physicochemical properties of each nonwaxy rice used in the present analysis were based on Bao et al. (2006c, 2007). It is not surprising that this set of association panel harbors wide genetic diversity in all starch physicochemical properties. AAC had significant correlation with pasting viscosity and gel texture traits except for PV, but had no relationship with thermal properties such as To, Tp, Tc and ΔHg. Tp had no correlation with HPV, SB and ADH, but had significant correlation with PV ($P<0.05$), CPV ($P<0.05$), CS ($P<0.01$), Stab ($P<0.01$), and SBratio ($P<0.01$) (Supplementary Table 1). From the correlation analysis, all the traits could be divided into two groups, AAC related traits (pasting viscosity and gel texture) and gelatinization temperature (GT) related traits (thermal property). Retrogradation

trait could be related with both groups. As a result, after normalization of all the trait value to between 0 and 1, a cluster analysis based on Ward's method showed that all the traits could be classified into three groups (Fig. 2). Group 1 includes To, Tp, Tc and PT, Group 2 includes BD, ΔHg, COH, $\Delta T_{1/2}$, and ADH, while Group 3 includes HPV, CPV, PV, AAC, Stab, SB, CS, SBratio, ΔHr, R% and HD. Thus, Group 1 represents the GT-related traits, Group 3 represents AAC-related traits, while Group 2 is a mixture of AAC and GT related traits (Fig. 2).

Since the AAC and GT were the most important factors affecting other traits, the AAC-GT combination for each rice accession could be clearly visualized in the plot for both AAC and GT (Fig. 3). For the low AAC rice accessions (AAC<20%), the GT could be divided into high GT and low GT classes. The high GT rice had the peak temperature (Tp) ranging from 77.6 to 79.8 ^{o}C, while the low GT rice ranging from 65.8 to 71.0 ^{o}C. For the high AAC rice accessions, the GT could also be divided into two groups. One is intermediate GT group with GT ranging from 72.8 to 76.6 ^{o}C, and the other is the low GT group with GT ranging from 63.2 to 67.7 ^{o}C. It should be noted that the two low GT groups with contrasting AAC differed in the range of temperature, with the high-AAC rice having much lower GT than the low-AAC rice (Fig. 3).

All the rice accessions have been divided into seven groups or subpopulations (Jin et al. 2010). The difference in each starch property parameter among these seven subpopulations is listed in Table 2. For apparent amylose content and pasting viscosity parameters, POP3 and POP6 had higher AAC as well as higher PV, HPV, and CPV than other subpopulations. POP7 had the lowest AAC and SB, but had the highest PV and BD among the seven subpopulations. For gel texture parameters, POP3 had the highest HD and the lowest ADH and COH, and by contrast, POP5 and POP7 had the lowest HD but the highest ADH and COH. For pasting temperature (PT) and thermal property parameters, POP1 and POP2 had similarly lower PT than the other five

288 subpopulations. Similar results were also found for the thermal property parameters,

289 i.e. POP 1 and POP2 had lower To, Tp, Tc and ΔHg than the others. POP5 had the

290 highest mean $\Delta T_{1/2}$ and POP3 had the lowest mean $\Delta T_{1/2}$. For retrogradation property

291 parameters, POP3 had the highest mean ΔHr and R%, whereas POP5 and POP7 had

292 the lowest ΔHr and R% (Table 2).

293

294 **QTLs for starch physicochemical properties**

295 Association mapping of starch physicochemical property parameters were performed

296 using the 35 SSRG markers and 108 other markers (mostly SSRs) based on the Q+K

297 model. The identified QTLs are hereafter called main-effect QTLs. To uncover more

298 QTLs concealed by the *Wx* and *SSIIa* loci for the starch physicochemical traits,

299 further association mapping were conducted with *Wx* (G/A) and SSIIa (GC/TT) SNPs

300 as covariate based on the same Q+K model. The QTLs identified are called QTLs in

301 the *Wx* or *SSIIa* background. The results are summarized in Table 3 and Fig. 4.

302 AAC

303 A total of five main-effect QTLs were detected for AAC. *Wx* gene locus was detected

304 as a major QTL $(P=1.45\times10^{-95})$. *SSI* locus, RM122, RM346 and RM222 were also

305 detected. Using *Wx* SNP (A/G) as a covariate, six QTLs were detected including four

306 SBRGs, *GBSSII*, *BE1*, *SSIIc*, and *SSIIa*. Using *SSIIa* SNP (GC/TT) as a covariate, the

307 same five main-effect QTLs were detected.

308

309 Pasting viscosity

310 As expected, the *Wx* locus was detected as a main-effect QTL for all the pasting

311 viscosity parameters. SS1 was detected for SB and CS; *SSIIa* was detected for BD, CS,

312 Stability and SBratio. In addition, RM346 was detected for CPV, SB, CS, and

313 SBratio.

314 Under the *Wx* locus background, no main-effect QTL was detected for PV, HPV and

315 CPV. *SSIIa* was still detected with much smaller *P* value for BD (P=3.72×10^{-8}),

316 SBratio (P=0.0062) and Stability (P=8.24×10^{-8}). No other SSRGs were detected for

317 pasting viscosities. More SSRs were detected for BD (RM237, RM276, RM340,

318 RM48 and RM507) and Stability (RM209, RM237, RM276, and RM507).

319 Under the *SSIIa* locus background, the QTLs identified for each trait were similar to

320 those of main-effect QTLs; the only difference was that one less QTL (*SS1*) for CS

321 and one more QTL (*GS3*) for SBratio were detected.

322

323 Gel texture

324 *Wx* was the common main-effect QTL for HD, ADH and COH, while *SS1* was

325 detected as the main-effect QTL for HD and COH. PUL was detected for HD.

326 Under the *Wx* locus background, *GBSSII*, RM252 and RM3 were detected for HD,

327 and only RM252 was detected for COH, while no QTL was detected for ADH.

328 Under the *SSIIa* locus background, the QTLs identified for each trait were the same as

329 those of main-effect QTLs.

330 Thermal property and pasting temperature (PT)

331 For the thermal property and PT, *SSIIa* was a common main-effect QTL. Other

332 SSRGs, *SSIIIb* (PT), *AGPL2* (ΔHg), and *Wx* (ΔT$_{1/2}$) were also detected. In addition,

333 some SSRs were detected for PT (RM276, RM253 and RM346), To (RM346), Tc

334 (RM484), ΔHg (RM1 and RM48) and ΔT$_{1/2}$ (RM161 and RM346).

335 Under the *Wx* locus background, all the QTLs identified for each trait were the same

336 as those of main-effect QTLs except that one more QTL for Tc (RM484) and ΔHg

337 (RM152), and one less (RM346) for $\Delta T_{1/2}$.

338 Under the *SSIIa* locus background, it was interesting to find that *Wx* locus was

339 detected for Tc ($P=0.0018$) and ΔHg ($P=0.0016$). However, *Wx* was not detected for

340 $\Delta T_{1/2}$, and instead, other SSRGs, such as *SSIIb* and *AGPL3* were detected for $\Delta T_{1/2}$.

341 *AGPL2* was also detected for ΔHg. RM346 was still detected for PT, To and Tp, and

342 RM1 and RM161 were still detected for ΔHg and $\Delta T_{1/2}$, respectively. More additional

343 SSRs were identified for PT, To, Tp, Tc and $\Delta T_{1/2}$.

344 Retrogradation property

345 For the retrogradation property, five common SSRGs, *Wx*, *SSIIa*, *ISA1*, *AGPL3* and

346 *BEIIa*, were identified as main-effect QTLs for both ΔHr and R%. RM346 and

347 RM161 for ΔHr and RM346 for R% were also detected.

348 Under the *Wx* locus background, *ISA1* and *SSIIa* were still identified as QTLs for both

349 ΔHr and R%. By contrast, *BEIIa* was only significant for R%. Another SSRG

350 (*AGPL2*) and RM87 were detected for both traits.

351 Under the *SSIIa* locus background, the QTLs identified were similar to those of

352 main-effect QTLs: *Wx*, *ISA1*, *AGPL3* and *RM346* were significant for both traits, but

353 *BEIIa* was only detected for R%.

354

Discussion

356 Association mapping has become a robust technology for quickly identifying the

357 genotype-phenotype relationships among diverse germplasms. The success of

358 association mapping depends on the diversity of the germplasms being investigated,

359 the marker coverage of the target genome, and the appropriate methodologies used.

New advances in association mapping has been made in plants recently, with some great achievements coming from rice, such as mapping genes for cooking quality (Tian et al. 2009) and agronomic traits (Huang et al. 2010; Zhao et al. 2011).

Due to the diverse origins of the rice accessions used in different studies, a wide range of variations has always been found in different populations, for example, from the African and USA germplasm (Asante et al. 2013), Italian germplasm (Caffagni et al. 2013) and Korean germplasm (Lu et al. 2012a, 2012b; Zhao et al. 2013). The present study covers more than 20 starch physicochemical property traits that have been measured for 379 rice accessions. Compared with other studies on eating and cooking quality of rice, our research ranks the highest in both number of traits and accessions analyzed. In addition to the wide genetic diversity revealed for each trait, we found that all the traits could be classified into three groups: AAC-related traits, GT-related traits, and the mixture (Fig. 2). In another linkage mapping study, Wang et al. (2007) also found that eating and cooking traits of rice could be divided into two groups: the first class consists of AC, GC, and most of the paste viscosity parameters that form a major determinant of eating quality, the second class includes alkali spreading value, pasting temperature and pasting time, which characterize the cooking process. Furthermore, with plotting AAC and GT (measured as the Tp), we have another important finding that both AAC and GT could be divided into two classes, and in combination, there are four classes among nonwaxy rice (Fig. 3). For the low AAC rice accessions (AAC<20%), the GT could be divided into high GT, and low GT class. For the high AAC rice accessions, the GT could also be divided into two groups. The GT of low-AAC- low-GT group are much higher than the high-AAC-low-GT group (Fig. 3). The GT of waxy rice is similar to that of low-AAC rice that have high and low GT classes (Xu et al. 2013). Juliano and Villareal (1993), Juliano (1998) have long before indicated that high-AAC rice usually has intermediate or low-GT; low-AAC rice or waxy rice usually has high or low-GT among rice accessions. By contrast, it is difficult to find the combinations of high-AAC and high-GT rice, and low-AAC and intermediate-GT rice (Juliano and Villareal 1993; Juliano, 1998). No other genetic analyses have indicated this fact. The tight link between AAC and GT suggests that there might be interaction between AAC and GT. However, in depth screening of rice germplasm may find other rare combinations of AAC and GT. For example, Juliano et al. (2009) found the combination of high-AAC and high-GT rice.

394 These diverse materials provide precious resources for further genetic studies and

395 molecular analysis of the related genes.

396

397 Many studies have focused on the genetic basis of the starch physicochemical

398 properties in relation to the cooking and eating quality of rice. AAC is mainly

399 controlled by the *Wx* region on chromosome 6 (He et al. 1999; Tan et al. 1999; Bao et

400 al. 2000; Lanceras et al. 2000; Septiningsih et al. 2003; Aluko et al. 2004). GT and

401 thermal properties are genetically determined by a major QTL, i.e. the alkali

402 degeneration (*alk*) locus on chromosome 6, also known as *SSIIa* (He et al. 1999;

403 Aluko et al. 2004; Bao et al. 2004c; Fan et al. 2005; Tian et al. 2005; Wang et al.

404 2007). Genetic analysis with QTL mapping approach showed that the RVA

405 parameters are mainly controlled by the *Wx* gene (Bao et al. 1999, 2000, 2003; Larkin

406 and Park 2003; Wang et al. 2007; Traore et al. 2011). Gel texture parameters were

407 also mainly controlled by the *Wx* locus (Bao et al. 2000; 2004a). The aforementioned

408 results mostly came from linkage analyses. Association mapping for these traits have

409 been conducted recently (Chen et al. 2008a, 2008b; Tian et al. 2009; Lu et al. 2012a,

410 2012b; Zhao et al. 2013). Similar results of main-effect QTLs have been reported, i.e.

411 *Wx* and *SSIIa* controlled AAC-related traits and GT-related traits, respectively (Chen

412 et al. 2008a, 2008b; Tian et al. 2009; Caffagni et al. 2013). However, due to different

413 germplasms used, some of the studies have not identified the *Wx* for AAC (Lu et al.

414 2012a, 2012b; Zhao et al. 2013). Lu et al. (2012a, 2012b) detected *AGPS1*, *AGPL4*

415 and *SSIIb* for AAC and pasting viscosity in their rice materials. Kharabian-Masouleh

416 et al. (2012) reported that a SNP of *glucose-6-phosphate translocator* gene was highly

417 associated with amylose content and retrogradation property. This study also found

418 some new loci for starch physicochemical properties, such as *SSIIIb* for PT, *PUL* for

419 HD, *AGPL2* for ΔHg; *BEIIa* and *AGPL3* for retrogradation properties (Table 3). The

420 retrogradation property traits were comprehensively studied for the first time, and we

421 identified five common SSRGs, *Wx*, *SSIIa*, *ISA1*, *AGPL3* and *BEIIa*, as main-effect

422 QTLs for both ΔHr and R%. Either under the *Wx* locus or *SSIIa* backgournd, *ISA1*

could also be identified for both ΔHr and R%. Thus, it can be concluded that retrogradation properties are mainly controlled by *Wx*, *SSIIa* and *ISA1*. Their relative effects are in the order of *SSIIa* > *Wx* > *ISA1*. However, the effects of other SSRGs such as *BEIIa*, *AGPL2* and *AGPL3* could not be neglected.

Genetic linkage mapping studies showed that *Wx* not only controls AAC, gel consistency and pasting viscosity, but also affects GT (Wang et al. 2007; Lapitan et al. 2009), and the QTL cluster at *SSIIa* locus also contains individual QTL for gel consistency and some paste viscosity parameters (Wang et al. 2007). Tian et al. (2009) reported by association mapping that *Wx* not only affects AAC and gel consistency as a major gene but also regulates GT as a minor one; *SSIIa* plays an essential role not only in controlling GT, but also AC and gel consistency (Tian et al. 2009). In this study, we analyzed the effects of *Wx* and *SSIIa* background QTLs using the two functional SNPs for *Wx* (G/A) and *SSIIa* (GC/TT) as an additional covariate on the physicochemical property traits by association mapping. We found that more QTLs that were concealed by the *Wx* and *SSIIa* could be discovered. In the *SSIIa* background, the AAC-related traits were found to be controlled by similar QTLs as the main-effect QTLs, but it is possible to detect more other QTLs for the GT-related traits. Specifically, the Tc and ΔHg were found to be controlled by *Wx*. RM447 on chromosome 8 and RM17 on chromosome 12 were found to control PT, To, Tp and Tc. *SSIIb* and *AGPL3* were identified for $\Delta T_{1/2}$. Similarly, under the background of *Wx*, the GT-related traits were found to be controlled by similar main-effect QTLs, while it is possible to detect more QTLs for the AAC-related traits. Specifically, AAC was found to be controlled by more SSRGs, such as *SSIIa*, *BE1*, *SSIIc* and *GBSSII*. The Stab was found to be controlled by more SSRs. Zhao et al. (2013) also detected a locus near *SSIIa* (RM276) for AAC. All these findings confirm the importance of *Wx* and *SSIIa* in determining the eating and cooking quality of rice, and suggest that there might be epistatic interaction between *Wx* and *SSIIa*. Tian et al. (2009) introduced an antisense and sense *Wx* RNA to a high AAC rice and waxy rice respectively, and they found that GT changed simultaneously with significant decrease or increase in AAC. Tian et al. (2009) and Gao et al. (2011) introduced *SSIIa* gene to low-GT rice by transgenic engineering and found that it also affected the AAC, gel consistency and

17

pasting properties, suggesting that *SSIIa* is a modifier gene for AAC, gel consistency, and pasting properties in rice. These genetic transformation studies provide supporting evidence for the hypothesized interaction between *Wx* and *SSIIa*. However, it is also possible that the complex effects of *Wx* and *SSIIa* are derived from their close physical position in the chromosome 6, as suggested by strong linkage disequilibrium for these starch related traits. Thus, the exact roles played by *Wx* and *SSIIa* remain to be untangled.

Biochemically, starch biosynthesis in the cereal endosperm involves complex interactions among multiple isoforms of starch synthase, branching and debranching enzymes, leading to a fine amylopectin structure (Jeon et al. 2010; Tetlow et al. 2011). Multi-enzyme complexes (protein-protein interactions) have been indentified in wheat and maize endosperms during the period of grain filling (Tetlow et al. 2004, 2008; Hennen-Bierwagen et al. 2008, 2009). The multi-enzyme complex components in the normal maize endosperm include SSI, SSIIa, BEI, BEIIa, BEIIb, and SP (starach phosphorylase) (Liu et al. 2009). Based on the facts that *Wx* and *SSIIa* exert joint control over both AAC and GT, and the complex AAC and GT combinations exist in rice germplasm, Bao (2012) proposed a multi-enzyme complex model to explain the interaction between GBSSI (Wx protein) and SSIIa, and how they contribute to diverse combination of AAC and GT in rice germplasm. The model hypothesizes that BEIIb, SSI and SSIIa are the major multi-enzyme complex components in the intermediate-GT (SSIIa active) rice, the high activity of GBSSI in the stroma negatively regulates the function of the multi-enzyme complex, resulting in the synthesis of high-AAC intermediate-GT starch. In low-AAC high-GT rice, the lower GBSSI activity and hence the higher activity of the multi-enzyme complex, allows the synthesis of more chains with DP>12, thus producing high-GT starch. Low-GT rice can have high, intermediate, low or zero (waxy rice) AAC. Due to the loss of function of SSIIa, the components that comprise the multi-enzyme complex are unknown and thus need to be further studied. However, the multi-enzyme complex in SSIIa-active rice grains should be clarified first before a more realistic hypothesis can be put forward.

Undoubtedly, our results have direct applications to rice quality breeding programs. The well-known markers of *Wx* and *SSIIa* genes have been used in rice breeding in

which they greatly facilitate the precise picking of the desirable alleles from the good quality parent using marker-assisted selection (Wang et al. 2007; Jin et al. 2010). This study also points to a new direction in rice grain quality research, that is, to identify the protein-protein interactions among the related enzymes during grain filling. The knowledge gained from these new researches will enhance our understanding of starch biosynthesis, and ultimately contribute to the improvement of rice cooking and eating quality.

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1. Legends for Figures

747

748 **Fig. 1** The position of markers in physical distance on 12 chromosomes of rice
749 genome

750

751 **Fig. 2** Dendrogram generated with the starch properties parameters using Ward's

752 method based on squared Euclidean distance

753

754 **Fig. 3** Plot of AAC-GT combination for nonwaxy rice accessions

755

756 **Fig. 4** Plots of main-effect QTLs (**A**), QTLs identified in the *Wx* background (**B**)

757 and *SSII*a background (**C**) for 20 starch physicochemical properties. The SSRGs with

758 the largest $-\log(P)$ values were highlighted with red (*Wx*), blue (*SSIIa*), green (*ISA1*)

759 and pink (*SSIIb*) colors
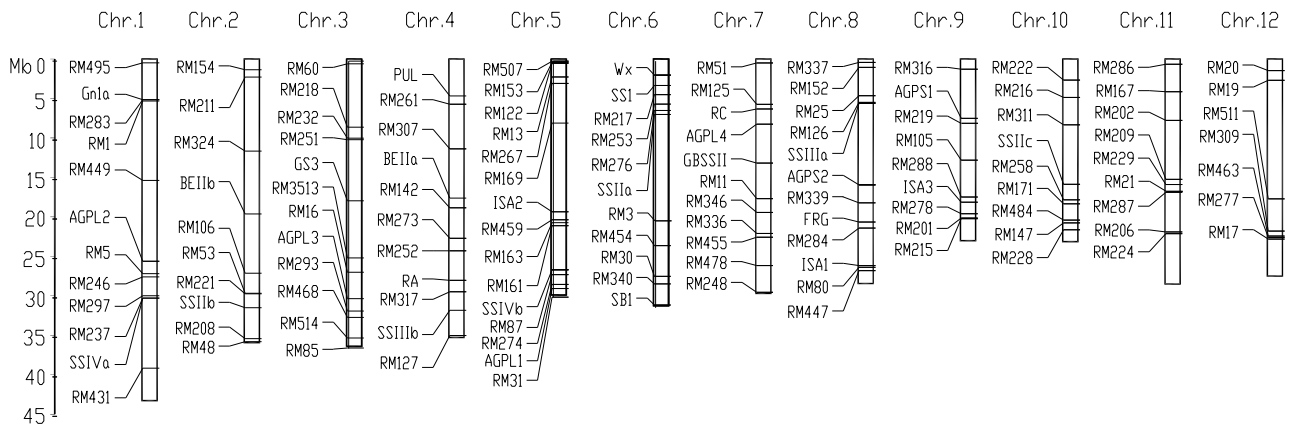
760

Figure
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Fig 1

Fig 2

Fig 3

Fig 4.

A

B
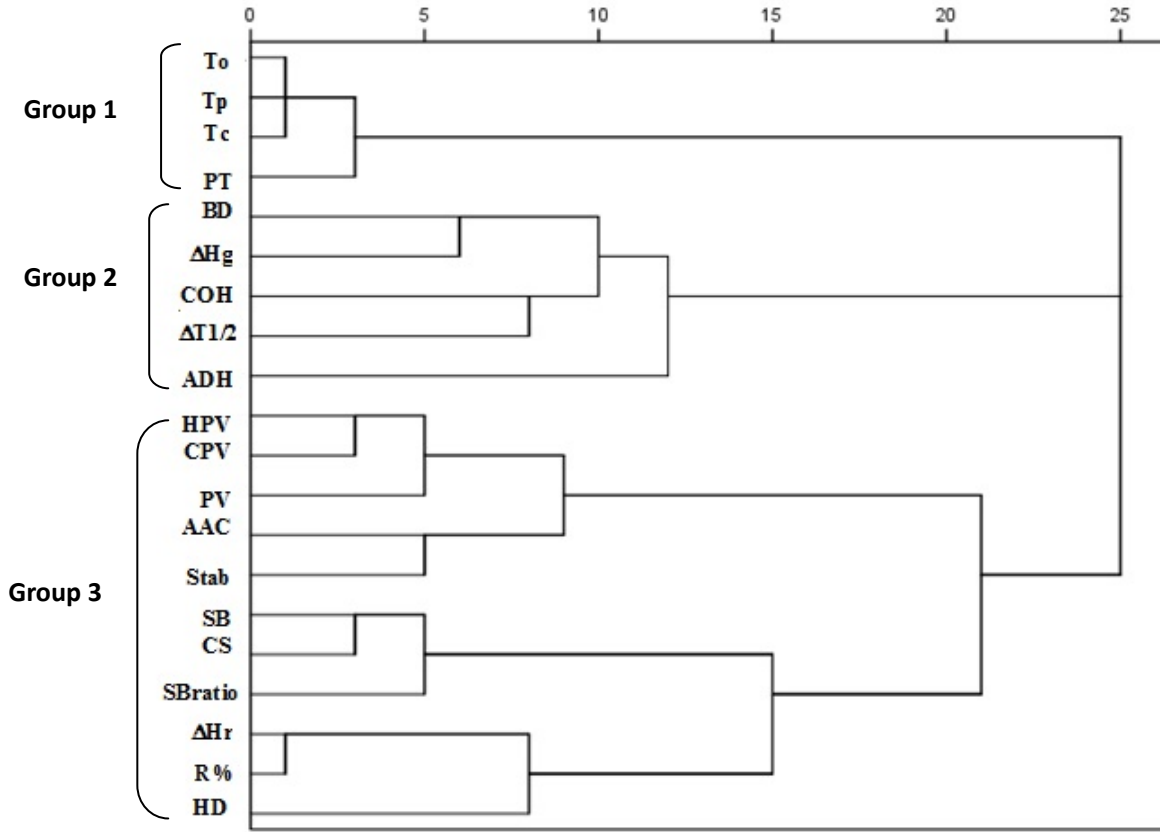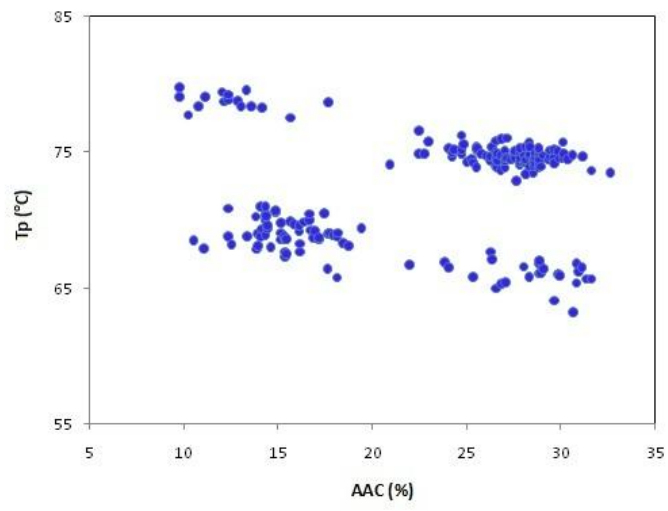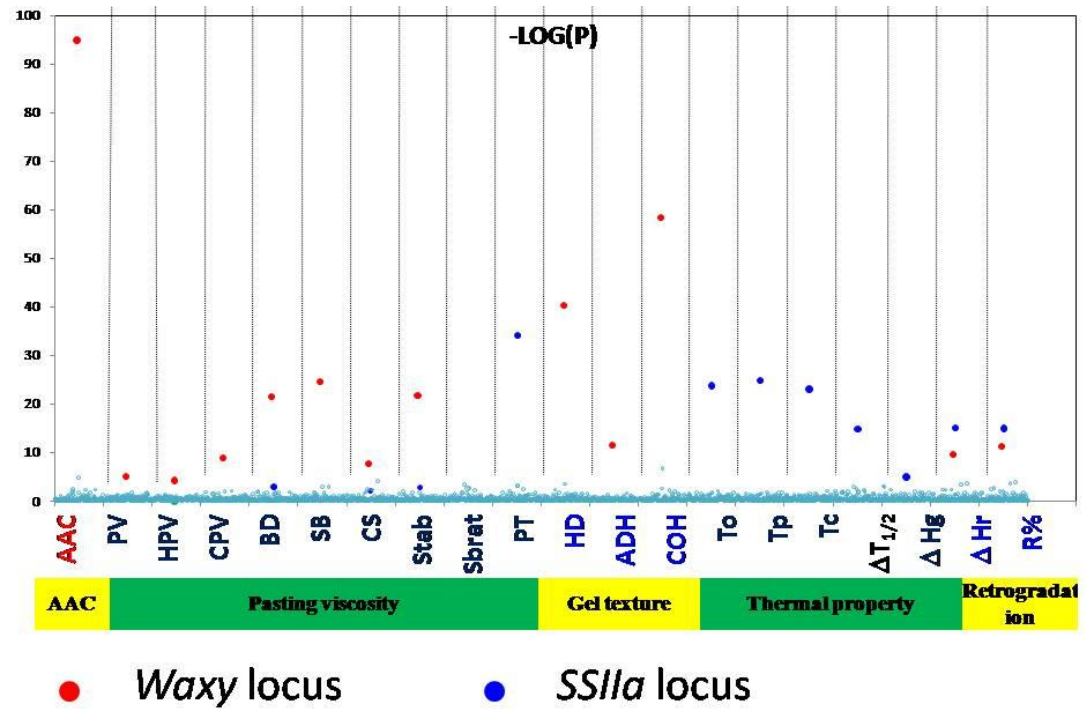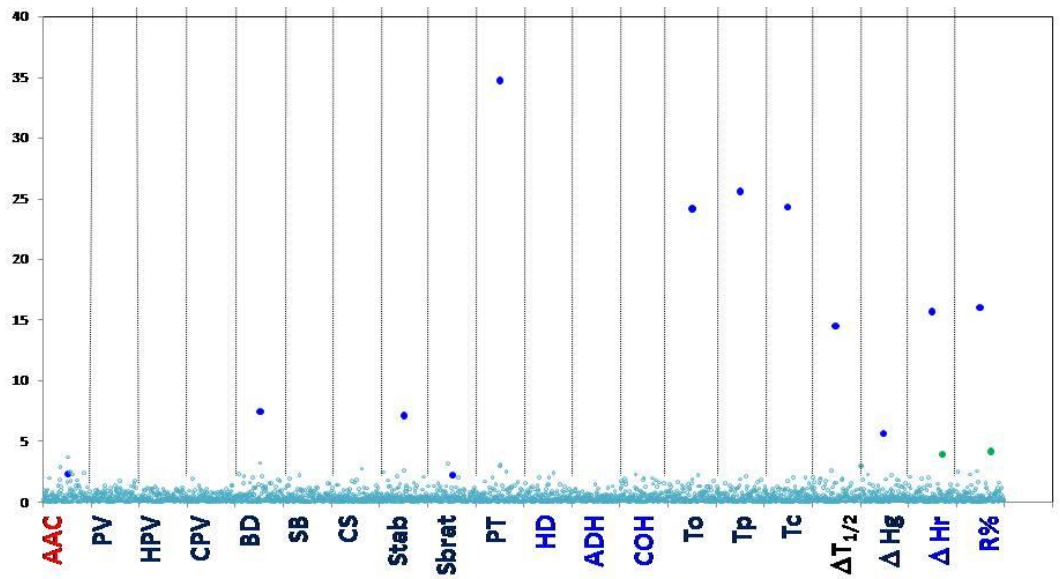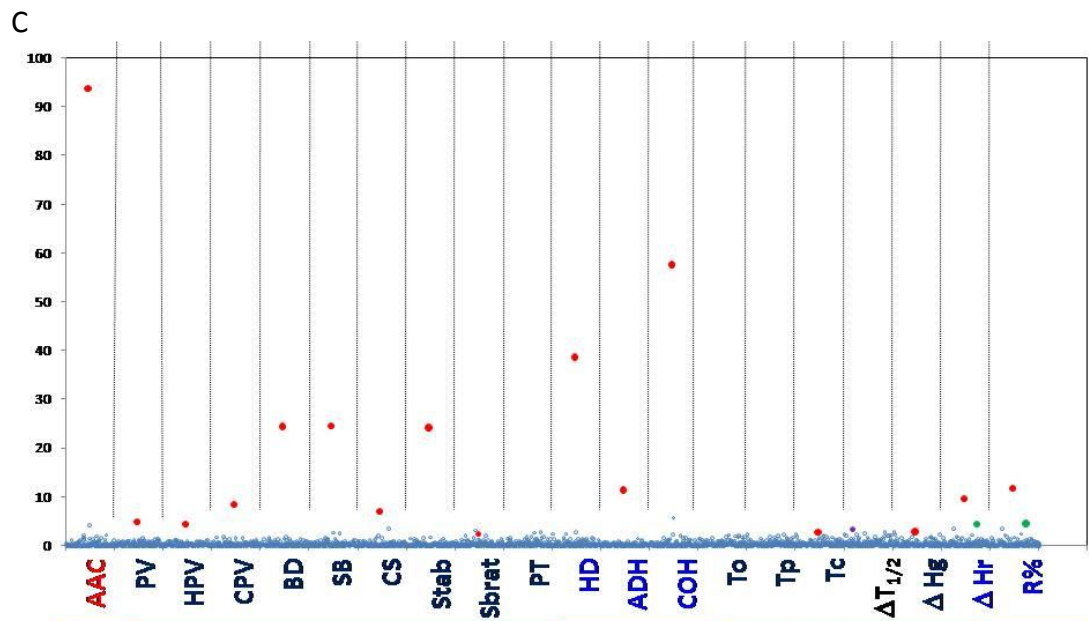
Table 1 Summary of CAPS, dCAPS and InDel markers for starch synthesizing genes used in this study

Genes	Chromosome	Forward primer	Reverse primer	R.E. [1]	Allele size (bp)	PIC[1]
GBSSII	7	TGACCTGAAAATCATATTATTAC	CACTTTCGTTTGGTGCATCTG	SpeI	180 (A), 158 (T)	0.473
SSIIc (*SSII-1*)	10	CGGTGGCAAGGAGAGCCGGGGT	GCGGCACGGATCTGGAGAAG	MboII	210 (G); 181 (T)	0.109
SSIIb (*SSII-2*)	2	TTCGCAAAGCATGAGACAATAAG	GAGGCCCAAGTCATTCAACAA	-	InDel (155; 172)	0.280
SSIIIb (*SSIII-1*)	4	AGCAGAATGAATCTGACAATCTAG	CGTGATTTCCACCATAAGAGCAA	XbaI	182 (AG), 164+20(AGAG)	0.302
SSIIIa (*SSIII-2*)	8	TGTTAAAATTTCCCCCCAAGTAC	GCATAATGTTCAACTGTAGATAAAGAAG	MobII	203(C); 166+37(T)	0.471
SSIVa (*SSIV-1*)	1	GTCGCTTCCTAGGAGGGCAACGT	GATAACTGCTAAGATATTGAGAG	Acl	198 (C); 176 (T)	0.260
SSIVb (*SSIV-2*)	5	CCAAGTGGGGATCATCAACCTC	CCCGCAAAAATGAAGCTAAGC	-	InDel (252; 230)	0.005
	5	CTTCTGATTGATGGTTGGTTGC[2]	GGAAGAATAATCTCTACTAGGTGGC	SphI	728 (G); 523+205 (A)	0.381
BEIIb (*SBE3*)	2	GCATCCTCAACCTAAAAGACCA	GAATCAACCATCCAGCAAAGG	Scal	301 (G); 140+160 (A)	0.462
BEIIa (*SBE4*)	4	CTGGGTGCTCCTGTTTGTTTCT	CGTGCTTATTCGCTGTATTCCT	MnlI	192 (C); 163 (G)	0.358
ISA1	8	CGCACTGGATTTCAAGATGAGC	TCCATAGATGCTTTCGGCTGT	AluI	213 (C); 191 (T)	0.305
ISA2	5	CAGGCGTGTAGCAAGATCACTCAT	TGACCCGGTTCTTTCCATGAC	Nla III	181 (A); 157(G)	0.500
ISA3	9	TGACTGATTGGATGCTGCTAAAC	GCCGCTCTTGTTTGGAAATG	HinfI	246 (A); 174+72 (G)	0.336
PUL	4	AGAGAAGGAGAAAGAAGTGGAGAC[2]	GTCCAAACTGAATCACTCAATCG	-	InDel (128; 115)	0.334

1. R.E: restriction enzyme; PIC: polymorphism information content.

2. The CAPS (*SSIV-2*) and InDel (*PUL*) markers were adapted from Tian et al. (2010) and Yan et al. (2011).

Table 2 Comparison of the mean values of the phenotypic traits among seven subpopulations

Subpop	AAC	PV	HPV	CPV	BD	SB	CS	Stab	SBratio	PT	HD	ADH	COH	To	Tp	Tc	ΔT1/2	ΔHg	ΔHr	R%
POP1	24.5 b	241.1 b	180.8 ab	319.6 ab	60.3 c	78.5 a	138.8 b	0.7 ab	1.8 bcd	73.7 b	29.2 cd	-30.0 bc	0.6 b	63.0 c	69.2 c	75.9 c	7.2 b	7.0 d	1.1 de	14.8 de
POP2	21.2 c	224.1 c	158.3 d	309.5 b	65.8 bc	85.3 a	151.2 a	0.7 cd	2.0 a	73.9 b	25.0 d	-6.2 ab	0.6 b	67.0 b	72.2 ab	78.7 ab	6.4 cd	7.8 c	1.8 cd	21.7 cd
POP3	28.4 a	257.4 a	189.8 a	336.9 a	67.7 bc	79.4 a	147.1 ab	0.7 abc	1.8 bcd	76.2 a	41.6 a	-36.1 d	0.5 c	69.4 c	74.1 a	80.1 a	5.9 d	9.1 a	3.6 a	38.9 a
POP4	24.9 b	245.3 b	176.4 bc	323.9 ab	68.9 bc	78.6 a	147.6 ab	0.7 bcd	1.8 b	75.9 a	32.2 c	-36.3 d	0.6 b	68.5 ab	73.8 a	80.0 a	6.4 cd	8.4 b	2.5 bc	28.7 bc
POP5	18.0 d	235.5 bc	163.9 d	280.5 c	71.6 b	45.0 b	116.6 c	0.7 d	1.7 d	76.3 a	17.1 e	-21.6 a	0.7 a	64.1 c	70.7 bc	77.9 b	8.0 a	7.8 c	0.9 e	10.5 e
POP6	27.6 a	244.4 b	184.3 ab	329.7 a	60.1 c	85.3 a	145.4 ab	0.8 a	1.8 bc	76.3 a	37.0 b	-33.3 cd	0.5 c	68.5 ab	73.6 a	79.8 a	6.3 cd	8.5 ab	2.9 ab	34.0 ab
POP7	17.7 d	258.4 a	166.3 cd	288.8 c	92.1 a	30.3 c	122.4 c	0.6 e	1.8 cd	75.1 a	18.6 e	-21.4 a	0.7 a	66.6 b	72.3 ab	78.6 ab	6.7 bc	8.2 bc	1.0 e	11.5 e

Different letters in the same column were significant at $P < 0.05$.

Table 3 The marker loci associated with starch physicochemical property traits detected with Q+K model and with *Wx* or *SSIIa* as additional covariate.

Trait	Main effect loci			*Wx* covariate			*SSIIa* covariate		
	Locus	chro.[1]	p_Marker	Locus	Chro.	p_Marker	Locus	Chro.	p_Marker
AAC									
AAC	RM122	5	3.8×10^{-3}	RM507	5	1.3×10^{-3}	RM122	5	5.1×10^{-3}
	Wx	6	1.45×10^{-95}	*SSIIa*	6	5.3×10^{-3}	*Wx*	6	2.14×10^{-94}
	SS1	6	1.57×10^{-5}	RM276	6	2.1×10^{-4}	*SS1*	6	4.67×10^{-5}
	RM346	7	4.6×10^{-3}	*BE1*	6	3.2×10^{-3}	RM346	7	9.0×10^{-3}
	RM222	10	5.2×10^{-3}	*GBSSII*	7	5.5×10^{-3}	RM222	10	5.6×10^{-3}
				SSIIc	10	4.1×10^{-3}			
Pasting viscosity									
PV	*Wx*	6	1.18×10^{-5}				*Wx*	6	1.52×10^{-5}
HPV	*Wx*	6	6.46×10^{-5}				*Wx*	6	4.92×10^{-5}
CPV	*Wx*	6	1.52×10^{-9}				*Wx*	6	4.88×10^{-9}
	RM346	7	7.9×10^{-3}						
BD	*Wx*	6	3.92×10^{-22}	RM237	1	4.9×10^{-3}	*Wx*	6	3.95×10^{-25}
	SSIIa	6	1.2×10^{-3}	RM48	2	7.5×10^{-3}			
				RM507	5	9.3×10^{-3}			
				RM276	6	6.3×10^{-4}			
				SSIIa	6	3.72×10^{-8}			
				RM340	6	8.2×10^{-3}			
SB	*Wx*	6	2.87×10^{-25}	RM317	4	6.2×10^{-3}	*Wx*	6	2.83×10^{-25}
	SS1	6	1.2×10^{-3}				*SS1*	6	1.6×10^{-3}
	RM346	7	1.5×10^{-3}				RM346	7	2.6×10^{-3}

Trait									
CS	RM161	5	4.5×10^{-3}	RM346	7	1.8×10^{-3}	RM161	5	5.9×10^{-3}
	Wx	6	2.29×10^{-8}				*Wx*	6	1.26×10^{-7}
	SS1	6	9.9×10^{-3}				RM346	7	2.65×10^{-4}
	SSIIa	6	5.3×10^{-3}						
	RM346	7	8.77×10^{-5}						
PT	*SSIIIb*	4	9.1×10^{-3}	*SSIIIb*	4	9.9×10^{-3}	RM346	7	1.9×10^{-3}
	RM253	6	9.39×10^{-4}	RM253	6	1.1×10^{-3}	RM447	8	1.4×10^{-3}
	RM276	6	8.59×10^{-4}	RM276	6	8.02×10^{-4}	RM17	12	3.2×10^{-3}
	SSIIa	6	8.28×10^{-35}	*SSIIa*	6	2.06×10^{-35}			
	RM346	7	4.3×10^{-3}	RM346	7	3.1×10^{-3}			
Stability	*Wx*	6	2.45×10^{-22}	RM237	1	3.5×10^{-3}	*Wx*	6	6.67×10^{-25}
	SSIIa	6	1.3×10^{-3}	RM507	5	7.5×10^{-3}			
				RM276	6	2.4×10^{-3}			
				SSIIa	6	8.24×10^{-8}			
				RM209	11	8.4×10^{-3}			
SBratio	RM161	5	3.74×10^{-4}	RM161	5	7.37×10^{-4}	GS3	3	6.6×10^{-3}
	Wx	6	3.0×10^{-3}	*SSIIa*	6	6.2×10^{-3}	RM161	5	5.77×10^{-4}
	SSIIa	6	2.5×10^{-3}	RM346	7	1.0×10^{-2}	*Wx*	6	4.4×10^{-3}
	RM346	7	7.5×10^{-3}				RM346	7	7.7×10^{-3}
Gel texture									
HD	*PUL*	4	4.6×10^{-3}	RM252	4	9.8×10^{-3}	*PUL*	4	3.0×10^{-3}
	Wx	6	5.68×10^{-14}	RM3	6	9.3×10^{-3}	*Wx*	6	2.48×10^{-39}
	SS1	6	3.32×10^{-4}	*GBSSII*	7	4.2×10^{-3}	*SS1*	6	1.4×10^{-3}
ADH	*Wx*	6	3.58×10^{-12}				*Wx*	6	4.9×10^{-12}
COH	*Wx*	6	4.96×10^{-59}	RM252	4	5.6×10^{-3}	*Wx*	6	2.83×10^{-58}
	SS1	6	2.9×10^{-3}				*SS1*	6	4.7×10^{-3}

	Marker	Chr	p	Marker	Chr	p	Marker	Chr	p
	RM346	7	6.4×10^{-3}				RM346	7	7.6×10^{-3}
Thermal property									
To	*SSIIa*	6	2.0×10^{-24}	*SSIIa*	6	7.37×10^{-25}	RM346	7	5.5×10^{-3}
	RM346	7	9.1×10^{-3}	RM346	7	6.4×10^{-3}	RM447	8	8.7×10^{-3}
							RM215	9	6.5×10^{-3}
							RM17	12	4.4×10^{-3}
Tp	*SSIIa*	6	1.91×10^{-25}	*SSIIa*	6	2.67×10^{-26}	RM346	7	7.9×10^{-3}
				RM346	7	7.3×10^{-3}	RM447	8	4.1×10^{-3}
							RM17	12	4.7×10^{-3}
Tc	*SSIIa*	6	9.87×10^{-24}	*SSIIa*	6	5.64×10^{-25}	*Wx*	6	1.8×10^{-3}
	RM484	10	8.5×10^{-3}	RM346	7	9.9×10^{-3}	RM125	7	7.6×10^{-3}
				RM484	10	9.1×10^{-3}	RM17	12	3.4×10^{-3}
ΔHg	RM1	1	9.90×10^{-4}	RM1	1	1.0×10^{-3}	RM1	1	1.5×10^{-3}
	AGPL2	1	1.4×10^{-3}	*AGPL2*	1	1.1×10^{-3}	*AGPL2*	1	4.6×10^{-3}
	RM48	2	5.1×10^{-3}	RM48	2	5.5×10^{-3}	*Wx*	6	1.6×10^{-3}
	SSIIa	6	1.24×10^{-5}	*SSIIa*	6	2.38×10^{-6}	RM224	11	3.9×10^{-3}
				RM152	8	6.2×10^{-3}			
$\Delta T_{1/2}$	Gn1a	1	8.1×10^{-3}	Gn1a	1	9.3×10^{-3}	Gn1a	1	8.1×10^{-3}
	RM161	5	1.1×10^{-3}	RM161	5	2.5×10^{-3}	*SSIIb*	2	5.13×10^{-4}
	Wx	6	4.5×10^{-3}	*SSIIa*	6	3.47×10^{-15}	*AGPL3*	3	3.3×10^{-3}
	SSIIa	6	1.46×10^{-15}				RM161	5	2.3×10^{-3}
	RM346	7	5.5×10^{-3}				RM278	9	9.1×10^{-3}
							RM215	9	1.3×10^{-3}
							RM286	11	6.6×10^{-3}
							RM202	11	6.0×10^{-3}
							RM224	11	5.3×10^{-3}

		Gene	Chro.	p-value	Gene	Chro.	p-value	Gene	Chro.	p-value
Retrogradation										
	ΔHr	*AGPL3*	3	5.0×10^{-3}	*AGPL2*	1	8.8×10^{-3}	*AGPL3*	3	2.69×10^{-4}
		BEIIa	4	5.2×10^{-3}	RM87	5	1.02×10^{-2}	*Wx*	6	2.30×10^{-10}
		RM161	5	5.4×10^{-3}	*SSIIa*	6	2.22×10^{-16}	RM346	7	4.9×10^{-3}
		Wx	6	3.18×10^{-10}	*ISA1*	8	1.18×10^{-4}	*ISA1*	8	4.67×10^{-5}
		SSIIa	6	1.06×10^{-15}						
		RM346	7	4.33×10^{-4}						
		ISA1	8	2.03×10^{-4}						
	R%	*AGPL3*	3	4.2×10^{-3}	*AGPL2*	1	3.4×10^{-3}	*AGPL3*	3	2.31×10^{-4}
		BEIIa	4	2.5×10^{-3}	*BEIIa*	4	5.5×10^{-3}	*BEIIa*	4	5.1×10^{-3}
		Wx	6	7.28×10^{-12}	RM87	5	2.9×10^{-3}	*Wx*	6	2.25×10^{-12}
		SSIIa	6	1.32×10^{-15}	*SSIIa*	6	1.08×10^{-16}	RM346	7	3.2×10^{-3}
		RM346	7	2.25×10^{-4}	*ISA1*	8	7.18×10^{-5}	*ISA1*	8	3.59×10^{-5}
		ISA1	8	1.62×10^{-4}						

Chro.: chromosome.