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Improving rice eating and cooking quality by coordinated expression of the major starch synthesis-related genes, SSII and Wx, in endosperm

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Abstract

Key Message **Coordinated regulation of amylose and amylopectin synthesis via manipulation of *SSII-2*, *SSII-3* and *Wx* expression in endosperm can improve rice eating and cooking quality.**

Abstract With increasing rice consumption worldwide, many researchers are working to increase the yield and improve grain quality, especially eating and cooking quality (ECQ). The rice ECQ is mainly controlled by the expression of starch synthesis-related genes (SSRGs) in endosperm. Although the *Wx* and *SSII-3/SSIIa/ALK* genes, two major SSRGs, have been manipulated to improve rice ECQ via various breeding approaches, new methods to further improve ECQ are desired. In our previous study, we enhanced rice ECQ by knocking down *SSII-2* expression in the *japonica* Nipponbare cultivar (carrying the Wx^b allele) via RNA interference. Herein, the *SSII-2* RNAi was introduced into two Nipponbare-derived near-isogenic lines (NILs), Nip(Wx^a) and Nip(*wx*), carrying Wx^a and *wx* alleles respond for high and no amylose levels, respectively. Analysis of physicochemical properties revealed that the improved grain quality of *SSII-2* RNAi transgenic lines was achieved by coordinated downregulating the expression of *SSII-2*, *SSII-3* and *Wx*. To further confirm this conclusion, we generated *ssii-2*, *ssii-3* and *ssii-2ssii-3* mutants via CRISPR/Cas9 technique. The amylopectin structure of the resulting *ssii-2sii-3* mutants was similar to that in *SSII-2* RNAi transgenic lines, and the absence of *SSII-2* decreased the amylose content, gelatinisation temperature and rapid visco-analyser profile, indicating essential roles for *SSII-2* in the regulation of amylopectin biosynthesis and amylose content in rice endosperm. The effect of *SSII-2* was seen only when the activity of *SSII-3* was very low or lacking. Our study provides novel approaches and valuable germplasm resources for improving ECQ via plant breeding.

Keywords *Oryza sativa* L. · Eating and cooking quality · Amylose content · *SSII* · *Wx* · CRISPR/Cas9

Introduction

Rice is a major staple food worldwide, and its yield has been greatly increased over recent decades, but grain quality still requires improving. Rice grain quality includes eating and cooking quality (ECQ) (Huang et al. 2020a), nutrition quality (Yang et al. 2019; Zhu et al. 2018), grain appearance (Zhao et al. 2018) and milling quality (Wang et al. 2016). Among these, ECQ is of foremost consideration for both consumers and rice breeders. Starch, accounts for ~90 % of the edible part of milled rice, and is considered the primary determinant of rice ECQ because it influences the palatability, appearance, hardness, stickiness and digestibility of cooked rice. Starch is a semi-crystalline particle consisting of two glucose polymers, linear amylose and highly branched amylopectin. Both the composition and fine structure of starch determine its physicochemical properties, and

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hence the eventual rice ECQ. Previous studies demonstrated that rice cultivars with low-to-intermediate amylose content (AC) tend to show better palatability, stickiness and hardness than those of both glutinous and high-AC rice types (Huang et al. 2020b; Tian et al. 2009; Zhang et al. 2019a; Zhou et al. 2020). In addition, an increase in the ratio of amylopectin A chains (Ap-A) with degree of polymerization (DP) 6–12 to B1 chains (Ap-B1) with DP 13–24 may reduce gelatinisation temperature (GT) and hardness, and improve the stickiness of cooked rice, resulting in better texture and cooking properties (Li et al. 2016, 2020; Zhu 2018). Therefore, a moderate reduction in AC and an increase in ratio of amylopectin A chains to B1 chains are keys to improving rice ECQ.

The physicochemical properties of starch, including the apparent amylose content (AAC), gel consistency (GC), gelatinisation temperature (GT) and rapid visco-analyser (RVA) score, have been utilised to evaluate rice ECQ (Tan et al. 1999; Tian et al. 2009). Starch synthesis-related genes (SSRGs), including genes encoding ADP glucose pyrophosphorylase (AGPase), soluble starch synthase (SS), granule-bound starch synthase (GBSS), starch branching enzyme (SBE) and starch debranching enzyme (DBE), function cooperatively to determine these traits (Jeon et al. 2010). The *Waxy* (*Wx*) gene encoding granule-bound starch synthase I (GBSSI) is the major determinant of AC and GC, and a minor contributor to GT (Sano 1984; Su et al. 2011; Tian et al. 2009; Wang et al. 1990; Zhang et al. 2019a). *SSII-3* (also known as *ALK* and *SSIIa*) is a major gene regulating GT, and it also contributes to AC and GC (Tian et al. 2009; Umemoto et al. 2002). *Wx* and *SSII-3* genes are closely located on chromosome 6, hence they have been co-selected during rice domestication (Tian et al. 2009).

To increase the proportion of amylopectin short chains with DP 6–12, *SSII* genes encoding soluble starch synthase II (SSII), which elongates amylopectin intermediate chains with DP 13–24 by extending amylopectin short chains with DP 6–12, have been knocked down with some success (Chen et al. 2020; Commuri and Keeling 2001; Fujita et al. 2006; Morell et al. 2003). There are three SSII isoforms in rice; SSII-1 (SSIIc), SSII-2 (SSIIb) and SSII-3 (SSIIa). The amino acid sequence identity among the three isoforms is greater than 50 %, and they share the consensus motif KXGGL, which is believed to form part of the ADPG binding site of SSII (Furukawa et al. 1990; Jiang et al. 2004; Ohdan et al. 2005b). These three *SSII* genes in rice are thought to result from two replications of large genomic fragments; the first replication produced two *SSII* genes, one of which differentiated into *SSII-1*, and the other subsequently produced *SSII-2* and *SSII-3* in a second replication (Jiang et al. 2004). The gene organisation near the *SSII-2* and *SSII-3* loci is similar; both contain the PLAGXNVMNX motif that is conserved in many starchy crops (Harn et al. 1998; Li et al. 1999). Studies showed that the *ssii-3* mutant contained more short amylopectin A chains (DP 6−12) and fewer medium-length amylopectin B1 chains (DP 13−24), leading to beneficial starch properties for improving rice ECQ (Gao et al. 2003; Miura et al. 2018; Umemoto et al. 2002). Key amino acid variations in SSII-3 can affect its enzyme activity and cause GT differences among rice cultivars, and at least three *SSII-3* alleles have been reported (Bao et al. 2006; Chen et al. 2020; Nakamura et al. 2005; Umemoto et al. 2002). The activity of *japonica* types SSIIa^{j} is only ~ 10 % that of *indica* type SSIIa^{i}/*ALK*^{c}, resulting in a lower GT (Chen et al. 2020; Nakamura et al. 2005). The recent study showed that the *SSIIa*^{j} gene from *japonica* cultivars could be further divided into two alleles *ALK*^{a} and *ALK*^{b}, and the GT of *ALK*^{b} is lower than that of *ALK*^{a} (Chen et al. 2020). The *ssiia*^{j}-deficient mutant displayed a lower GT than those of both *ALK*^{a} and *ALK*^{b} (Chen et al. 2020; Miura et al. 2018). In addition to *SSII-3*, the other two *SSII* genes, especially *SSII-2*, also have potential as targets for regulating the chain-length distribution (CLD) of amylopectin in rice endosperm (Li et al. 2018).

AC is the chief determinant of rice ECQ (Tian et al. 2009). Natural allelic variation in *Wx*, including *Wx*^{lv}, *Wx*^{a}, *Wx*^{in}, *Wx*^{b}, *Wx*^{mw}, *Wx*^{mp}, *Wx*^{mq}, *Wx*$^{op/hp}$ and *wx*, has led to a wide variety of AC values, ranging from 30 to 0 % in different rice cultivars (Cai et al. 1998; Liu et al. 2009; Mikami et al. 1999, 2008; Sato et al. 2002; Zhang et al. 2019a). *Wx*^{a} (AC ~ 25 %) and *Wx*^{b} (AC 15−18 %) are the two major *Wx* alleles in *indica* and *japonica* rice cultivars, respectively. The *wx* allele is a null *Wx* type present in glutinous rice (Cai et al. 1998; Hirano et al. 1998). Recent studies showed that the *Wx* alleles have undergone a high-to-low AC selection trend during rice domestication (Anacleto et al. 2019; Zhang et al. 2019a). Application of the elite *Wx* alleles, particularly *Wx*^{in}, *Wx*^{b} and *Wx*^{mp} corresponding to intermediate-to-low AC, has been the major method employed to improve rice ECQ in modern rice breeding programs (Phing Lau et al. 2016). For example, introgression of *Wx*^{mp} or *Wx*^{mq} alleles (AC 8−12 %) into commercial rice cultivars can be used to breed elite 'soft rice' with high ECQ and soft grains after cooking (Zhang et al. 2019b).

RNA interference (RNAi) is a powerful tool for knocking down target gene expression by inducing the degradation of corresponding mRNAs (Fire et al. 1998). More recently, CRISPR/Cas9 has been used to knockout target genes efficiently and specifically (Miao et al. 2013; Zhang et al. 2014). Gene knockout is typically achieved by frame-shift mutation via CRISPR/Cas9, and the inserted transfer DNA (T-DNA) can be removed by separation, hence this simple approach has been widely adopted in modern molecular plant breeding programs (Chen et al. 2019; Donohoue et al. 2018).

In our previous studies, we suppressed the expression of *SSII-2*, a neglected SSRG, in *japonica* rice cultivar Nipponbare using RNAi, which markedly reduced AC and GT,

and increased GC, thereby significantly improving rice ECQ (Li et al. 2018; Xu et al. 2020b). *SSII-2* RNAi transgenic rice exhibited similar ECQ as traditional soft rice, but the appearance was much better than that of soft rice. Thus, this represents a promising approach for generating novel elite soft rice. To confirm this notion and further evaluate its potential for cooperatively improving rice quality using various *Wx* alleles, we herein introduced the *SSII-2* RNAi construct into Wx^a and *wx* near-isogenic lines (NILs) derived from Nipponbare (Wx^b). In addition, we further created *ssii-2* and/or *ssii-3* single and double mutants using CRISPR/Cas9 to explore the underlying mechanism by which *SSII-2* influences the regulation of rice grain quality, and to provide novel germplasm resources for breeding high-ECQ rice.

Materials and methods

Plant materials

The *japonica* rice cultivar Nipponbare (Nip) and its derived NILs, transgenic lines and mutants were used in this study (Table 1). Nipponbare itself has the Wx^b allele with a relate low AC (~ 16 %), herein named Nip(Wx^b) or wild-type (WT). The two NILs with Wx^a and *wx* alleles in the Nipponbare background are called Nip(Wx^a) and Nip(*wx*), respectively, corresponding to high and zero AC (Zhang et al. 2019a). Nip(Wx^a) (BC_8F_7) and Nip(*wx*) (BC_9F_8) were generated after at least eight rounds of backcrossing with the recurrent Nip and their genetic backgrounds were genotyped by whole-genome sequencing (Zhang et al. 2019a). The Wx^a allele is from the *indica* cultivar Longtefu, while the *wx* allele is from the *japonica* waxy cultivar Suyunuo. Nip(Wx^b)_Ri-1 and Nip(Wx^b)_Ri-2 are two representative *SSII-2* RNAi transgenic lines in the Nipponbare background, corresponding to *SSSII-2 RNAi* Line 3 and *SSSII-2 RNAi* Line 4 in our previous study, respectively (Li et al. 2018). *SSII-2* RNAi was introduced into the NILs Nip(Wx^a) and Nip(*wx*) by crossing, and four homozygous lines, Nip(Wx^a)_Ri-1, Nip(Wx^a)_Ri-2, Nip(*wx*)_Ri-1 and Nip(*wx*)_Ri-2, were selected in their selfing progenies by genotyping (Table 1).

Nipponbare was also used to generate the related *ssii-2* and *ssii-3* mutants via CRISPR/Cas9 (Table 1). All the above rice lines were grown under the same climatic and management conditions during the summer in a paddy field at Yangzhou University, Yangzhou, China (32°23′N). Each line was grown in triplicate and planted randomly in each plot.

Construction of CRISPR/Cas9 vectors and screening of homozygous mutants

The CRISPR/Cas9 system used in this study included two vectors, intermediate vector *SK-gRNA* and the CRISPR-Cas9 binary vector *pC1300-Cas9* (Wang et al. 2015). The target sites in *SSII-2* and *SSII-3* genes (Fig. S1A, B) were designed via the CRISPR-GE (http://skl.scau.edu.cn/) online toolkit and individually cloned into *SK-gRNA* (Xie et al. 2017). Genomic DNA (gRNA) fragments containing the target sites were then ligated into *pC1300-Cas9* to obtain the *SSII-2* or *SSII-3* single-knockout vectors and the *SSII-2SSII-3* double-knockout vector via the isocaudamers-based system (Fig.

Table 1 Genotypes of rice lines used in this study

Lines	*Wx* allele	*ALK/SSII-3 allele*	*SSII-2* RNAi structure	Cas9 edited	Genotype of *SSII-2* and *SSII-3*
Nip(Wx^a)	Wx^a	ALK^b	−	−	*SSII-2SSII-2/SSII-3SSII-3*
Nip(Wx^a)_Ri-1	Wx^a	ALK^b	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
Nip(Wx^a)_Ri-2	Wx^a	ALK^b	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
Nip(Wx^b)	Wx^b	ALK^a	−	−	*SSII-2SSII-2/SSII-3SSII-3*
Nip(Wx^b)_Ri-1	Wx^b	ALK^a	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
Nip(Wx^b)_Ri-2	Wx^b	ALK^a	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
Nip(*wx*)	*wx*	ALK^a	−	−	*SSII-2SSII-2/SSII-3SSII-3*
Nip(*wx*)_Ri-1	*wx*	ALK^a	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
Nip(*wx*)_Ri-2	*wx*	ALK^a	+	−	$ssii\text{-}2^Dssii\text{-}2^D$/*SSII-3SSII-3*
s2-1	Wx^b	ALK^a	−	+	*ssii-2ssii-2/SSII-3SSII-3*
s2-2	Wx^b	ALK^a	−	+	*ssii-2ssii-2/SSII-3SSII-3*
s3-1	Wx^b	alk^a	−	+	*SSII-2SSII-2/ssii-3ssii-3*
s3-2	Wx^b	alk^a	−	+	*SSII-2SSII-2/ssii-3ssii-3*
s23-1	Wx^b	alk^a	−	+	*ssii-2ssii-2/ssii-3ssii-3*
s23-2	Wx^b	alk^a	−	+	*ssii-2ssii-2/ssii-3ssii-3*

^{D}denotes downregulation by RNA interference

S1C, D). Confirmed constructs were introduced into Nipponbare by *Agrobacterium*-mediated transformation. The primers used are listed in Table S1.

The target DNA region of *SSII-2* and *SSII-3* were verified by sequencing, and the sequencing data were analysed by DSDecodeM (http://skl.scau.edu.cn/dsdecode/) or manual decoding (Liu et al. 2015). The homozygous *ssii-2* and/or *ssii-3* mutants without the T-DNA insertion were screened and selected for following analysis. Six homozygous mutated lines, two from each construction, were selected for further experimental analysis, and named *s2*-1, *s2*-2, *s3*-1, *s3*-2, *s23*-1 and *s23*-2 (Table 1), where *s2* and *s3* are single null *ssii-2* or *ssii-3* mutants, while *s23* indicates double mutants with both *ssii-2* and *ssii-3* mutations.

Total RNA extraction and quantitative real-time PCR (qRT-PCR) expression analysis

Total RNA was extracted from rice caryopses (with glumes removed) 10 days after flowering (DAF) using an RNAplant Plus Reagent kit (Tiangen, Beijing, China). First-strand cDNA was synthesised using a PrimeScript RT reagent kit (Takara, Kusatsu, Japan), and qRT-PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad) using AceQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). The *Actin01* gene was used for normalisation, and each experiment included three biological replicates. The primers used are listed in Table S1, and were tested in our previous study (Fan et al. 2019).

Western blotting

Rice flour from mature grains was weighted and suspended in total protein extraction buffer comprising 125 mM Tris-HCl pH 6.8, 4 M urea, 4 % sodium dodecyl sulphate (SDS) and 5 % 2-mercaptoethanol at 37 °C at a ratio of 1:15 (1 mg flour to 15 µL buffer). The buffer volume for glutinous rice was doubled due to its high stickiness. The extracted total proteins were mixed with SDS loading buffer (4×), denatured at 99 °C for 10 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The antibody specifically recognizing GBSSI (Clontech) was used for examining the accumulation of GBSSI, and the acid subunit of glutelin was set as loading control.

Measuring rice grain physicochemical properties

Air-dried mature seeds were dehusked using a SY88-TH instrument (Sangyong, Korea), polished (Pearlest, Kett, Japan), ground into powder using a FOSS 1093 Cyclotec Sample Mill (Sweden), and passed through a 100-mesh screen (pore size 0.15 mm). The starch content of the milled rice flour was analysed using a K-TSTA total starch assay kit (Megazyme, Ireland). The crude protein content was calculated from the nitrogen content of the rice flour using a FOSS TECATOR Kjeltec2300 instrument (according to AOAC standard method 990.03) (Zhang et al. 2013). AAC, GC, GT and RVA were determined according to our previously reported method (Zhang et al. 2016). In brief, AAC was calculated using an iodine staining method, GC was determined by measuring the length of rice gel after gelatinisation and cooling, and GT was measured using a DSC 200 F3 differential scanning calorimeter (Netzsch, Germany). The obtained DSC curves included the temperature of onset (To), peak (Tp) and end (Te), and the enthalpy of gelatinisation (ΔH). RVA values were measured using a Techmaster RVA instrument (Newport Scientific, Warriewood, Australia). The primary RVA parameters include peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV), setback viscosity (SBV), breakdown viscosity (BDV), peak time (PeT) and pasting temperature (PaT). All tests were performed in duplicate or triplicate.

Gel permeation chromatography (GPC) and fluorophore-assisted carbohydrate electrophoresis (FACE) analyses

Starch was extracted from milled rice endosperm using the neutral protease method (Wang and Wang 2001) with slight modifications. In brief, purified rice starch was debranched with isoamylase (EC3.2.1.68, E-ISAMY, Megazyme), dissolved in dimethyl sulphoxide (DMSO), and used to determine the relative molecular weight distribution by GPC using a PL-GPC 220 instrument (Agilent, USA). The three well-resolved fractions from GPC curves represent A and B1 chains of amylopectin (Ap1), B2 to B4 chains of amylopectin (Ap2), and amylose (Am), respectively.

Debranched starch was also quantitatively analysed using FACE with a PA-800 Plus instrument (Beckman, USA) and APTS-labelled linear glucans to determine the chain-length distribution (CLD) of amylopectin, denoted *N*de(*X*) (Gu et al. 2019). The Δ*N*de (*X*) value, representing the change in CLD, was then calculated from the *N*de(*X*) value for transgenic lines minus the *N*de(*X*) value for the corresponding WT, where, de indicates linear glucans and X indicates the degree of polymerisation (DP).

Statistical analysis

At least two replicates were performed for each experiment. All data are presented as means ± standard deviations (means ± SD). One-way analysis of variance (ANOVA) was used to determine the level of significance (* and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively).

Different lower-case letters indicate statistically significant differences at $p < 0.05$.

Results

SSII-2 RNAi simultaneously regulates amylose content and amylopectin structure to improve the quality of non-waxy rice

In our previous study, we successfully generated *SSII-2* RNAi transgenic rice in Nip (Wx^b) (Li et al. 2018; Xu et al. 2020b). Both AAC and GT were decreased in these transgenic rice, while GC was increased, which led to significantly improved rice ECQ without any defects in endosperm transparency. This novel soft rice has a far better appearance than traditional soft rice. To determine whether the effect of *SSII-2* is dependent on the specific allele type of the *Wx* gene, a key determinant of rice ECQ, we further introduced the same *SSII-2* RNAi construct into NILs Nip(Wx^a) carrying the representative Wx^a alleles in non-waxy rice, as shown in Table 1. The main agronomic traits of all transgenic lines were similar to those of their WT counterparts (Fig. S2), consistent with our previous studies (Li et al. 2018; Xu et al. 2020b). Therefore, neither suppression of *SSII-2* expression nor changes in *Wx* alleles affected rice growth and development.

In the high AC Nip(Wx^a) background, knockdown of *SSII-2* expression had no effect on the total starch or protein content (Fig. S3A, B). However, both AAC and GT data from DSC, including To, Tp and Te, were dramatically decreased in transgenic lines (Fig. 1 A and C and Fig. S3C, D), while GC values were significantly increased (Fig. 1B). RVA profile of rice flours revealed an overall downward trend for transgenic lines compared with WT (Fig. 1D, E). In more detail, PKV, HPV, CPV, SBV, PeT and PaT were significantly decreased, while only BDV was increased (Table S2). Low AAC, soft GC, high BDV and low SBV are all representative characteristics favored by Chinese rice consumers. These results indicate that suppression of *SSII-2* in the Wx^a background had the same effect on grain physicochemical properties as those in Wx^b rice, suggesting that knockdown of *SSII-2* expression could be a universal strategy for improving rice quality.

Next, GPC and FACE were used to examine the starch fine structure and CLD of *SSII-2* RNAi transgenic rice in both Nip(Wx^a) and Nip(Wx^b) backgrounds. The GPC results indicated that the amylose proportion (Am) in isolated grain starch from transgenic lines was significantly decreased, while the amylopectin proportion (Ap), especially Ap1, was increased (Fig. 1G, H). The FACE data showed that the proportion of amylopectin A chains with a DP value from 7 to 11 was significantly increased, and the proportion of B1 chains with a DP value from 13 to 20 was slightly decreased in *SSII-2* RNAi transgenic rice (Fig. 1J). These results implied that suppression of *SSII-2* expression in non-waxy rice could simultaneously modulate both AC and amylopectin structure, thereby leading to improved rice ECQ.

SSII-2 RNAi improves the amylopectin structure of glutinous rice

To explore the specific effect of *SSII-2* on the amylopectin structure, we also generated *SSII-2* RNAi/Nip(*wx*) transgenic rice, and grains contained similar amounts of starch and crude protein to those in the WT Nip(*wx*) counterpart (Fig. S3A, B). GT, estimated from To, Tp and Te using DSC analysis, was dramatically decreased in both transgenic lines. The above results are consistent with those for the Nip(Wx^b) and Nip(Wx^a) backgrounds. Regarding AAC and GC, no remarkable changes were observed (Fig. 1 A−C and Fig. S3E). In addition, the overall RVA profile of *SSII-2* RNAi/Nip(*wx*) transgenic rice showed a general downward trend (Fig. 1F). The only difference was that the PKV of *SSII-2* RNAi/Nip(*wx*) rice was slightly increased, unlike that of the Nip(Wx^a) and Nip(Wx^b) backgrounds (Table S2).

From the GPC data, no amylose peak was observed either of the *SSII-2* RNAi transgenic lines or the Nip(*wx*) control (Fig. 1I). This is a typical feature of waxy rice due to the absence of the GBSSI enzyme. However, the Ap1 fraction of amylopectin was significantly increased in *SSII-2* RNAi/Nip(*wx*) transgenic rice compared with the WT counterpart, while the Ap2 fraction was decreased. As expected, consistent changes in amylopectin CLD were observed for the *SSII-2* RNAi/Nip(*wx*) transgenic lines (Fig. 1J). The results indicate that RNA interference of *SSII-2* only influenced the amylopectin structure of waxy rice.

Interestingly, when we compared the altered amylopectin CLD in *SSII-2* RNAi transgenic lines with different *Wx* alleles and AAC, the amylopectin structure in *SSII-2* RNAi/Nip(Wx^b) showed greater change than that of *SSII-2* RNAi/Nip(Wx^a), while the change in *SSII-2* RNAi/Nip(*wx*) was greater than that of *SSII-2* RNAi/Nip(Wx^b), indicating that the lower the AC, the greater the change in amylopectin structure (Fig. 1J). These results further confirmed that downregulating *SSII-2* in rice endosperm could help to coordinate the synthesis and structure of amylose and amylopectin.

SSII and *Wx* are downregulated in the endosperm of *SSII-2* RNAi transgenic rice

To clarify the mechanism by which *SSII-2* affects the physicochemical properties and structure of starch, we examined the expression of *SSII* subfamily genes and *Wx* gene, especially the two major genes (*Wx* and *SSII-3*) respectively

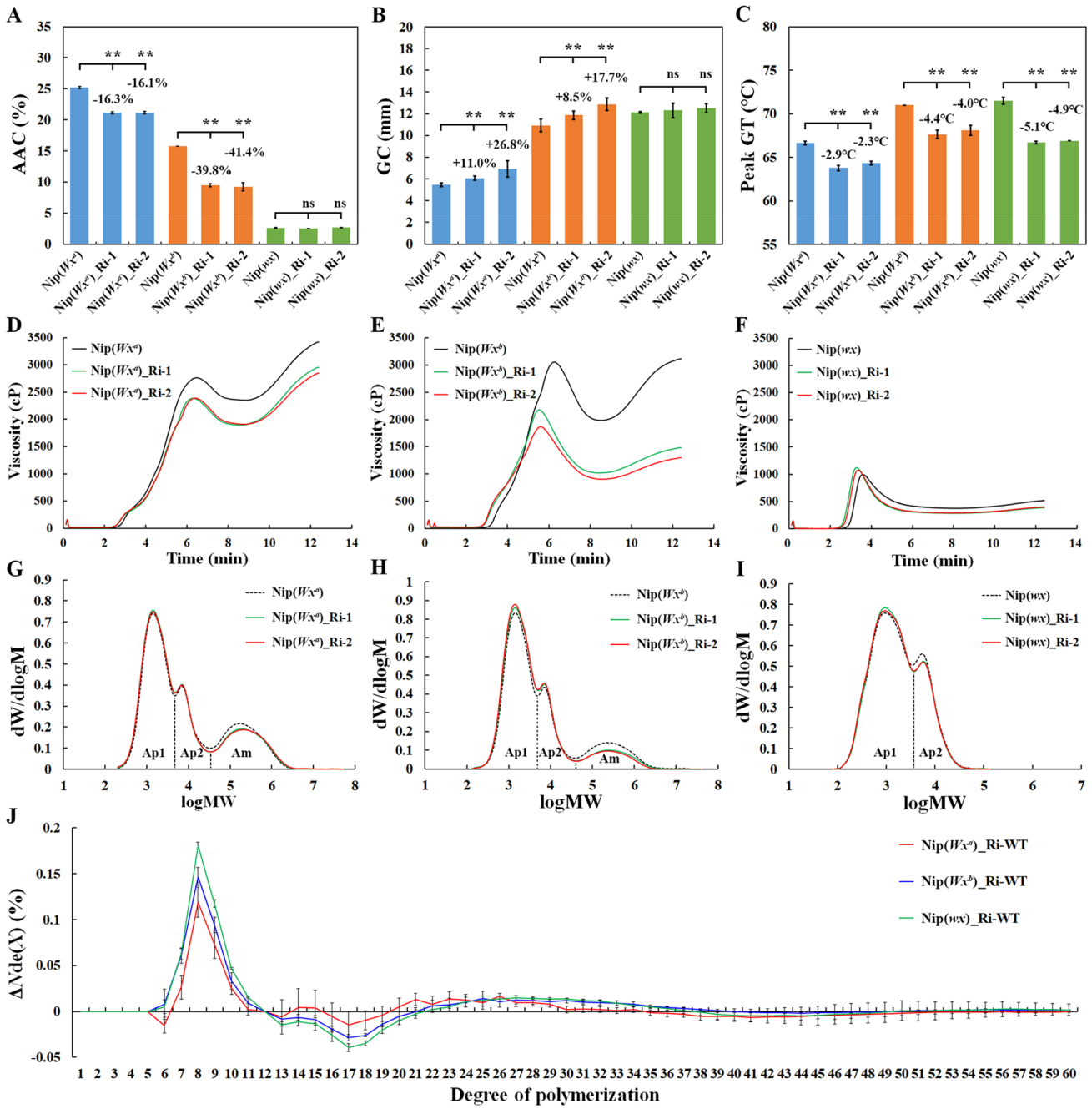

Fig. 1 Comparison of physicochemical properties and starch fine structures of mature seeds between *SSII-2* RNAi transgenic rice and their corresponding *Wx* NIL wild types. **A** Apparent amylose content (AAC) of rice flours. **B** Gel consistency (GC) of rice flours. **C** Peak gelatinisation temperature (GT) of rice flours determined by DSC. **D**–**F** Rapid visco-analyser (RVA) profile of rice flours in Nip(Wx^a), Nip(Wx^b) and Nip(*wx*) backgrounds, respectively. (G–I) Fine structures of starches determined by gel permeation chromatography (GPC) in Nip(Wx^a), Nip(Wx^b) and Nip(*wx*) backgrounds, respectively. **J** Changes in chain length distribution of amylopectin determined by fluorophore-assisted carbohydrate electrophoresis (FACE). Error bars represent SD (*$p < 0.05$ and **$p < 0.01$; *ns* no significant difference)

controlling AC and GT. The qRT-PCR data showed that expression of *SSII-2* was dramatically decreased in the developing seeds of *SSII-2* RNAi lines with different *Wx* alleles, demonstrating that suppressing *SSII-2* expression via RNAi is a promising strategy (Fig. 2A). In addition, both the transcript and protein abundance of *Wx* were significantly reduced in the caryopses of *SSII-2* RNAi non-waxy rice, while no *Wx* expression was detected in any of the Nip(*wx*)-related lines (Fig. 2B, C). Therefore, interfering with the expression of *SSII-2* simultaneously reduced the expression

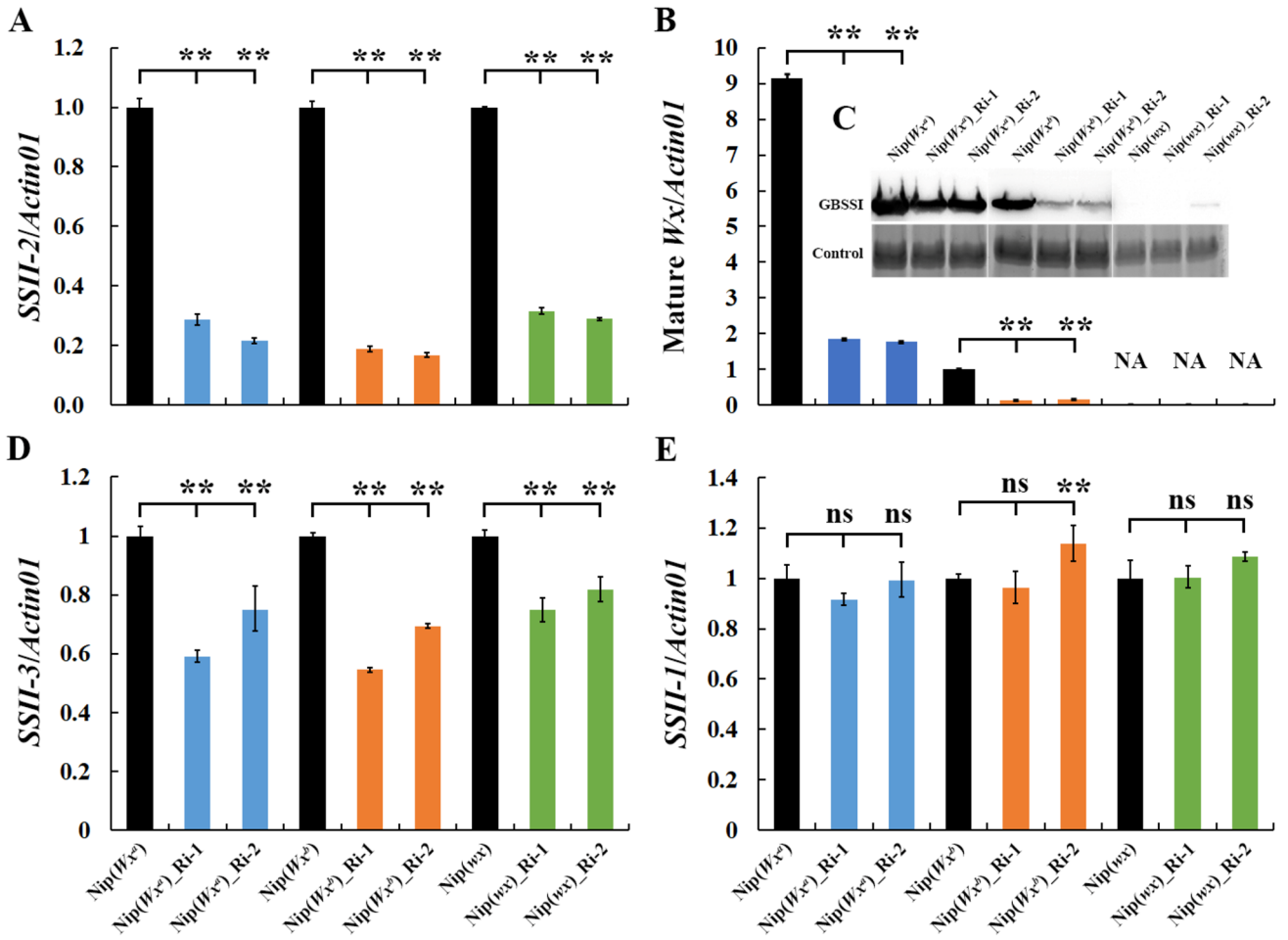

Fig. 2 Expression analysis of *SSII* and *Wx* genes in *SSII-2* RNAi transgenic rice and the wild type controls. **A**–**E** qRT-PCR expression analysis of *SSII-2*, *Wx*, *SSII-1* and *SSII-3*. **C** Western blotting analysis of the GBSSI protein. Rabbit anti-GBSSI antibody was used to detect GBSSI. The acid subunit of glutelin served as a loading control. NA, not applicable. Error bars represent SD (*$p < 0.05$ and **$p < 0.01$; ns, no significant difference)

of *Wx*, which may largely account for the decline in AC in non-waxy *SSII-2* RNAi transgenic rice. Regarding the other two *SSII* members, expression of *SSII-3* was also markedly reduced in all *SSII-2* RNAi lines (Fig. 2D), while *SSII-1* expression was almost unchanged (Fig. 2E). Given that *SSII-3* is the major gene controlling rice GT, the reduced *SSII-3* expression could explain the decrease in GT and modified amylopectin CLD in the *SSII-2* RNAi transgenic lines.

Generation of null *ssii-2* and *ssii-3* mutants by CRISPR/Cas9

Since the expression of *SSII-3*, the close isoform of *SSII-2*, was also decreased, the CRISPR/Cas9 system was used to further confirm the effects of *SSII-2*, and avoid the potential off-target effect caused by the RNAi method. Thus, *ssii-2* and *ssii-3* single mutants and the *ssii-2ssii-3* double mutant were generated via CRISPR/Cas9 (Fig. S1). Ten mutants without T-DNA insertions were selected after several generations of screening, including five *ssii-2* single mutants (*s2*), two *ssii-3* single mutants (*s3*) and three *ssii-2ssii-3* double mutants (*s23*). All these mutants had undergone one insertion or deletion event in the target site that caused a frameshift mutation in the target gene (Fig. 3A). Except for a slight decrease in kernel weight, none of the other agronomic traits of the mutants were altered (Fig. S4). Therefore, two typical mutants of each type were selected for subsequent analyses.

Effects of *SSII-2* and *SSII-3* mutations on *SSII* and *Wx* expression in endosperm

The abundance of *SSII* and *Wx* transcripts in developing endosperm of mutants and WT plants was analysed. The qRT-PCR results showed that expression of both *SSII-2* and *SSII-3* was dramatically decreased in *s2* mutants

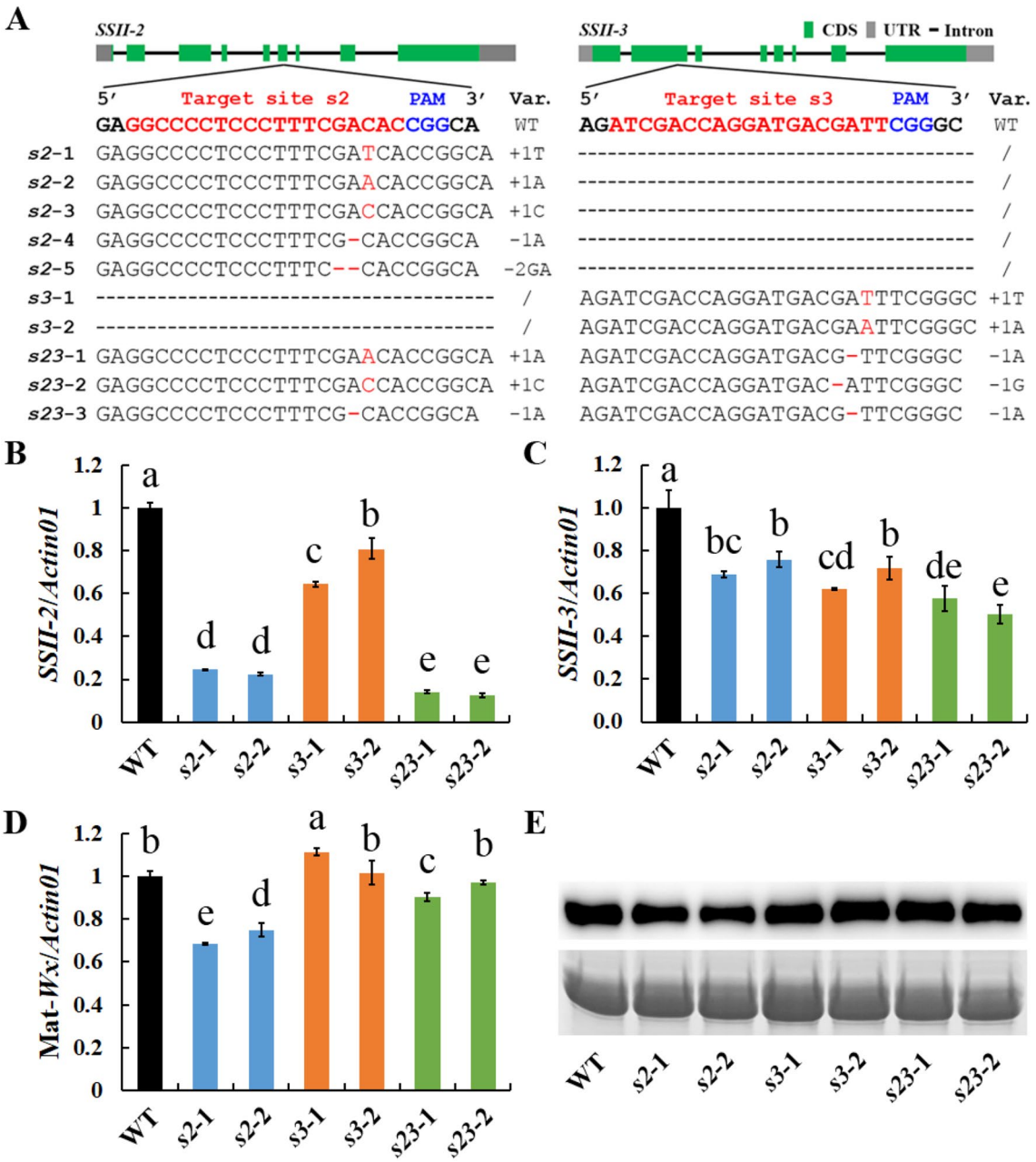

Fig. 3 Generation of *s2*, *s3* and *s23* mutants, and expression analysis of *SSII* and *Wx* genes in *s2*, *s3* and *s23* mutants. **A** Mutation information for the target sites of *SSII-2* and *SSII-3* genes. Red nucleotide sequences and red dashes in different lines indicate sequence changes. PAM, proto-spacer-motif; Var., variants of identified mutations; WT, wild type. **B**–**D** Relative expression of *SSII-2*, *SSII-3* and *Wx*. **E** The accumulation of GBSSI in *s2*, *s3* and *s23* mutants. Different lowercase letters indicate statistically significant differences at $p < 0.05$

(Fig. 3B, C). In *s3* mutants, expression of both *SSII-2* and *SSII-3* was slightly lower than in controls. In *s23* double mutants, both *SSII-2* and *SSII-3* expression was also greatly decreased (Fig. 3B, C). Furthermore, the *Wx* expression was significantly decreased in *s2* mutants, slightly decreased in *s23* double mutants but a little bit increased in *s3* mutants (Fig. 3D), while only the amount of GBSSI protein was decreased in *s2* mutants and no significant change was observed in *s3* and *s23* mutants (Fig. 3E). These results indicate that knockout of *SSII-2* can markedly reduce the expression of *SSII-2*, *SSII-3* and *Wx* genes, while knockout of *SSII-3* alone or both *SSII-2* and *SSII-3* mainly affected the expression of *SSII-2* and *SSII-3*, with minimal or no effect on *Wx* expression.

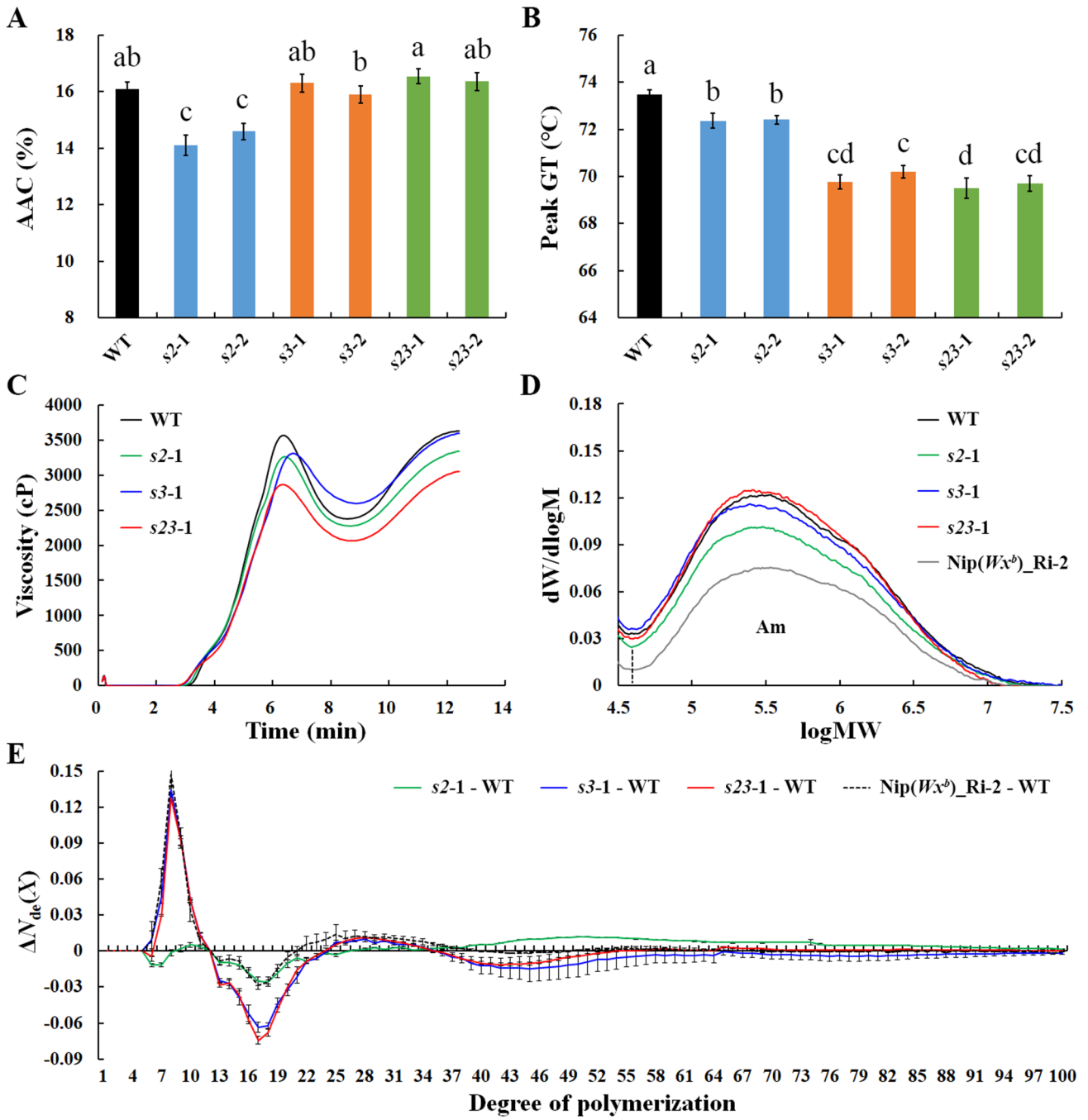

Fig. 4 Physicochemical properties and starch fine structure of mature seeds from *s2*, *s3* and *s23* mutants. **A** Apparent amylose content (AAC) of rice flours. **B** Peak gelatinisation temperature (GT) of rice flours determined by DSC. **C** Rapid visco-analyser (RVA) profile of rice flours. **D** Fine structures of starches determined by gel permeation chromatography (GPC). **E** Changes in chain length distribution of amylopectin determined by fluorophore-assisted carbohydrate electrophoresis (FACE). Different lower-case letters indicate statistically significant differences at $p < 0.05$

Improvements in grain quality and starch structure in *ssii-2* and *ssii-3* null mutants are similar to those in *SSII-2* RNAi transgenic lines

The grain physicochemical properties of the mutants were analysed and compared in detail. First, AAC analysis revealed a significant reduction in AAC for *s2* single mutants, while *s3* and *s23* mutants exhibited minimal change (Fig. 4A). In the case of GT, the To, Tp and Te values of all mutant grains were significantly decreased (Fig. 4B and Table S3). However, the decrease in GT in *s3* mutants was greater than that in *s2* mutants. Interestingly, the *s23* double

mutants displayed the lowest grain GT values among the mutants, suggesting an additive effect for *SSII-2* and *SSII-3* on grain GT. Regarding starch viscosity properties, the RVA profiles of *s2* mutants showed an overall downward trend compared with those of WT samples, reflected by the lower PKV, HPV, BDV, CPV, SBV and PaT values. The RVA profiles of *s3* mutants also displayed a decrease in PKV, BDV, CPV and PaT, but HPV and SBV were increased (Fig. 4C). Moreover, the overall RVA profile of *s23* double mutants were lower than those of *s2* and *s3* single mutants, and similar to those of *SSII-2* RNAi lines (Fig. 1D).

The GPC results showed that the AC of *s2* mutants was significantly decreased, while that of *s3* and *s23* mutants was not altered (Fig. 4D). Regarding amylopectin, only Ap1 of *s2* mutants was significantly increased, while Ap1 of *s3* and *s23* mutants and Ap2 of all mutants were not changed (Table S5). Furthermore, data from FACE analysis indicated that the absence of *SSII-2* alone caused a minor change in amylopectin fine structure, including a slight increase in the proportion of chains with DP 10−12 and > 34, and a decrease in chains with DP 5−9 and 13−33 (Fig. 4E). The chain-length distributions of amylopectin from *s3* and *s23* mutants were similar, both showing a significant increase in DP 5−12 and a significant decrease in DP 13−24 chains (Fig. 4E). Compared with *SSII-2* RNAi lines, the amylopectin structure of *s2* grains showed a consistent change in chains with DP 13−24, while that of *s3* and *s23* grains showed a consistent change in chains with DP 7−11.

Taken together, the above results revealed that mutating *SSII-2* can indeed decrease rice AC and GT, albeit to a lesser extent than that caused by *SSII-2* RNAi. Meanwhile, *ssii-3* single mutants and *ssii-2ssii-3* double mutants exhibited similar amylopectin CLD and GT levels to those in *SSII-2* RNAi rice, but there was no change in AC. In conclusion, *SSII-2* is a promising biotechnological target for improving rice ECQ via either RNAi or CRISPR/Cas9 strategies during rice breeding programs.

Discussion

Synergetic downregulation of *Wx*, *SSII-2* and *SSII-3* expression in the endosperm of *SSII-2* RNAi lines

Three NILs within the *Wx* locus were generated in the Nipponbare background, among which non-waxy Nip(Wx^a) and Nip(Wx^b) rice contained different amounts of mature *Wx* mRNA and GBSSI, while no mature *Wx* mRNA or GBSSI protein were detected in the glutinous Nip(*wx*) rice. The *SSII-2* RNAi construct was subsequently introduced into the three NILs, and qRT-PCR and western blotting results indicated that expression of the *Wx* gene was markedly lower in the resulting *SSII-2* RNAi lines. It is intriguing how suppression of *SSII-2* could trigger such a striking decrease in expression of the *Wx* gene. Gene sequence analysis indicated that the *SSII-2* fragment used for RNAi shared low sequence identity with the *Wx* gene, with a maximum of only 9 consecutive identical bases. Additionally, the *Wx* gene was not in the list of potential off-target genes predicted by dsCheck (http://dsCheck.RNAi.jp/) (Naito et al. 2005), suggesting that the dramatic decrease in *Wx* expression was not caused by off-target effects (Supplemental Table 6). To further confirm this notion, we analysed the expression of *Wx* in *ssii-2* mutants, which were generated by the CRISPR/Cas9 editing system. The results showed that both the expression of *Wx* transcripts and GBSSI proteins were significantly reduced in the mutants, suggesting that the decrease in *Wx* expression was indeed caused by *SSII-2* RNAi. Therefore, we speculate that either knockdown or knockout of *SSII-2* expression could decrease *Wx* expression, and consequently AC. Note that the reduction in *Wx* transcripts and GBSSI proteins in *ssii-2* mutants was lower than in *SSII-2* RNAi lines (Figs. 2B, C and 3D, E), indicating that other factors might be involved in the grain quality formation in *SSII-2* RNAi transgenic lines. For *SSII-3* gene, mutation of *SSII-3*, the closest isoform of *SSII-2*, caused slightly increased expression of *Wx* transcripts but no remarkable change in GBSSI protein accumulation (Fig. 3 C and E).

Moreover, expression levels of both *SSII-2* and *SSII-3* were dramatically decreased in *SSII-2* RNAi transgenic lines, but *SSII-1* expression was not altered, suggesting that *SSII-2* and *SSII-3* both contributed to the improved grain quality of *SSII-2* RNAi transgenic rice. Mutation of either *SSII-2* or *SSII-3* alone via CRISPR/Cas9 could lead to a decrease in expression of the other, indicating interdependency and reciprocal regulation of these two *SSII* isoforms.

Simultaneous modulation of amylose content and amylopectin structure in *SSII-2* RNAi lines

Analysis of grain physicochemical properties revealed that both AC and amylopectin fine structure were improved in *SSII-2* RNAi/Nip(Wx^a) and *SSII-2* RNAi/Nip(Wx^b) transgenic rice, resulting in increased GC, decreased GT and RVA profile, and hence enhanced rice ECQ. Meanwhile in *SSII-2* RNAi/Nip(*wx*) rice, only amylopectin synthesis was affected, also leading to decreased GT and improved stickiness, hardness and cooking properties.

The absence of *SSII-2* resulted in minor changes in amylopectin CLD, consistent with its low expression in rice endosperm (Ohdan et al. 2005a), and this distinguished it from *SSII-3* knockout lines. The proportion of amylopectin with DP 5−8 was decreased while the portion with DP 9−12 was increased in short chains, and the proportion with DP 13−30 was decreased while the proportion with DP ≥ 31 was increased in intermediate chains for *ssii-2* mutants,

compared with WT (Figs. 4E and S5). We speculate that the increase in amylopectin A chain with DP 9−12 and the decrease in amylopectin A chain with DP 5−8 and B chain with DP 13−30 together lowered GT in *ssii-2* rice. Previous studies indicate that short-chain amylopectin with DP ≤ 12 can only form short double helices, whereas long parallel chains of amylopectin can form longer double helices (Miura et al. 2018).

Both *ssii-3* and *ssii-2ssii-3* mutants displayed similar GT and CLD for amylopectin to those of *SSII-2* RNAi lines, but for RVA profiles, a consistent shape was only observed for *ssii-2ssii-3* double mutants and *SSII-2* RNAi lines, suggesting that both *SSII-2* and *SSII-3* were responsible for improving ECQ in *SSII-2* RNAi rice. Thus, the formation of grain quality in *SSII-2* RNAi lines and *ssii-2* mutants was likely caused by a moderate decrease in Am and Ap-B1, and an increase in Ap-A via coordinated downregulation of the expression of *Wx*, *SSII-2* and *SSII-3* (Fig. 5). We speculated that the downregulation of *SSII-3* expression was not only caused by suppressing of *SSII-2*, but also the possible off-target effect of RNAi to *SSII-3* expression, even though *SSII-3* was not in the list of potential off-target genes predicted by dsCheck (Supplemental Table 6).

By specific knockout of *SSII-2* and *SSII-3* using CRISPR/Cas9, the *ssii-2ssii-3* (*s23*) mutants were created trying to reproduce the moderate AC reduction as in *SSII-2* RNAi lines. But the GBSSI accumulation as well as the AC showed no significant change in *ssii-2ssii-3* mutants. Enzyme complexes were observed during amylopectin synthesis and SSII-3 was reported to form complexes with SSI and SBEs in rice, maize and wheat (Crofts et al. 2015; Hennen-Bierwagen et al. 2008; Tetlow et al. 2008). SSII-3 is the core of the SSI/SSII-3/SBEII (SBE3/SBEIIb or SBE4/SBEIIa) trimer, located between SSI and SBEII. In addition to extending the chain length of amylopectin, SSII-3 also transports SSI and SBEII to starch granules (Liu et al. 2012). The *ssii-3* mutant in maize, typically known as *sugary 2* (*su2*), showed decreased SSI and SSIIb proteins on starch granules. Besides, new enzymes complexes were observed after mutation of one component of the original enzyme complex (Liu et al. 2011). In present study, the expression of *SSII-3* was knockdown in *SSII-2* RNAi lines, while the SSII-3 protein was fully absence in *ssii-3* mutants. This could be a possible reason for the AC difference between these lines. By using the genome editing system, the AC reduction in *ssii-2ssii-3* mutants can be further achieved by application of natural *Wx* allelic variants, as well as the novel edited *Wx* alleles (Huang et al. 2020a; Xu et al. 2020a; Zeng et al. 2020; Zhang et al. 2019a).

Moreover, the *SSII-3* was successfully knockout in *ssii-3* mutants resulting from the CLD and dramatically decreased GT, while slight AC changes were observed in *ssii-3* mutants created by CRISPR/Cas9. It distinguishes from the increased AC in *ssii-3* mutants in previous studies (Chen et al. 2020; Miura et al. 2018). The reason need to be further explained.

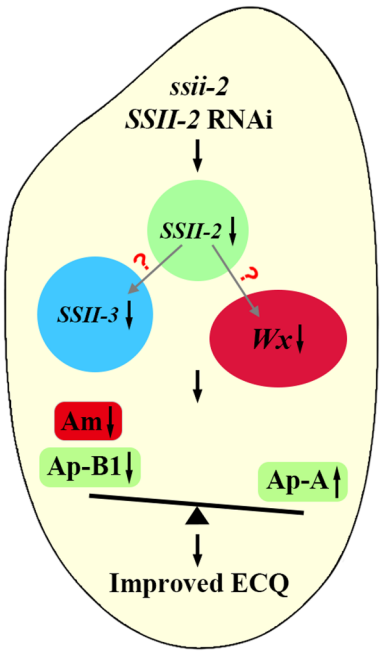

Fig. 5 Proposed mechanism by which *SSII-2*, *SSII-3* and *Wx* regulate rice grain quality. Either knockdown or knockout of *SSII-2* may suppress the expression of *SSII-2*, *SSII-3* and *Wx*, resulting in decreased amylose (Am) and amylopectin B1 chains (Ap-B1), and increased amylopectin A chains (Ap-A), and a consequent improvement in rice ECQ

SSII-2 is a novel biotechnological target for improving rice ECQ

The present work revealed that the *SSII-2* gene is involved in the synthesis of both rice amylose and amylopectin by modulating the expression of *Wx* and *SSII-3* genes. *SSII-2* knockdown or knockout rice displayed decreased AC and GT, but there were almost no negative effects on other agronomic traits, including grain appearance, which may be beneficial for high-quality rice breeding applications. In the past few decades, tremendous effort has been expended to improve rice grain quality without lowering the yield, and regulation of rice starch biosynthesis appears to be a promising approach. However, most previous studies focused on modulating SSRGs with high or seed-specific expression, including *Wx*, *SSI*, *SSII-3*, *SSIII-2*, *SBE1*, *SBE3*, *ISA1* and *Pull* (Jeon et al. 2010; Nishi et al. 2001; Zhou et al. 2016; Zhu et al. 2012). Other SSRGs with low expression abundance, including *SSII-2*, have received less attention, and their functions typically remain unknown. For example, *SSII-2* is believed to be involved in the biosynthesis of transient starch in plant leaves, based on its expression pattern in maize and rice (Harn et al. 1998; Jiang et al. 2004). Various studies on rice ECQ improvement have successfully

identified regulators, including transcription factors and starch/enzyme-binding proteins such as RSR1, OsbZIP58, OsMADS7, and the NF-YB1-YC12-bHLH144 complex (Bello et al. 2019; Fu and Xue 2010; Wang et al. 2013; Zhang et al. 2018), as well as PTST1, PTST2 (flo6) and OsGBP (Peng et al. 2014; Seung et al. 2015; Wang et al. 2019). These transcription factors mainly affect AC, GC and GT in rice endosperm, but accompanying defects in rice yield or grain appearance greatly limits their application in rice breeding programs. At present, application of natural allelic variation in *Wx* and *SSII-3* remains the major approach for improving rice ECQ (Phing Lau et al. 2016). Excitingly, our current study suggests that *SSII-2*, a neglected *SSII* isoform, could be a novel beneficial target for improving rice ECQ during breeding programs.

Conclusions

In summary, our study revealed the underlying mechanism of *SSII-2* RNAi-mediated improvements in rice ECQ, and highlighted the key role of the coordinated regulation of amylose and amylopectin synthesis via suppression of the expression of *SSII-2*, *SSII-3* and *Wx*. Furthermore, we also uncovered the function of *SSII-2* in the regulation of both amylose and amylopectin synthesis by studying *ssii-2* mutants. The findings provide new strategies and germplasm resources for breeding high-ECQ rice.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-021-01162-8.

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Author contributions QQL, QFL and HL conceived the project; LH, ZG, ZC, JY, RC, HT, DZ and XF performed the experiments and analysed the data; LH, QQL and QFL wrote the manuscript.

Data availability All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

References

Anacleto R, Badoni S, Parween S, Butardo VM Jr., Misra G, Cuevas RP et al (2019) Integrating a genome-wide association study with a large-scale transcriptome analysis to predict genetic regions influencing the glycaemic index and texture in rice. Plant Biotechnol J 17:1261–1275

Bao JS, Corke H, Sun M (2006) Nucleotide diversity in starch synthase IIa and validation of single nucleotide polymorphisms in relation to starch gelatinization temperature and other physicochemical properties in rice (*Oryza sativa* L.). Theor Appl Genet 113:1171–1183

Bello BK, Hou Y, Zhao J, Jiao G, Wu Y, Li Z et al (2019) NF-YB1-YC12-bHLH144 complex directly activates *Wx* to regulate grain quality in rice (*Oryza sativa* L.). Plant Biotechnol J 17:1222–1235

Cai X, Wang Z, Xing Y, Zhang J, Hong M (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5′ UTR and decreased expression of *Waxy* gene in rice cultivars of intermediate amylose content. Plant J 14:459–465

Chen K, Wang Y, Zhang R, Zhang H, Gao C (2019) CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu Rev Plant Biol 70:667–697

Chen Z, Lu Y, Feng L, Hao W, Li C, Yang Y et al (2020) Genetic dissection and functional differentiation of *ALKa* and *ALKb*, two natural alleles of the *ALK*/*SSIIa* gene, responding to low gelatinization temperature in rice. Rice 13:39

Commuri PD, Keeling PL (2001) Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. Plant J 25:475–486

Crofts N, Abe N, Oitome NF, Matsushima R, Hayashi M, Tetlow IJ et al (2015) Amylopectin biosynthetic enzymes from developing rice seed form enzymatically active protein complexes. J Exp Bot 66:4469–4482

Donohoue PD, Barrangou R, May AP (2018) Advances in industrial biotechnology using CRISPR-Cas systems. Trends Biotechnol 36:134–146

Fan X, Li Y, Lu Y, Zhang C, Li E, Li Q et al (2019) The interaction between amylose and amylopectin synthesis in rice endosperm grown at high temperature. Food Chem 301:125258

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806–811

Fu FF, Xue HW (2010) Coexpression analysis identifies rice starch regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. Plant Physiol 154:927–938

Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y (2006) Function and characterization of starch synthase I using mutants in rice. Plant Physiol 140:1070–1084

Furukawa K, Tagaya M, Inouye M, Preiss J, Fukui T (1990) Identification of lysine 15 at the active site in *Escherichia coli* glycogen synthase. Conservation of Lys-X-Gly-Gly sequence in the bacterial and mammalian enzymes. J Biol Chem 265:2086–2090

Gao Z, Zeng D, Cui X, Zhou Y, Yan M, Huang D et al (2003) Map-based cloning of the *ALK* gene, which controls the gelatinization temperature of rice. Sci China C Life Sci 46:661–668

Gu F, Gong B, Gilbert RG, Yu W, Li E, Li C (2019) Relations between changes in starch molecular fine structure and in thermal properties during rice grain storage. Food Chem 295:484–492

Harn C, Knight M, Ramakrishnan A, Guan H, Keeling PL, Wasserman BP (1998) Isolation and characterization of the zSSIIa and zSSIIb starch synthase cDNA clones from maize endosperm. Plant Mol Biol 37:639–649

Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ et al (2008) Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. Plant Physiol 146:1892–1908

Hirano HY, Eiguchi M, Sano Y (1998) A single base change altered the regulation of the *Waxy* gene at the posttranscriptional level during the domestication of rice. Mol Biol Evol 15:978–987

Huang L, Li Q, Zhang C, Chu R, Gu Z, Tan H et al (2020a) Creating novel *Wx* alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system. Plant Biotechnol J 18:2164–2166

Huang L, Sreenivasulu N, Liu Q (2020b) *Waxy* editing: old meets new. Trends Plant Sci 25:963–966

Jeon JS, Ryoo N, Hahn TR, Walia H, Nakamura Y (2010) Starch biosynthesis in cereal endosperm. Plant Physiol Biochem 48:383–392

Jiang H, Dian W, Liu F, Wu P (2004) Molecular cloning and expression analysis of three genes encoding starch synthase II in rice. Planta 218:1062–1070

Li Z, Chu X, Mouille G, Yan L, Kosar-Hashemi B, Hey S et al (1999) The localization and expression of the class II starch synthases of wheat. Plant Physiol 120:1147–1156

Li H, Prakash S, Nicholson TM, Fitzgerald MA, Gilbert RG (2016) Instrumental measurement of cooked rice texture by dynamic rheological testing and its relation to the fine structure of rice starch. Carbohydr Polym 146:253–263

Li QF, Huang LC, Chu R, Li J, Jiang MY, Zhang CQ et al (2018) Down-regulation of *SSSII-2* gene expression results in novel low-amylose rice with soft, transparent grains. J Agric Food Chem 66:9750–9760

Li C, Wu A, Yu W, Hu Y, Li E, Zhang C, Liu Q (2020) Parameterizing starch chain-length distributions for structure-property relations. Carbohydr Polym 241:116390

Liu L, Ma X, Liu S, Zhu C, Jiang L, Wang Y et al (2009) Identification and characterization of a novel *Waxy* allele from a Yunnan rice landrace. Plant Mol Biol 71:609–626

Liu F, Ahmed Z, Lee EA, Donner E, Liu Q, Ahmed R et al (2011) Allelic variants of the amylose extender mutation of maize demonstrate phenotypic variation in starch structure resulting from modified protein–protein interactions. J Exp Bot 63:1167–1183

Liu F, Romanova N, Lee Elizabeth A, Ahmed R, Evans M, Gilbert Elliot P et al (2012) Glucan affinity of starch synthase IIa determines binding of starch synthase I and starch-branching enzyme IIb to starch granules. Biochem J 448:373–387

Liu W, Xie X, Ma X, Li J, Chen J, Liu Y (2015) DSDecode: a web-based tool for decoding of sequencing chromatograms for genotyping of targeted mutations. Mol Plant 8:1431–1433

Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X et al (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 23:1233–1236

Mikami I, Aikawa M, Hirano HY, Sano Y (1999) Altered tissue-specific expression at the *Wx* gene of the opaque mutants in rice. Euphytica 105:91–97

Mikami I, Uwatoko N, Ikeda Y, Yamaguchi J, Hirano HY, Suzuki Y, Sano Y (2008) Allelic diversification at the *Wx* locus in landraces of Asian rice. Theor Appl Genet 116:979–989

Miura S, Crofts N, Saito Y, Hosaka Y, Oitome NF, Watanabe T et al (2018) Starch synthase IIa-deficient mutant rice line produces endosperm starch with lower gelatinization temperature than *japonica* rice cultivars. Front Plant Sci 9:645–645

Morell MK, Kosar-Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S et al (2003) Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties. Plant J 34:173–185

Naito Y, Yamada T, Matsumiya T, Ui-Tei K, Saigo K, Morishita S (2005) dsCheck: highly sensitive off-target search software for double-stranded RNA-mediated RNA interference. Nucleic Acids Res 33:W589–W591

Nakamura Y, Francisco PB, Hosaka Y, Sato A, Sawada T, Kubo A, Fujita N (2005) Essential amino acids of starch synthase IIa differentiate amylopectin structure and starch quality between *japonica* and *indica* rice varieties. Plant Mol Biol 58:213–227

Nishi A, Nakamura Y, Tanaka N, Satoh H (2001) Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. Plant Physiol 127:459–472

Ohdan T, Francisco PB Jr, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y (2005a) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. J Exp Bot 56:3229–3244

Ohdan T, Francisco PJT, Hirose T, Terao T, Satoh H, Nakamura Y (2005b) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. J Exp Bot 56:3229–3244

Peng C, Wang Y, Liu F, Ren Y, Zhou K, Lv J et al (2014) *FLOURY ENDOSPERM6* encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. Plant J 77:917–930

Phing Lau WC, Latif MA, Rafii MY, Ismail MR, Puteh A (2016) Advances to improve the eating and cooking qualities of rice by marker-assisted breeding. Crit Rev Biotechnol 36:87–98

Sano Y (1984) Differential regulation of *Waxy* gene expression in rice endosperm. Theor Appl Genet 68:467–473

Sato H, Suzuki Y, Sakai M, Imbe T (2002) Molecular characterization of *Wx-mq*, a novel mutant gene for low-amylose content in endosperm of rice (*Oryza sativa* L.). Breed Sci 52:131–135

Seung D, Soyk S, Coiro M, Maier BA, Eicke S, Zeeman SC (2015) PROTEIN TARGETING TO STARCH is required for localising GRANULE-BOUND STARCH SYNTHASE to starch granules and for normal amylose synthesis in *Arabidopsis*. PLoS Biol 13:e1002080

Su Y, Rao Y, Hu S, Yang Y, Gao Z, Zhang G et al (2011) Map-based cloning proves *qGC-6*, a major QTL for gel consistency of *japonica/indica* cross, responds by *Waxy* in rice (*Oryza sativa* L.). Theor Appl Genet 123:859

Tan Y, Yu S, Xing Y, Xu C, Zhang Q, Li J (1999) The three important traits for cooking and eating quality of rice grains are controlled by a single locus in an elite rice hybrid, Shanyou 63. Theor Appl Genet 99:642–648

Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS et al (2008) Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. Plant Physiol 146:1878–1891

Tian Z, Qian Q, Liu Q, Yan M, Liu X, Yan C et al (2009) Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. Proc Natl Acad Sci USA 106:21760–21765

Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y (2002) Mapping of a gene responsible for the difference in amylopectin structure between *japonica*-type and *indica*-type rice varieties. Theor Appl Genet 104:1–8

Wang L, Wang YJ (2001) Comparison of protease digestion at neutral pH with alkaline steeping method for rice starch isolation. Cereal Chem 78:690–692

Wang Z, Wu Z, Xing Y, Zheng F, Guo X, Zhang W, Hong M (1990) Nucleotide sequence of rice *Waxy* gene. Nucleic Acids Res 18:5898–5898

Wang JC, Xu H, Zhu Y, Liu QQ, Cai XL (2013) OsbZIP58, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm. J Exp Bot 64:3453–3466

Wang C, Shen L, Fu Y, Yan C, Wang K (2015) A simple CRISPR/Cas9 system for multiplex genome editing in rice. J Genet Genom 42:703–706

Wang X, Pang Y, Wang C, Chen K, Zhu Y, Shen C et al (2016) New candidate genes affecting rice grain appearance and milling quality detected by genome-wide and gene-based association analyses. Front Plant Sci 7:1998

Wang W, Wei X, Jiao G, Chen W, Wu Y, Sheng Z et al (2019) *GBSS-BINDING PROTEIN*, encoding a CBM48 domain-containing protein, affects rice quality and yield. J Integr Plant Biol 62:948–966

Xie X, Ma X, Zhu Q, Zeng D, Li G, Liu Y (2017) CRISPR-GE: a convenient software toolkit for CRISPR-based genome editing. Mol Plant 10:1246–1249

Xu Y, Lin Q, Li X, Wang F, Chen Z, Wang J et al (2020a) Fine-tuning the amylose content of rice by precise base editing of the *Wx* gene. Plant Biotechnol J 19:11–13

Xu Z, Yu M, Yin Y, Zhu C, Ji W, Zhang C et al (2020b) Generation of selectable marker-free soft transgenic rice with transparent kernels by downregulation of *SSSII-2*. Crop J 8:53–61

Yang Y, Guo M, Sun S, Zou Y, Yin S, Liu Y et al (2019) Natural variation of *OsGluA2* is involved in grain protein content regulation in rice. Nature Commun 10:1949

Zeng D, Liu T, Ma X, Wang B, Zheng Z, Zhang Y et al (2020) Quantitative regulation of *Waxy* expression by CRISPR/Cas9-based promoter and 5'UTR-intron editing improves grain quality in rice. Plant Biotechnol J 18:2385–2387

Zhang C, Zhu L, Shao K, Gu M, Liu Q (2013) Toward underlying reasons for rice starches having low viscosity and high amylose: physiochemical and structural characteristics. J Sci Food Agric 93:1543–1551

Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z et al (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12:797–807

Zhang C, Zhou L, Zhu Z, Lu H, Zhou X, Qian Y et al (2016) Characterization of grain quality and starch fine structure of two *japonica* rice (*Oryza Sativa*) cultivars with good sensory properties at different temperatures during the filling stage. J Agric Food Chem 64:4048–4057

Zhang H, Xu H, Feng M, Zhu Y (2018) Suppression of *OsMADS7* in rice endosperm stabilizes amylose content under high temperature stress. Plant Biotechnol J 16:18–26

Zhang C, Zhu J, Chen S, Fan X, Li Q, Lu Y et al (2019a) *Wxlv*, the ancestral allele of rice *Waxy* gene. Mol Plant 12:1157–1166

Zhang H, Zhou L, Xu H, Wang L, Liu H, Zhang C et al (2019b) The *qSAC3* locus from *indica* rice effectively increases amylose content under a variety of conditions. BMC Plant Biol 19:275

Zhao DS, Li QF, Zhang CQ, Zhang C, Yang QQ, Pan LX et al (2018) GS9 acts as a transcriptional activator to regulate rice grain shape and appearance quality. Nat Commun 9:1240

Zhou H, Wang L, Liu G, Meng X, Jing Y, Shu X et al (2016) Critical roles of soluble starch synthase SSIIIa and granule-bound starch synthase Waxy in synthesizing resistant starch in rice. Proc Natl Acad Sci USA 113:12844–12849

Zhou H, Xia D, He Y (2020) Rice grain quality—traditional traits for high quality rice and health-plus substances. Mol Breed 40:1

Zhu F (2018) Relationships between amylopectin internal molecular structure and physicochemical properties of starch. Trends Food Sci Technol 78:234–242

Zhu L, Gu M, Meng X, Cheung SC, Yu H, Huang J et al (2012) High-amylose rice improves indices of animal health in normal and diabetic rats. Plant Biotechnol J 10:353–362

Zhu Q, Zeng D, Yu S, Cui C, Li J, Li H et al (2018) From golden rice to aSTARice: bioengineering astaxanthin biosynthesis in rice endosperm. Mol Plant 11:1440–1448