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

Feng Yang[1#], Yaling Chen[2#], Chuan Tong[2], Yan Huang[2], Feifei Xu[2], Kehu Li[1], Harold Corke[1], Mei Sun[1*], Jinsong Bao[2*]

[1] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, P.R. China

[2] Institute of Nuclear Agricultural Sciences, Zhejiang University, Hua Jiachi Campus, Hangzhou, 310029, P. R. China;

[#]These authors contributed equally to this work.

*Corresponding author, E-mail: meisun@hku.hk; and jsbao@zju.edu.cn; Tel:+86-571-86971932; Fax: +86-571-86971421

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

thermal property, and retrogradation, might be controlled by one or a few genes with major effects (He et al. 1999; Bao et al. 2000; Bao et al. 2004a; Wan et al. 2004; Fan et al. 2005). Linkage mapping of the quantitative trait locus (QTL) for AAC and pasting viscosity (Bao et al. 1999; He et al. 1999; Bao et al. 2000, 2003; Larkin et al. 2003; Septiningsih et al. 2003; Aluko et al. 2004; Wan et al. 2004; Fan et al. 2005; Wang et al. 2007; Lapitan et al. 2009; Traore et al. 2011) shows that they are largely controlled by the *Wx* locus on chromosome 6. Since *GBSSI* is responsible for amylose synthesis, *GBSS1* alleles correlated with variation in AAC of rice grain is not surprising. Linkage mapping studies have identified only one major QTL, i.e. the alkali degeneration (*alk*) locus on chromosome 6, as having major responsibility for different gelatinization temperatures in diverse rice germplasm (He et al. 1999; Aluko et al. 2004; Bao et al. 2004b; Fan et al. 2005; Tian et al. 2005; Wang et al. 2007; Lapitan et al. 2009). Map-based cloning of the *alk* locus reveals that it encodes SSIIa, which is the major gene responsible for GT (Gao et al. 2003). QTL mapping shows that the amylopectin chain length distribution is also controlled by the *SSIIa* locus (Umemoto et al. 2002). The function of *SSIIa* is to elongate the short A and B1 chains with degree of polymerization (DP) < 10 to form long B1 chains of amylopectin (Nakamura et al. 2005). Although the functions of several genes in determining the rice eating and cooking qualities have been gradually clarified, such as *Wx* and *SSIIa*, many of others still remain unknown even though their functions in starch biosynthesis have been revealed.

In addition to *Wx* and *SSIIa*, the contribution of other genes to rice eating and cooking quality is derived from analysis of allele variations and their association with the quality parameters. Association analysis is a popular method to test the relationship between specific sequence polymorphisms in candidate genes and phenotypic variations (Thornsberry et al. 2001; Gupta et al. 2005). Molecular markers specific to a simple sequence repeat polymorphism with respect to (CT)n repeats and to a single nucleotide polymorphism of *Wx* gene have been successfully designed to distinguish 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) $\times$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

CAPS (dCAPS) follow the methods of Konieczny and Ausubel (1993) and Neff et al. (1998).

The primers for PCR were synthesized by the Shanghai Shenggong BioTech Co. Ltd. (Table 1). The PCR was carried out in a total volume of 20 μL containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM $MgCl_2$, 0.1 mM dNTPs, 200 nM primers, 1 unit of *Taq* polymerase, and 30 ng of genomic DNA. All amplifications were performed on a PTC-100 thermal cycler (MJ Research, Inc.) under the following conditions: 5 min at 95ºC; 35 cycles of 1 min at 95 ºC, 45 s at 55 ºC and 1 min at 72 ºC; and a final extension step at 72 ºC for 10 min.

Amplified PCR products were digested using suitable restriction endonucleases in a total volume of 20 μL according to the manufacturer's instructions (Table 1). The digests were resolved by electrophoresis in 1.5–2.0% agarose gel and visualized using a VersaDoc imaging system (Bio-Rad) after staining with ethidium bromide.

Statistical analysis

The polymorphism information content (PIC) values were determined using PowerMarker version 3.25 (Liu and Muse 2005). Nei's genetic distance (Nei et al. 1983) was calculated and used for unweighted pair-group method with arithmetic means (UPGMA) analysis as implemented in PowerMarker, with the UPGMA tree viewed using MEGA 4.0 (Tamura et al. 2007).

Analysis of variance (ANOVA) and principal component analysis were performed using the SAS System for Windows version 8 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was conducted for comparison of means at $P < 0.05$. Cluster analysis of the starch properties parameters were performed in SPSS Statistics

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

Association mapping

The population structure (Q) was determined using the STRUCTURE program (Pritchard et al. 2000), with 100 simple sequence repeat (SSR) markers (Jin et al. 2010), and the kinship coefficients (K) between accessions were estimated using the SPAGeDi program (v. 1.2g) (Hardy and Vekemans, 2002) with the same set of SSR markers (Shao et al. 2011). Association analysis between marker alleles and different starch physicochemical properties was performed with TASSEL Version 2.1 software, taking the gross level population structure (Q) and kinship (K) into account (Yu et al. 2006; Bradbury et al. 2007). The *P*-value determining whether a marker is associated with a trait was set at $P<0.01$.

Results

Marker development for starch biosynthesizing genes and their genotypes

In addition to available SNP, InDel, and SSR markers tagged for *Wx*, *SSI*, *BEI*, *SSIIa*, and all *AGP* genes (two small subunits and four large subunits) by Bao et al. (2002, 2006a, 2006b, 2012), we further developed CAPS, dCAPS and InDel markers for tagging other starch biosynthesis related genes, i.e. *GBSSII*, *SSIIc*, *SSIIb*, *SSIIIa*, *SSIIIb*, *SSIVa*, *SSIVb*, *BEIIa*, *BEIIb*, *ISA1-3* and *PUL* (Table 1) in this study. Most of SNPs tagged were derived from the study of Tian et al. (2009) who reported many functional SNPs for starch genes. All 13 new gene-specific markers (Table 1) produced two or more alleles each, with the polymorphic information content (PIC) ranging from 0.005 (InDel marker *SSIVb*) to 0.500 (marker *ISA2*). A deletion of 23bp in *SSIVb* was reported by Tian et al. (2009) in rice Suyunuo; we developed the primers to genotype this InDel in our rice accessions, and also found only Suyunuo had this deletion. Due to its low PIC, we adapted another CAPS marker from Tian et al. (2010) and Yan et al. (2011) to genotype this gene and found this marker had the PIC value of 0.381 among all rice accessions.

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

subpopulations. Similar results were also found for the thermal property parameters, i.e. POP 1 and POP2 had lower To, Tp, Tc and ΔHg than the others. POP5 had the highest mean $\Delta T_{1/2}$ and POP3 had the lowest mean $\Delta T_{1/2}$. For retrogradation property parameters, POP3 had the highest mean ΔHr and R%, whereas POP5 and POP7 had the lowest ΔHr and R% (Table 2).

QTLs for starch physicochemical properties

Association mapping of starch physicochemical property parameters were performed using the 35 SSRG markers and 108 other markers (mostly SSRs) based on the Q+K model. The identified QTLs are hereafter called main-effect QTLs. To uncover more QTLs concealed by the *Wx* and *SSIIa* loci for the starch physicochemical traits, further association mapping were conducted with *Wx* (G/A) and SSIIa (GC/TT) SNPs as covariate based on the same Q+K model. The QTLs identified are called QTLs in the *Wx* or *SSIIa* background. The results are summarized in Table 3 and Fig. 4.

AAC

A total of five main-effect QTLs were detected for AAC. *Wx* gene locus was detected as a major QTL (P=1.45×10^{-95}). *SSI* locus, RM122, RM346 and RM222 were also detected. Using *Wx* SNP (A/G) as a covariate, six QTLs were detected including four SBRGs, *GBSSII*, *BE1*, *SSIIc*, and *SSIIa*. Using *SSIIa* SNP (GC/TT) as a covariate, the same five main-effect QTLs were detected.

Pasting viscosity

As expected, the *Wx* locus was detected as a main-effect QTL for all the pasting viscosity parameters. SS1 was detected for SB and CS; *SSIIa* was detected for BD, CS,

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

Under the *Wx* locus background, no main-effect QTL was detected for PV, HPV and CPV. *SSIIa* was still detected with much smaller *P* value for BD ($P=3.72\times10^{-8}$), SBratio ($P=0.0062$) and Stability ($P=8.24\times10^{-8}$). No other SSRGs were detected for pasting viscosities. More SSRs were detected for BD (RM237, RM276, RM340, RM48 and RM507) and Stability (RM209, RM237, RM276, and RM507).

Under the *SSIIa* locus background, the QTLs identified for each trait were similar to those of main-effect QTLs; the only difference was that one less QTL (*SS1*) for CS and one more QTL (*GS3*) for SBratio were detected.

Gel texture

Wx was the common main-effect QTL for HD, ADH and COH, while *SSI* was detected as the main-effect QTL for HD and COH. PUL was detected for HD.

Under the *Wx* locus background, *GBSSII*, RM252 and RM3 were detected for HD, and only RM252 was detected for COH, while no QTL was detected for ADH.

Under the *SSIIa* locus background, the QTLs identified for each trait were the same as those of main-effect QTLs.

Thermal property and pasting temperature (PT)

For the thermal property and PT, *SSIIa* was a common main-effect QTL. Other SSRGs, *SSIIIb* (PT), *AGPL2* (ΔHg), and *Wx* ($\Delta T_{1/2}$) were also detected. In addition, some SSRs were detected for PT (RM276, RM253 and RM346), To (RM346), Tc (RM484), ΔHg (RM1 and RM48) and $\Delta T_{1/2}$ (RM161 and RM346).

Under the *Wx* locus background, all the QTLs identified for each trait were the same as those of main-effect QTLs except that one more QTL for Tc (RM484) and ΔHg (RM152), and one less (RM346) for $\Delta T_{1/2}$.

Under the *SSIIa* locus background, it was interesting to find that *Wx* locus was detected for Tc (*P*=0.0018) and ΔHg (*P*=0.0016). However, *Wx* was not detected for $\Delta T_{1/2}$, and instead, other SSRGs, such as *SSIIb* and *AGPL3* were detected for $\Delta T_{1/2}$. *AGPL2* was also detected for ΔHg. RM346 was still detected for PT, To and Tp, and RM1 and RM161 were still detected for ΔHg and $\Delta T_{1/2}$, respectively. More additional SSRs were identified for PT, To, Tp, Tc and $\Delta T_{1/2}$.

Retrogradation property

For the retrogradation property, five common SSRGs, *Wx*, *SSIIa*, *ISA1*, *AGPL3* and *BEIIa*, were identified as main-effect QTLs for both ΔHr and R%. RM346 and RM161 for ΔHr and RM346 for R% were also detected.

Under the *Wx* locus background, *ISA1* and *SSIIa* were still identified as QTLs for both ΔHr and R%. By contrast, *BEIIa* was only significant for R%. Another SSRG (*AGPL2*) and RM87 were detected for both traits.

Under the *SSIIa* locus background, the QTLs identified were similar to those of main-effect QTLs: *Wx*, *ISA1*, *AGPL3* and *RM346* were significant for both traits, but *BEIIa* was only detected for R%.

Discussion

Association mapping has become a robust technology for quickly identifying the genotype-phenotype relationships among diverse germplasms. The success of association mapping depends on the diversity of the germplasms being investigated, 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.

These diverse materials provide precious resources for further genetic studies and molecular analysis of the related genes.

Many studies have focused on the genetic basis of the starch physicochemical properties in relation to the cooking and eating quality of rice. AAC is mainly controlled by the *Wx* region on chromosome 6 (He et al. 1999; Tan et al. 1999; Bao et al. 2000; Lanceras et al. 2000; Septiningsih et al. 2003; Aluko et al. 2004). GT and thermal properties are genetically determined by a major QTL, i.e. the alkali degeneration (*alk*) locus on chromosome 6, also known as *SSIIa* (He et al. 1999; Aluko et al. 2004; Bao et al. 2004c; Fan et al. 2005; Tian et al. 2005; Wang et al. 2007). Genetic analysis with QTL mapping approach showed that the RVA parameters are mainly controlled by the *Wx* gene (Bao et al. 1999, 2000, 2003; Larkin and Park 2003; Wang et al. 2007; Traore et al. 2011). Gel texture parameters were also mainly controlled by the *Wx* locus (Bao et al. 2000; 2004a). The aforementioned results mostly came from linkage analyses. Association mapping for these traits have been conducted recently (Chen et al. 2008a, 2008b; Tian et al. 2009; Lu et al. 2012a, 2012b; Zhao et al. 2013). Similar results of main-effect QTLs have been reported, i.e. *Wx* and *SSIIa* controlled AAC-related traits and GT-related traits, respectively (Chen et al. 2008a, 2008b; Tian et al. 2009; Caffagni et al. 2013). However, due to different germplasms used, some of the studies have not identified the *Wx* for AAC (Lu et al. 2012a, 2012b; Zhao et al. 2013). Lu et al. (2012a, 2012b) detected *AGPS1*, *AGPL4* and *SSIIb* for AAC and pasting viscosity in their rice materials. Kharabian-Masouleh et al. (2012) reported that a SNP of *glucose-6-phosphate translocator* gene was highly associated with amylose content and retrogradation property. This study also found some new loci for starch physicochemical properties, such as *SSIIIb* for PT, *PUL* for HD, *AGPL2* for ΔHg; *BEIIa* and *AGPL3* for retrogradation properties (Table 3). The retrogradation property traits were comprehensively studied for the first time, and we identified five common SSRGs, *Wx*, *SSIIa*, *ISA1*, *AGPL3* and *BEIIa*, as main-effect 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

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.

Acknowledgement

This work is supported in part by the National High Technology Development Project of China (2006AA10Z193), Natural Science Foundation of China (30300227；30771327) and Zhejiang Province (LZ13C130001), and a Hong Kong Research Grants Council GRF award (7507/06M) and CRCG grants from the University of Hong Kong.

References

Aluko G, Martinez C, Tohme J, Castano C, Bergman C, Oard JH (2004) QTL mapping of grain quality traits from the interspecific cross *Oryza sativa* × *O. glaberrima*. Theor Appl Genet 109: 630-639.

Asante M, Offei SK, Gracen V, Adu-Dapaah H, Danquah EY, Bryant R, McClung AM (2013) Starch physicochemical properties of rice accessions and their association with molecular markers. Starch-Stärke 65: 1022-1028.

Ayres NM, McClung AM, Larkin PD, Bligh HFJ, Jones CA, Park WD (1997) Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. Theor Appl Genet 94: 773-781.

Bao JS, Xia YW (1999) Genetic control of the paste viscosity characteristics in indica rice (*Oryza sativa* L.). Theor Appl Genet 98: 1120-1124.

Bao JS, Zheng XW, Xia YW, He P, Shu QY, Lu X, Chen Y, Zhu LH (2000) QTL mapping for the paste viscosity characteristics in rice (*Oryza sativa* L.). Theor Appl Genet 100: 280-284.

Bao JS, Corke H, Sun M (2002) Microsatellites in starch-synthesizing genes in relation to starch physicochemical properties in waxy rice (*Oryza sativa* L.).

523 Theor Appl Genet 105: 898-905.
524 Bao JS, Harold C, He P, Zhu LH (2003) Analysis of quantitative trait loci for starch
525 properties of rice based on an RIL population. Acta Bot Sin 45: 986-994.
526 Bao JS, Sun M, Zhu LH, Corke H (2004a) Analysis of quantitative trait locus for
527 some starch properties in rice (*Oryza sativa* L.), thermal properties, gel texture,
528 swelling volume. J Cereal Sci 39: 379-385.
529 Bao JS, Kong XL, Xie JK, Xu LJ (2004b) Analysis of genotypic and environmental
530 effects on rice starch. 1. Apparent amylose content, pasting viscosity, and gel
531 texture. J Agric Food Chem 52: 6010-6016.
532 Bao JS, Corke H, Sun M (2006a) Microsatellites, single nucleotide polymorphisms
533 and a sequence tagged site in starch-synthesizing genes in relation to starch
534 physicochemical properties in nonwaxy rice (*Oryza sativa* L.). Theor Appl Genet
535 113: 1185-1196.
536 Bao JS, Corke H, Sun M (2006b) Nucleotide diversity in starch synthase IIa and
537 validation of single nucleotide polymorphisms in relation to starch gelatinization
538 temperature and other physicochemical properties in rice (*Oryza sativa* L.).
539 Theor Appl Genet 113: 1171-1183.
540 Bao JS, Shen SQ, Sun M, Corke H (2006c) Analysis of genotypic diversity in the
541 physicochemical properties of nonwaxy rice: apparent amylose content, pasting
542 viscosity and gel texture. Starch- Stärke 58: 259-267.
543 Bao JS, Sun M, Corke H (2007) Analysis of genotypic diversity in starch thermal and
544 retrogradation properties in nonwaxy rice. Carbohydr Polym 67: 174-181.
545 Bao JS, Lu Y, Yang F, Zhang G, Shao YF, Corke H, Sun M (2012) Nucleotide
546 polymorphisms in *OsAGP* genes and their possible association with grain weight
547 of rice. J Cereal Sci 55: 312-317.
548 Bao JS (2012) Towards understanding of the genetic and molecular basis of eating
549 and cooking quality of rice. Cereal Foods World 57: 148-156.
550 Bradbury P J, Zhang Z, Kroon D E, Casstevens T M, Ramdoss Y, Buckler E S (2007)
551 TASSEL: software for association mapping of complex traits in diverse samples.
552 Bioinformatics 23: 2633-2635.
553 Caffagni A, Albertazzi G, Gavina G, Ravaglia S, Gianinetti A, Pecchioni N, Milc J
554 (2013) Characterization of an Italian rice germplasm collection with genetic
555 markers useful for breeding to improve eating and cooking quality. Euphytica
556 194: 383-399.

557 Chen MH, Bergman C, Pinson S, Fjellstrom R (2008a) Waxy gene haplotypes:
558 associations with apparent amylose content and the effect by the environment in
559 an international rice germplasm collection. J Cereal Sci 47: 536-545.
560 Chen MH, Bergman C, Pinson S, Fjellstrom R (2008b) Waxy gene haplotypes:
561 associations with pasting properties in an international rice germplasm collection.
562 J Cereal Sci 48: 781-788.
563 Doyle J (1991) DNA protocols for plants CTAB total DNA isolation. In: Hewitt GM,
564 Johnston A (eds) Molecular Techniques in Taxonomy, 283-293. Springer, Berlin.
565 Fan CC, Yu XQ, Xing YZ, Xu CG, Luo LJ, Zhang Q (2005) The main effects,
566 epistatic effects and environmental interactions of QTLs on the cooking and
567 eating quality of rice in a doubled-haploid line population. Theor Appl Genet 110:
568 1445-1452.
569 Fujita N, Kubo A, Suh DS, Wong KS, Jane JL, Ozawa K, Takaiwa F, Inaba Y,
570 Nakamura Y (2003) Antisense inhibition of isoamylase alters the structure of
571 amylopectin and the physicochemical properties of starch in rice endosperm.
572 Plant Cell Physiol 44: 607-618.
573 Gao Z, Zeng D, Cui X, Zhou Y, Yan M, Huang D, Li J, Qian Q (2003) Map-based
574 cloning of the ALK gene, which controls gelatinization temperature of rice. Sci
575 China C Life Sci 46: 661-668.
576 Gao Z, Zeng D, Cheng F, Tian Z, Guo L, Su Y, Yan M, Jiang H, Dong G, Huang
577 Y, Han B, Li J, Qian Q (2011) ALK, the key gene for gelatinization temperature,
578 is a modifier gene for gel consistency in rice. J Integr Plant Biol 53: 756-765.
579 Gupta PK, Rustgi S, Kulwal PL (2005) Linkage disequilibrium and association
580 studies in higher plants: present status and future prospects. Plant Mol Biol 57:
581 461-485.
582 Hannah LC, James M (2008) The complexities of starch biosynthesis in cereal
583 endosperms. Curr Opin Biotechnol 19: 160-165.
584 Hardy OJ, Vekemans X (2002) SPAGeDi: a versatile computer program to analyse
585 spatial genetic structure at the individual or population levels. Mol Ecol Notes 2:
586 618–620.
587 He P, Li SG, Qian Q, Ma YQ, Li JZ, Wang WM, Chen Y, Zhu LH (1999) Genetic
588 analysis of rice grain quality. Theor Appl Genet 98: 502-508.
589 Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ, Emes MJ, Jame
590 MG, Myers AM (2008) Starch biosynthetic enzymes from developing maize

591 endosperm associate in multisubunit complexes. Plant Physiol 146: 1892-1908.
592 Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers
593 AM (2009) Proteins from multiple metabolic pathways associate with starch
594 biosynthetic enzymes in high molecular weight complexes: a model for
595 regulation of carbon allocation in maize amyloplasts. Plant Physiol 149:
596 1541-1559.
597 Hirose T, Terao T (2004) A comprehensive expression analysis of starch synthase
598 gene family in rice (*Oryza sativa* L.) Planta 220: 9-16.
599 Huang X, Wei X, Sang T, Zhao Q, Feng Q, Zhao Y, Li C, Zhu C, Lu T, Zhang Z, Li M,
600 Fan D, Guo Y, Wang A, Wang L, Deng L, Li W, Lu Y, Weng Q, Liu K, Huang T,
601 Zhou T, Jing Y, Li W, Lin Z, Buckler ES, Qian Q, Zhang QF, Li J, Han B (2010)
602 Genome-wide association studies of 14 agronomic traits in rice landraces. Nat
603 Genet 42: 961-967.
604 James MG, Denyer K, Myers AM (2003) Starch synthesis in the cereal endosperm.
605 Curr Opin Plant Biol 6: 215-222.
606 Jeon JS, Ryoo N, Hahn TR, Walia H, Nakamura Y (2010) Starch biosynthesis in
607 cereal endosperm 48: 383-392.
608 Jin L, Lu Y, Xiao P, Sun M, Corke H, Bao J (2010) Genetic diversity and population
609 structure of a diverse set of rice germplasm for association mapping. Theor Appl
610 Genet 121: 474-487.
611 Juliano BO, Villareal CP (1993) Grain quality evaluation of world rices. International
612 Rice Research Institute, Manila, the Philippines.
613 Juliano BO (1998) Varietal impact on rice quality. Cereal Foods World 43: 207-222.
614 Juliano BO, Perez C M, Resurreccion AP (2009) Apparent amylose content and
615 gelatinization temperature types of Philippine rice accessions in the IRRI gene
616 bank. Philipp Agric Sci 92: 106-109.
617 Kharabian-Masouleh A, Waters DL, Reinke RF, Ward R, Henry RJ (2012) SNP in
618 starch biosynthesis genes associated with nutritional and functional properties of
619 rice. Sci Rep 2:557.
620 Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations
621 using co-dominant ecotype-specific PCR-based markers. Plant J 4: 403-410.
622 Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y (1999) The
623 starch-debranching enzymes isoamylase and pullulanase are both involved in
624 amylopectin biosynthesis in rice endosperm. Plant Physiol 121: 399-410.

625 Lapitan VC, Redona ED, Abe T, and Brar DS (2009) Mapping of quantitative trait
626 loci using a double-haploid population from the cross of indica and japonica
627 cultivars of rice. Crop Sci. 49: 1620-1628.
628 Lanceras JC, Huang ZL, Naivikul O, Vanavichit A, Ruanjaichon V, Tragoonrung S
629 (2000) Mapping of genes for cooking and eating qualities in Thai jasmine rice
630 (KDML 105). DNA Res 7: 93-101.
631 Larkin PD, McClung AM, Ayres NM, Park WD (2003) The effect of the Waxy locus
632 (Granule bound starch synthase) on pasting curve characteristics in specialty rice
633 (*Oryza sativa* L.). Euphytica 131: 243-253.
634 Larkin PD, Park WD (2003) Association of waxy gene single nucleotide
635 polymorphisms with starch characteristics in rice (*Oryza sativa* L.). Mol Breed
636 12: 335-339.
637 Lee SK, Hwang SK, Han M, Eom JS, Kang HG, Han Y, Choi SB, Cho MH, Bhoo SH,
638 An G, Hahn TR, Okita TW, Jeon JS (2007) Identification of the ADP-glucose
639 pyrophosphorylase isoforms essential for starch synthesis in the leaf and seed
640 endosperm of rice (*Oryza sativa* L.). Plant Mol Biol 65: 531-546.
641 Liu F, Makhmoudova A, Lee E A, Wait R, Emes M J, and Tetlow I J (2009) The
642 amylose extender mutant of maize conditions novel protein– protein interactions
643 between starch biosynthetic enzymes in amyloplasts. J Exp Bot 60: 4423-4440.
644 Lu FH, Park YJ (2012a) An SNP downstream of the OsBEIIb gene is significantly
645 associated with amylose content and viscosity properties in rice (*Oryza sativa* L.).
646 J Cereal Sci 56: 706-712.
647 Lu FH, Park YJ (2012b) Sequence variations in OsAGPase significantly associated
648 with amylose content and viscosity properties in rice (*Oryza sativa* L.). Genet
649 Res (Camb) 94: 179-189.
650 Liu K, Muse SV (2005) PowerMarker: an integrated analysis environment for genetic
651 marker analysis. Bioinformatics 21: 2128-2129.
652 Nakamura Y (1996) Some properties of starch debranching enzymes and their
653 possible role in amylopectin biosynthesis. Plant Sci 121: 1-18.
654 Nakamura Y (2002) Towards a better understanding of the metabolic system for
655 amylopectin biosynthesis in plants: Rice endosperm as a model tissue. Plant Cell
656 Physiol 43: 718-725.
657 Nakamura Y, Francisco Jr PB, Hosaka Y, Sato A, Sawada T, Kubo A, Fujita N (2005)
658 Essential amino acids of starch synthase IIa differentiate amylopectin structure

659 and starch quality between japonica and indica rice varieties. Plant Mol Biol 58:
660 213-227.
661 Nei M, Tajima F, and Tateno Y (1983) Accuracy of estimated phylogenetic trees from
662 molecular data. J Mol Evol 19: 153-170.
663 Neff MM, Neff JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the
664 genetic analysis of single nucleotide polymorphisms: experimental applications
665 in Arabidopsis thaliana genetics. Plant J 14: 387-392.
666 Ohdan T, Francisco PB Jr, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y (2005)
667 Expression profiling of genes involved in starch synthesis in sink and source
668 organs of rice. J Exp Bot 56: 3229-3244.
669 Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using
670 multilocus genotype data. Genetics 155: 945-959.
671 Shao Y, Jin L, Zhang G, Lu Y, Shen Y, Bao J (2011) Association mapping of grain
672 color, phenolic content, flavonoid content and antioxidant capacity in dehulled
673 rice. Theor Appl Genet 122: 1005-1016.
674 Septiningsih EM, Trijatmiko KR, Moeljopawiro S, McCouch SR (2003) Identification
675 of quantitative trait loci for grain quality in an advanced backcross population
676 derived from the *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*.
677 Theor Appl Genet. 107: 1433-1441.
678 Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary
679 Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.
680 Tan YF, Li JX, Yu SB, Xing YZ, Xu CG, Zhang QF (1999) The three important traits
681 for cooking and eating quality of rice grains are controlled by a single locus in an
682 elite rice hybrid, Shanyou 63. 99: 642-648.
683 Teng B, Zhang Y, Wu J, Cong X, Wang R, Han Y, Luo Z (2013) Association between
684 allelic variation at the waxy locus and starch physicochemical properties using
685 single-segment substitution lines in rice (*Oryza sativa* L.). Starch-Stärke 65:
686 1069-1077.
687 Tetlow IJ, Morell MK, Emes MJ (2004) Recent developments in understanding the
688 regulation of starch metabolism in higher plants. J Exp Bot 55: 2131-2145.
689 Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS, Wait R,
690 Morell MK, Emes MJ (2008) Analysis of protein complexes in wheat
691 amyloplasts reveals functional interactions among starch biosynthetic enzymes.
692 Plant Physiol 146: 1878-1891.

693 Tetlow IJ (2011) Starch biosynthesis in developing seeds. Seed Sci Res 21: 5-32.
694 Thornberry JM, Goodman MM, Doebley J, Kresovich S, Nielsen D, Buckler ES
695 (2001) Dwarf8 polymorphisms associate with variation in flowering time. Nature
696 Genet 28: 286-289.
697 Tian R, Jiang GH, Shen LH, Wang LQ, He YQ (2005) Mapping quantitative trait loci
698 underlying the cooking and eating quality of rice using a DH population. Mol
699 Breed 15: 117-124.
700 Tian Z, Quian Q, Liu Q, Yan M, Liu X, Yan C, Liu G, Gao Z, Tang S, Zeng D, Wang Y,
701 Yu J, Gu M, Li J (2009) Allelic diversities in rice starch biosynthesis lead to a
702 diverse array of rice eating and cooking qualities. Proc Natl Acad Sci USA 106:
703 21760-21765.
704 Tian Z, Yan C, Qian Q, Yan S, Xie H, Wang F, Xu J, Liu G, Wang Y, Liu Q, Tang S, Li
705 J, Gu MH (2010) Development of gene-tagged molecular markers for starch
706 synthesis-related genes in rice. Chin Sci Bull 55: 3768-3777.
707 Traore K, McClung AM, Chen MH, Fjellstrom R (2011) Inheritance of flour paste
708 viscosity is associated with a rice Waxy gene exon 10 SNP marker. J Cereal Sci
709 53: 37-44.
710 Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y (2002) Mapping of a gene
711 responsible for the difference in amylopectin structure between japonica-type
712 and indica-type rice varieties. Theor Appl Genet 104: 1-8.
713 Wan XY, Wan JM, Su CC, Wang CM, Shen WB, Li JM, Wang HL, Jiang L, Liu SJ,
714 Chen LM, Yasui H, Yoshimura A (2004) QTL detection for eating quality of
715 cooked rice in a population of chromosome segment substitution lines. Theor
716 Appl Genet 110: 71-79.
717 Wang LQ, Liu WJ, Xu Y, He YQ, Luo LJ, Xing YZ, Xu CG, Zhang QF (2007)
718 Genetic basis of 17 traits and viscosity parameters characterizing the eating and
719 cooking quality of rice grain. Theor Appl Genet 115: 463-476.
720 Wong KS, Kubo A, Jane JL, Harada K, Satoh H, Nakamura Y (2003) Structures and
721 properties of amylopectin and phytoglycogen in the endosperm of sugary-1
722 mutants of rice. J Cereal Sci 37(2):139-149.
723 Xu F, Zhang G, Tong C, Sun X, Corke H, Sun M, Bao J S (2013) Association mapping
724 of starch physicochemical properties with starch biosynthesizing genes in waxy
725 rice (*Oryza sativa* L.). J Agric Food Chem 61: 10110-10117.
726 Yan CJ, Tian ZX, Fang YW, Yang YC, Li J, Zeng SY, Gu SL, Xu CW, Tang SZ, Gu

MH (2011) Genetic analysis of starch paste viscosity parameters in glutinous rice (*Oryza sativa* L.). Theor Appl Genet 122:63-76.

Yu J, Pressoir G, Briggs WH, Bi IV, Yamasaki M, Doebley JF, McMullen MD, Gaut BS, Nielsen DM, Holland JB, Kresovich S, Buckler ES (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat Genet 38: 203-208.

Zhang G, Cheng Z, Zhang X, Guo X, Su N, Jiang L, Mao L, Wan J (2011) Double repression of soluble starch synthase genes SSIIa and SSIIIa in rice (*O. sativa* L.) uncovers interactive effects on physicochemical properties of starch. Genome 54: 448–459.

Zhao K, Tung CW, Eizenga GC, Wright MH, Ali ML, Price AH, Norton GJ, Islam MR, Reynolds A, Mezey J, McClung AM, Bustamante CD, McCouch SR (2011) Genome-wide association mapping reveals a rich genetic architecture of complex traits in *Oryza sativa*. Nat Commun 2: 467.

Zhao WG, Chung JW, Kwon SW, Lee JH, Ma KH, Park YJ (2013) Association analysis of physicochemical traits on eating quality in rice (*Oryza sativa* L.). Euphytica 191: 9-21.

1. Legends for Figures

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

Fig. 2 Dendrogram generated with the starch properties parameters using Ward's method based on squared Euclidean distance

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

Fig. 4 Plots of main-effect QTLs (**A**), QTLs identified in the *Wx* background (**B**) and *SSII*a background (**C**) for 20 starch physicochemical properties. The SSRGs with the largest –log(*P*) values were highlighted with red (*Wx*), blue (*SSIIa*), green (*ISA1*) and pink (*SSIIb*) colors

Figure
Click here to download Figure: Figures.doc

Fig 1

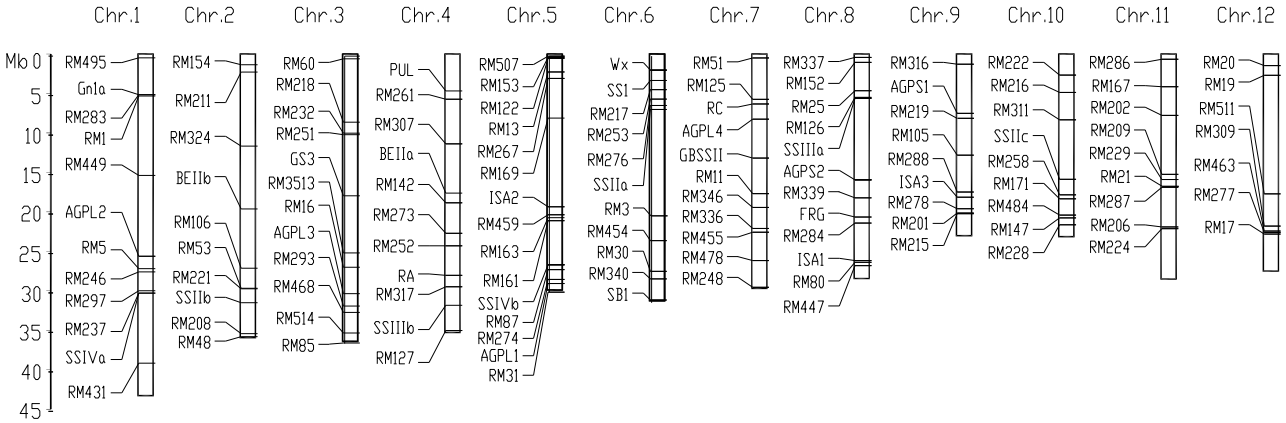


Fig 2

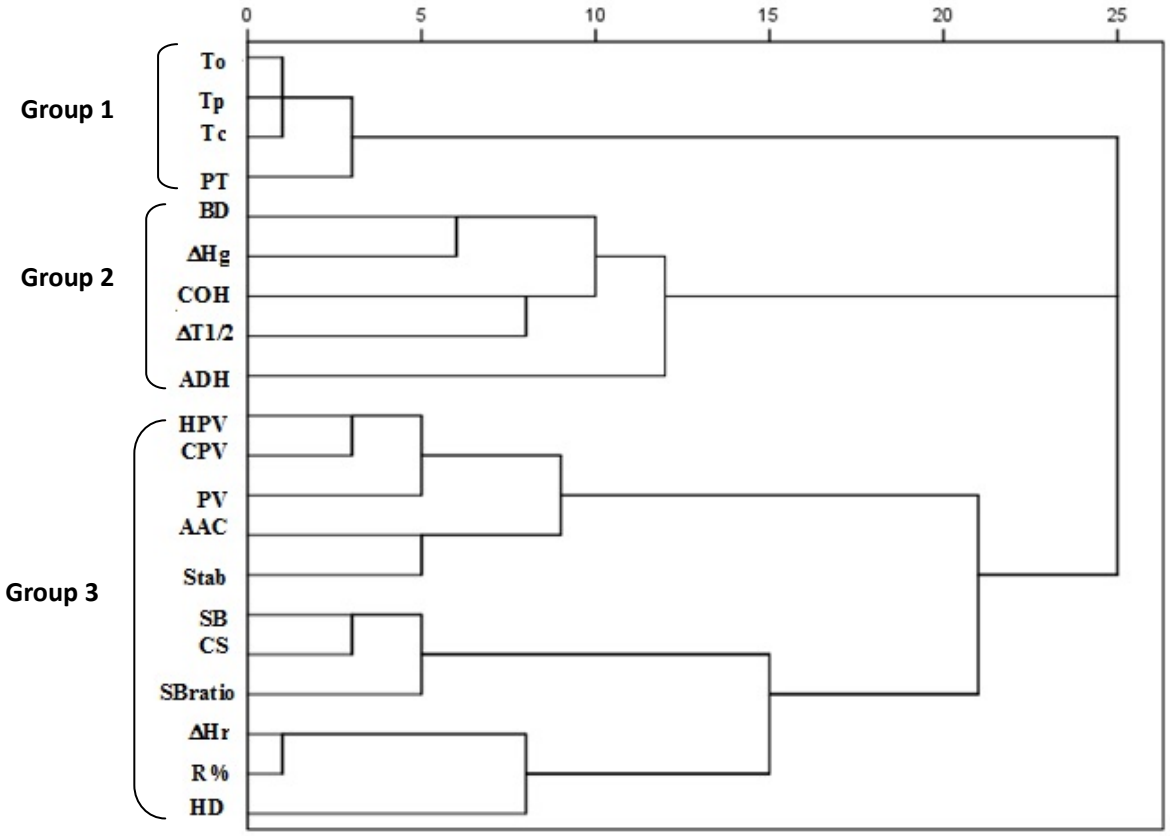

0
5
10
15
20
25
Group 1
To
Tp
Tc
PT
Group 2
BD
ΔHg
COH
ΔT1/2
ADH
Group 3
HPV
CPV
PV
AAC
Stab
SB
CS
SBratio
ΔHr
R%
HD

Fig 3

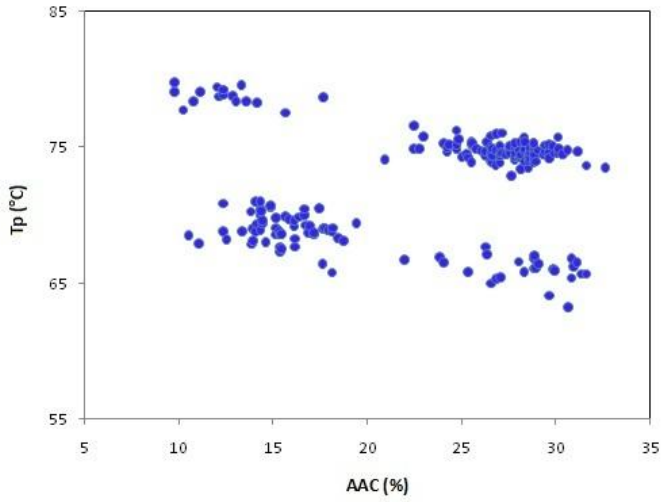
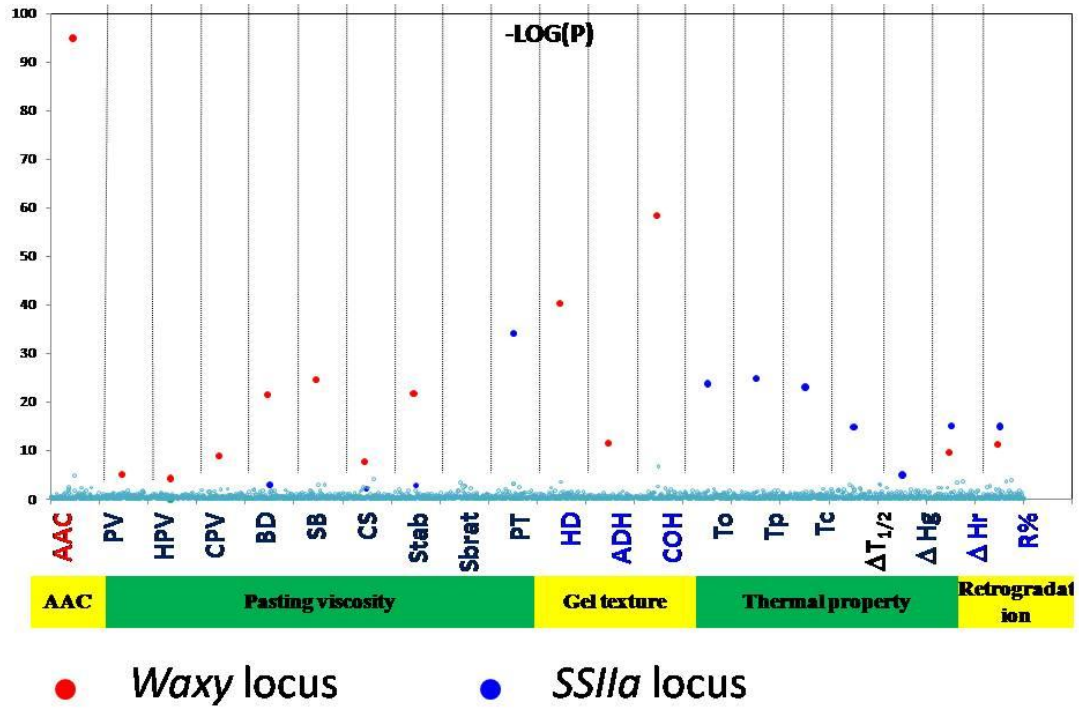
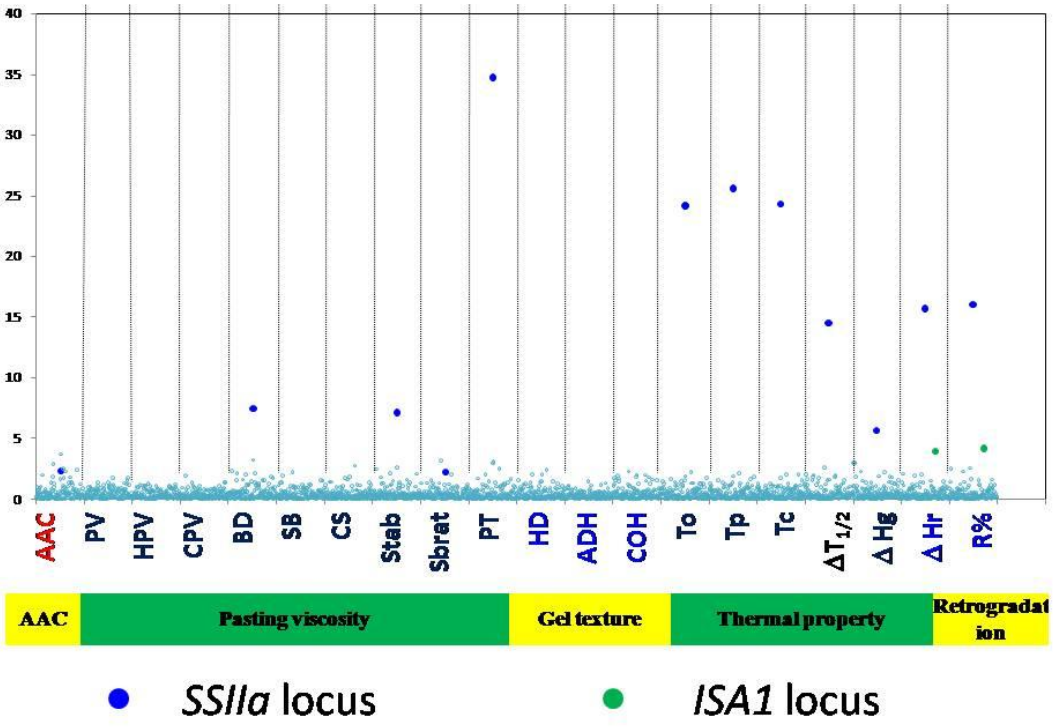


Fig 4.

A


-LOG(P)
100
90
80
70
60
50
40
30
20
10
0
AAC
PV
HPV
CPV
BD
SB
CS
Stab
Sbrat
PT
HD
ADH
COH
To
Tp
Tc
ΔT1/2
Δ Hg
Δ Hr
R%
AAC
Pasting viscosity
Gel texture
Thermal property
Retrogradation
Waxy locus
SSIIa locus

B


40
35
30
25
20
15
10
5
0
AAC
PV
HPV
CPV
BD
SB
CS
Stab
Sbrat
PT
HD
ADH
COH
To
Tp
Tc
ΔT1/2
Δ Hg
Δ Hr
R%
AAC
Pasting viscosity
Gel texture
Thermal property
Retrogradation
SSIIa locus
ISA1 locus

C

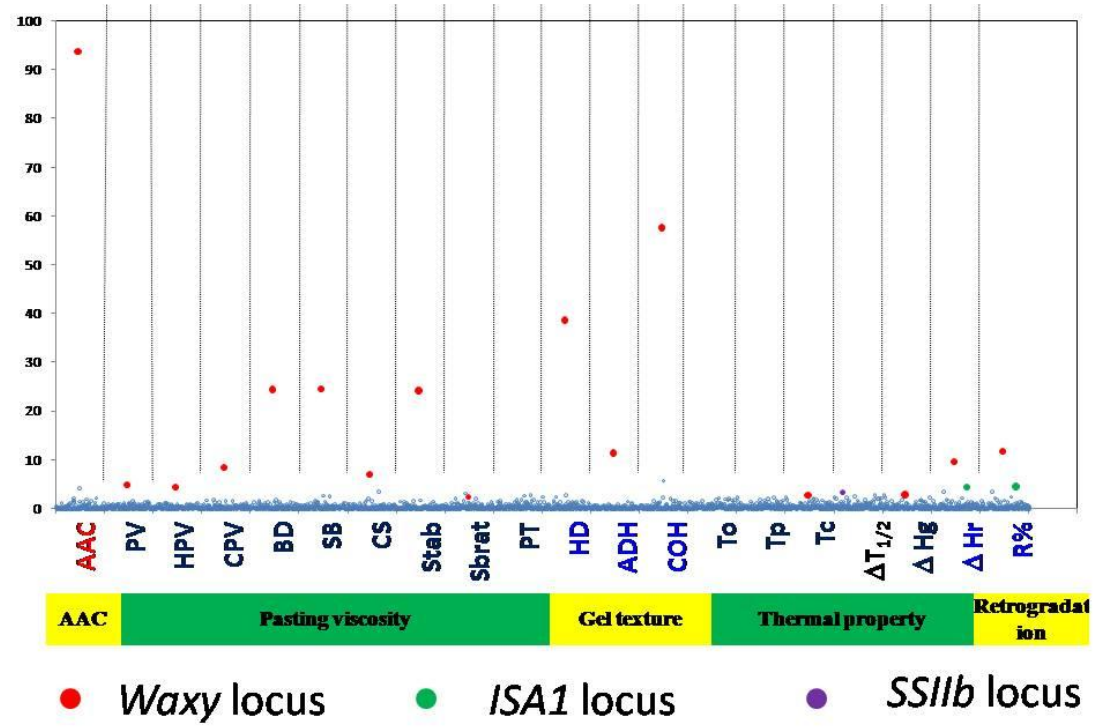

100
90
80
70
60
50
40
30
20
10
0
AAC
PV
HPV
CPV
BD
SB
CS
Stab
Sbrat
PT
HD
ADH
COH
To
Tp
Tc
ΔT1/2
Δ Hg
Δ Hr
R%
AAC
Pasting viscosity
Gel texture
Thermal property
Retrogradation
Waxy locus
ISA1 locus
SSIIb locus

Table
Click here to download Table: Tables.docx

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	AclI	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	ScaI	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.

		Main effect loci			*Wx* covariate			*SSIIa* covariate		
	Trait	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}

	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}

	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}

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.