Carbohydrate Polymers 306 (2023) 120595

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Generating waxy rice starch with target type of amylopectin fine structure and gelatinization temperature by *waxy* gene editing

Yuhao Fu [a,b], Yonghuan Hua [b,c], Tingting Luo [b], Chunyan Liu [a], Baoli Zhang [b], Xingyu Zhang [b], Yiping Liu [b], Zizhong Zhu [b], Yang Tao [a,b], Zhongyan Zhu [a,*], Ping Li [b,*], Jun Zhu [b,*]

[a] State Key Laboratory of Southwestern Chinese Medicine Resources, Innovative Institute of Chinese Medicine and Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China

[b] State Key Laboratory of Crop Gene Exploration and Utilization in Southwest China, Rice Research Institute of Sichuan Agricultural University, Chengdu 611137, China

[c] Meishan Dongpo District Agricultural and Rural Bureau, Meishan 620000, China

ARTICLE INFO

Keywords:
Waxy rice starch
Amylopectin fine structure
Gelatinization temperature
Amylose content
Waxy gene
CRISPR/Cas9

ABSTRACT

Waxy rice, which lacks amylose, is an important variant in rice, and its starches have been widely used. New waxy rice varieties generated via the CRISPR/Cas9 gene-editing system is described. Herein, four waxy rice starches with different physicochemical properties were successfully obtained by the CRISPR/Cas9 editing *Waxy* (*Wx*) gene. The results showed that the amylose content (AC) of *wx* mutant starches ranged from 0.26 to 1.78 %, and CZB*wx1* starches had the best gel consistency and highest water solubility among all *wx* mutants. Mutations of Wx^b allele produced more short-chains (degree of chain polymerization (DP) 6–11), and less medium- and long-chains (DP12–70) than that of Wx^a, while the AC of Wx^a allele mutants was higher than the mutations of Wx^b allele. The gelatinization temperature (GT) of wx^a mutant starches was higher than that of wx^b mutant starches. Moreover, we found that the GT and amylopectin fine structure type of waxy rice starch did not change after *Wx* gene editing. Based on these findings, it is possible to produce waxy rice starch with different physicochemical properties, containing target GT and chain length distributions of amylopectin.

1. Introduction

Waxy rice is regarded as a high-quality rice variant, also known as glutinous rice, which is very sticky when cooked (Juliano, 1998). Its starch content accounts for approximately 80 % of the dry weight of polished rice, and the majority of starch is amylopectin with very little or no amylose (0 %–2 %) (Bean et al., 1984). Due to the unique properties of waxy rice starch, it is extensively used in the chemical industry, medicine, and daily human life (Bao et al., 2004; Puchongkavarin et al., 2005; Wang & Wang, 2002).

Starch is comprised of two polymers, amylopectin, and amylose. Amylose is a mainly linear chain with α-1,4-linked glucosyl units, while amylopectin is a highly branched polymer with short α-1,4-linked glucosyl chains linked by α-1,6-linkages. A large number of studies on starch properties point to the fact that the fine structure of amylopectin is thought to be a significant factor leading to the different characteristics of starch (Huang & Lai, 2014; Singh et al., 2012; Vandeputte, Vermeylen, et al., 2003). Greater branch density of amylopectin leads to higher solubility, which is not accessible to retrograde (Precha Atsawanan et al., 2018). In four crop starches, amylopectin is thought to consist of 4 chains, and the average chain length of A chains, B1 chains, B2 chains, B3 chains, and B4 chains were 12–16, 20–24, 42–48, 69–75, 104–140, respectively. (Hizukuri, 1986). The chain-length distribution of amylopectin and the amylopectin chain ratio (ACR value) are related to the gelatinization temperature (GT) (Bao et al., 2009; Nakamura et al., 2002). Based on the GT, the amylopectin structure types of rice starch can be classified into two types: low GT-type (LGT; GT < 72 °C) and high GT-type (HGT; GT > 77 °C) (Fu et al., 2022).

Abbreviations: AC, Amylose content; DP, Degree of chain polymerization; GT, Gelatinization temperature; *Wx*, Waxy gene; ACR, Amylopectin chain ratio; WT, Wild-type; SEM, Scanning electron microscopy; ATR, Attenuated total reflectance; RVA, Rapid Viscosity Analyzer; PKV, Peak viscosity; HPV, Hot paste viscosity; CPV, Cool paste viscosity; BDV, Breakdown viscosity; SBV, Setback viscosity; T_o, Onset temperature; T_p, Peak temperature; T_c, Conclusion temperature; ΔH, Gelatinization enthalpy; HPAEC-PAD, High-performance anion-exchange chromatography with pulsed amperometric detection; HPGPC, High-performance size exclusion chromatography.

* Corresponding authors.
E-mail addresses: little_foos@aliyun.com (Y. Fu), zhuzhongyan123@163.com (Z. Zhu), liping6575@163.com (P. Li), Zhujun987@126.com (J. Zhu).

https://doi.org/10.1016/j.carbpol.2023.120595
Received 16 November 2022; Received in revised form 31 December 2022; Accepted 14 January 2023
Available online 16 January 2023
0144-8617/

The CRISPR/Cas9 system in waxy rice breeding among diverse germplasm has been fully elaborated (Ma et al., 2015; Zhang et al., 2018). The *Waxy* (*Wx*) gene was edited to produce null mutations by CRISPR/Cas9, to generate *waxy* (*wx*) mutant lines with a very low amylose content (AC), but without changing major agronomic traits (Zhang et al., 2018). From a recent study on the physicochemical properties of *wx* mutants grain via CRISPR/Cas9, five classes of waxy rice flour were obtained with dramatically different quality (Fu et al., 2022). Although these studies have provided a new fast strategy for waxy rice breeding and starch production, the starch quality of waxy rice is still difficult to predict, and more information is needed to obtain quality waxy rice starch before gene editing.

In the current study, two major *Wx* alleles (Wx^a and Wx^b) were selected, and in four elite cultivated rice varieties the *Wx* gene was edited. These varieties were QN (Wx^a), H36 (Wx^a), YX (Wx^b) and CZB (Wx^b). Starch in *wx* mutant lines was examined for functional and physicochemical characteristics, and the relationships between physicochemical characteristics of wild types and their corresponding *wx* mutant lines were determined. Moreover, we analyzed the major factors affecting various physicochemical characteristics only for *wx* mutant lines or waxy rice starch. Such information can not only contribute to producing waxy rice starch with specific characteristics but can also help to expand its application in the food and other industries.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, four elite rice varieties with two major *Wx* gene alleles were selected, including two Wx^a allele varieties (QN and H36) and two Wx^b allele varieties (YX and CZB). These rice varieties, which are mainly planted in eastern Asia and western China, were supplied by the Rice Research Institute of Sichuan Agricultural University. Unless indicated, all the rice lines were grown in paddy fields in Chengdu, China, during the normal rice-growing seasons.

2.2. Gene editing and cloning

The CRISPR/Cas9 targeted genome editing tool was constructed as previously described (Fu et al., 2022). The primer sequences used to construct the vector are shown in Fig. 1B and Table S1. The PCR mix being based on the Foregene PCR Fast Mix. Target site sequencing of all plants by direct or cloned sequencing of the PCR products were amplified using the primer set *Wx*-seq (Fig. 1A, B and Table S1). The PCR products were sequenced by the Sanger method.

2.3. Measurements of rice starch

Waxy rice and rice starch physicochemical properties were determined as follows: the seeds of a single *wx* mutant with T5 plant stable inheritance were dried at 37 °C for two weeks. The dried seeds were shelled, polished, and milled using a rice pearling mill, and sieved through a 74-μm mesh. Starch was prepared following the method of Zhang et al. (2016).

The mature seeds were harvested from homozygote plants and used for determination after storing at room temperature for >7 days. The AC was measured according to the method of Umemoto et al. (1995).

2.4. Scanning electron microscopy (SEM)

The morphology of *wx* mutant starches was examined by scanning electron microscopy (SEM, Phenom Pro, Phenom, Holland) operating at 5 kV, and gold coating was applied prior to SEM analysis (Zhu et al., 2020).

2.5. Crystalline characteristics

The crystalline characteristics of starch samples were analyzed using the wide-angle X-ray powder diffraction data (Rigaku Ultima IV, Japan). This instrument was operated at 40 kV and 100 mA with measurement angle (2θ) was in the range of 10 to 90° and scanning rate was 2°/min. The relative crystallinity (RC, %) was calculated using the Jade 6.0 software.

2.6. Fourier transforms infrared spectroscopy

FT-IR spectra of rice starches were obtained with a Thermo Scientific Nicolet iS50 spectrometer (Thermo Fisher Scientific, America) using an attenuated total reflectance (ATR). The Omnic version 8.2 software was chosen to analyze the ATR spectrum of the starch sample in the range of 400–4000 cm^{-1}. The ratio of 1047 to 1022 cm^{-1} obtained from the ATR spectrum refers to the ratio of the crystalline area to the amorphous area in starch.

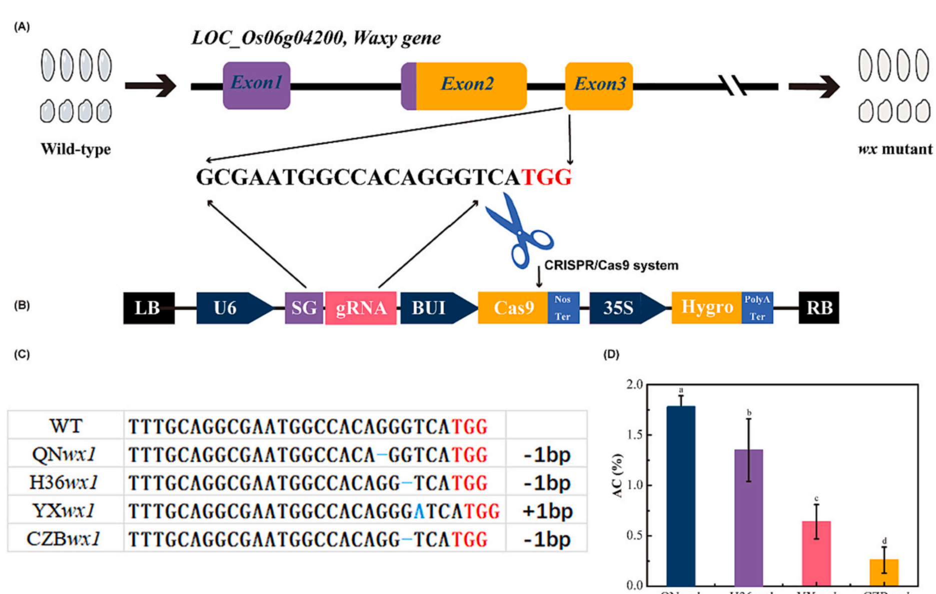

Fig. 1. Identification of *wx* mutant lines. (A) Schematic experimental design. From left to right: wild-type rice, *Waxy* gene (*LOC_Os06g04200*), target sequence, the CRISPR/Cas9 system and waxy rice. (B) CRISPR/Cas9 gene editing vector. LB, Left border; U6, Rice U6 promoter; SG, Guide RNA; UBI, UBI promoter; NOS Ter, NOS terminator; 35S, 35S promoter; Hygro, Hygromycin gene; PolyA Ter, PolyA terminator; RB, Right border. (C) Nucleotide variations at the targets (the protospacer adjacent motif in blank) of homozygous mutant lines from QN*wx1*, H36*wx1*, YX*wx1* and CZB*wx1*. "–", base deletion; "+", base insertion. The targeted sequence is highlighted in blue, and the protospacer adjacent motif (PAM) sequences are in red. (D) Amylose content of *wx* mutant starches and their corresponding WTs.

2.7. Water solubility

Starch samples were suspended in water at the concentration of 1 % (*w/v*) in centrifuge tubes. The starch samples were then heated in a water bath at 55, 75 and 95 °C each for 30 min, with frequent vortex mixing at 2 min intervals. Calculation of water solubility was performed according to the method of Precha Atsawanan et al. (2018).

2.8. Gel consistency

The gel consistency of starch was assessed according to the method of Vandeputte, Vermeylen, et al. (2003). Starch samples were placed in 13 × 100 mm tubes containing 200 μL 0.025 % thymol blue, and then 2.0 mL 0.2 M KOH in boiling water was added for 3 min. Gel consistency was measured in mL as the length when laid flat on the graph for 30 min.

2.9. Pasting properties

Pasting properties of rice starch were measured using a Rapid Viscosity Analyzer (RVA, model 3D, Newport Scientific, Warriewood, Australia) and Thermocline for Windows software (version 2.0) according to the methods of Park et al. (2007). The pasting parameters of starches included the peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown viscosity (BDV = PKV − HPV), setback viscosity (SBV = PKV − HPV), and peak time.

2.10. Thermal properties

Thermal properties of rice starch were determined using differential scanning calorimetry (DSC Q2000, TA Instruments, Ltd., Crawley, United Kingdom) according to the methods of Patindol et al. (2009). The thermal parameters of starches, such as the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and gelatinization enthalpy (ΔH) were evaluated.

2.11. Amylopectin chain-length distribution and molecular weight

The chain-length distribution of amylopectin was measured using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) according to the methods of Fu et al. (2022).

The molecular weight of starch was measured using a high-performance size exclusion chromatography (HPGPC) system according to the methods of Huang and Lai (2014).

2.12. Data analysis

All experiments were repeated three times independently. The results are expressed as mean ± standard deviation. Data analysis was performed with SPSS 25.0 (IBM, USA), and values in the same column with the same letters do not differ significantly ($p < 0.05$). SnapGene 4.3 was used to visualize PCR and cloning.

3. Results and discussion

3.1. Mutant isolation

Four elite cultivated rice varieties with two different major *Wx* alleles were selected QN (*Wx*^{a}), H36 (*Wx*^{a}), YX (*Wx*^{b}) and CZB (*Wx*^{b}), respectively, which are widely grown in Southwest China. We designed a CRISPR/Cas9 construct to accurately target the third exon of the *Wx* gene to generate a null mutation (Fig. 1A and B). We observed that over 80 % of T_0 transformants were mutants in the four varieties (Table S2), indicating that the CRISPR/Cas9 system showed high mutagenesis efficiency in rice (Ma et al., 2015). The *wx* trait mutations did not change the major agronomic traits of the T_1 generation lines, such as panicle number per plant, grain number per plant, and seed-setting frequency (Table S3). These results were consistent with previous studies (Fu et al., 2022; Huang et al., 2020; Zeng et al., 2020; Zhang et al., 2018). In addition, we identified single-base homozygous mutants of the four varieties, QN*wx1*, H36*wx1*, YX*wx1*, CZB*wx1*, and extracted their starch for further study (Fig. 1C).

3.2. AC and starch granular structure

The starch of *wx* mutant lines in this study represented in AC, ranged from 0.26 % to 1.78 % (Fig. 1D and Table 1). CZB*wx1* showed the lowest AC than the other three *wx* mutant lines, and the AC of the corresponding wild type (WT) was also lower than the other varieties (Table S3). The AC of rice with the *Wx*^{a} allele ranged from 21.6 % to 25 %, while that of rice with the *Wx*^{b} allele ranged from 14.2 % to 15.6 % (Sano, 1984). Rice varieties with *Wx*^{a} allele is generally produced more amylose than *Wx*^{b} allele. Similar results were found in *wx* mutants, *wx*^{a} mutant starches contained much amylose compared with *wx*^{b} mutant starches. Previous studies reported that the AC in waxy rice starch ranged from 0.2 % to 4.2 % (Chung et al., 2011; Kong, Zhu, et al., 2015; Precha Atsawanan et al., 2018). Furthermore, these other studies reported that the AC in waxy starch ranged from 0 % (waxy maize) up to approximately 9 % (waxy barley), and this was mainly related to plant species (Jane et al., 1999; Song & Jane, 2000). In the current study, the AC in *wx* mutant lines with similar to the range of AC in waxy rice, and could be called waxy rice starch.

The starch granular structure of *wx* mutant lines was different to WT lines in size and shape and showed a significant variation on SEM (Figs. 2A and S1). The granules ranged in size from 2.1 to 7.1 μm in different starches, and the granular size of *wx* mutant starches were bigger than their corresponding WTs due to lack of amylose. Our results showed that granular size and shape were consistent with previous reports (Kong, Zhu, et al., 2015; Singh et al., 2006; Singh Sodhi & Singh, 2003). The different genotypes of granule-bound starch synthase I (GBSSI) or other starch-synthesizing enzymes had an influence on starch granule diversity (Man et al., 2013; Shufen et al., 2019; Wani et al., 2012).

3.3. Crystalline structure

Figs. 2B and S2 show the X-ray diffraction patterns of *wx* mutant starches and their corresponding WTs. All the rice starches displayed an A-type pattern with a doublet at 17° and 18° and with individual peaks at 15°, 20° and 23°(Ong & Blanshard, 1995). The relative crystallinity of *wx* mutant starches was higher than WTs, ranging from 30.64 % to 45.62 % (Fig. S2). Similar, previous studies reported that the relative crystallinity in waxy rice starches or low amylose rice ranged from 40.3 % to 49.1 %, and waxy rice starches showed higher relative crystallinity as compared with normal rice starches (Kong, Kasapis, & Bao, 2015; Shi & Gao, 2011; Zeng et al., 2015). It had been reported that amylose content increased with a decrease in crystallinity in rice starches (Chung et al., 2011). In the current study, due to the AC decreasing significantly, the crystallinity of *wx* mutant starches increased significantly. Among these, the starch of H36*wx1* showed the highest relative crystallinity to other *wx* mutant starches, the starch of YX*wx1* and CZB*wx1* had a similar crystallinity, and the starch of QN*wx1* exhibited the lowest degree of crystallinity as compared with three *wx* mutants. Moreover, amylopectin fine structure is generally responsible for rice crystallinity, while amylose disrupts the crystallinity (Singh et al., 2007). Therefore, our study provided a good model for crystallinity research in rice starch, which eliminates amylose interference.

3.4. FTIR spectroscopy

Figs. 2C and S3 show the deconvoluted FTIR spectra of *wx* mutant starches and their corresponding WTs. The absorption peaks at 1047

Table 1
Amylose content, water solubility, gel consistency and RVA pasting of *wx* mutant starches.

Cultivar	AC (%)	Water solubility (%)	Gel consistency (cm)	PKV (cp)	HPV (cp)	BDV (cp)	HPV (cp)	SBV (cp)	Peak time (s)	PT (°C)
QN*wx1*	1.78 ± 0.11[a]	40.83 ± 1.14[a]	36 ± 2[a]	2768 ± 29[a]	1359 ± 13[a]	1408 ± 16[a]	1753 ± 43[a]	−1014 ± 15[a]	4.52 ± 0.01[a]	81.47 ± 0.15[a]
H36*wx1*	1.35 ± 0.31[b]	59.27 ± 0.81[b]	46 ± 2[b]	2473 ± 30[b]	1073 ± 34[b]	1401 ± 5[a]	1449 ± 36[b]	−1024 ± 7[a]	4.47 ± 0.01[b]	81.57 ± 0.10[a]
YX*wx1*	0.64 ± 0.17[c]	71.20 ± 1.10[c]	48 ± 1[b]	2779 ± 44[a]	1234 ± 33[c]	1546 ± 70[b]	1515 ± 40[b]	−1246 ± 84[b]	3.66 ± 0.01[c]	71.30 ± 0.06[c]
CZB*wx1*	0.26 ± 0.13[d]	78.83 ± 1.31[d]	74 ± 2[c]	2448 ± 23[b]	943 ± 14[d]	1505 ± 36[b]	1171 ± 20[c]	−1277 ± 22[b]	3.66 ± 0.01[c]	72.13 ± 0.08[d]

AC, PKV, HPV, BDV, HPV, SBV, PT and cp = peak viscosity, hot paste viscosity, cool paste viscosity, breakdown viscosity (BDV = PKV − HPV), pasting temperature, and centipoise, respectively.
Values in the same column with the same letters do not differ significantly ($p < 0.05$).

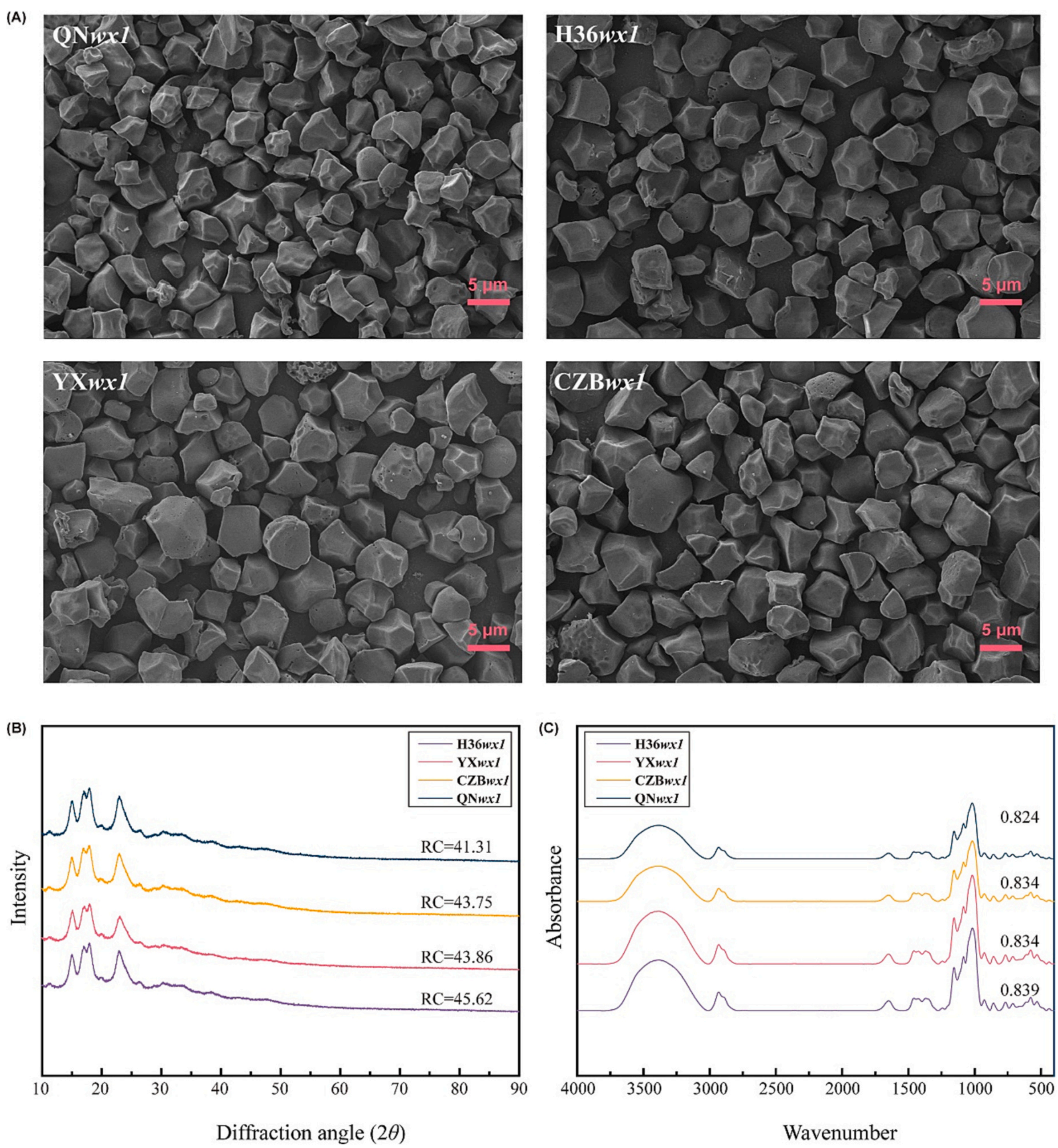

Fig. 2. Typical scanning electron micrographs (A), X-ray diffraction patterns (B) and deconvoluted Fourier transform infrared (FTIR) spectra (C) of *wx* mutant starches. The numbers of (B) at right are the relative crystallinity. The numbers of (C) at right are the ratio of 1047 cm^{-1} to 1022 cm^{-1}.

cm^{-1} and 1022 cm^{-1} reflect the crystallization and amorphous area in the rice starch granules, respectively. The ratio of the intensity of bans at 1047 cm^{-1} and 1022 cm^{-1} signifies the amount of crystallization to amorphous domains (Van Soest et al., 1995). All *wx* mutant starches had the higher value of ratio 1047/1022 compared with their corresponding WTs due to lack of amylose, ranging from 0.805 to 0.839 (Fig. S3) (Chung et al., 2011). H36*wx1* also showed the highest ratio value among all rice starches, while QN*wx1* had the lowest ratio value in all *wx* mutant starches. The FTIR results are in agreement with the XRD findings.

3.5. Water solubility

Water solubility is an important attribute in product fabrication, i.e., a highly solubilized product can be efficiently mixed with other ingredients and provide products with a uniform appearance. When starch is heated in excess water, the crystalline structure is disrupted, and water molecules become linked by hydrogen bonds to the exposed hydroxyl groups of amylose and amylopectin. This causes an increase in granule solubility (Lee et al., 2005). As shown in Table 1, when the starch concentration was 1 % (*w/v*) and the temperature was 95 °C, the highest solubility of CZB*wx1* was 77.8 %, while the solubility of H36*wx1* and QN*wx1* was 58.7 % and 39.9 %, respectively. Amylose has generally been considered to an insoluble starch. Our results showed that the water solubility decreased with an increase in AC in *wx* mutant starches.

3.6. Gel consistency

Fig. 3 shows the gel consistency of *wx* mutant starches at the concentration of 1 % (*w/v*), and the measured values of gel consistency are summarized in Table 1. Starch with a high AC is more likely to form hydrogen bonds between molecules, contributing to firm gels (Gani et al., 2013). The gel consistency of CZB*wx1* starch with the least AC was better than the other three *wx* mutants. However, this is not enough to explain the current experimental results. YX*wx1* also had a lower AC, but its gel consistency was similar to H36*wx1*. These results indicate that not only AC, but also other factors can affect gel consistency, such as the chain-length distribution and average chain length of amylopectin (Cagampang et al., 1973; Precha Atsawanan et al., 2018; Vandeputte, Derycke, et al., 2003). We obtained waxy rice starches with two different gel consistencies, which could be used in different areas.

3.7. Pasting properties

Figs. 4 and S4 show the RVA profiles of the four *wx* mutant starches

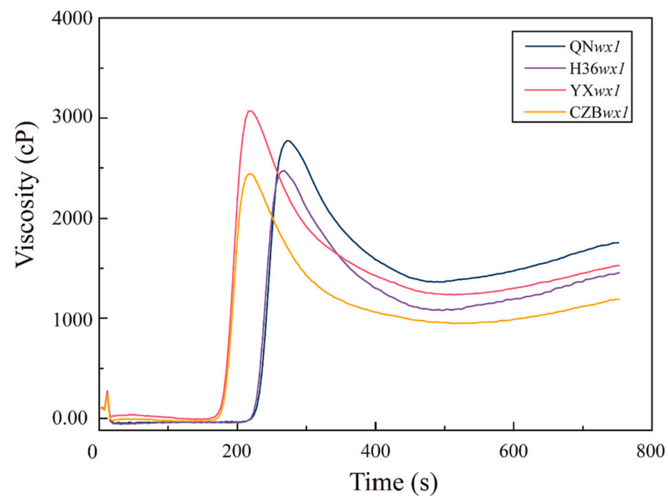

Fig. 4. RVA profiles of *wx* mutant starches.

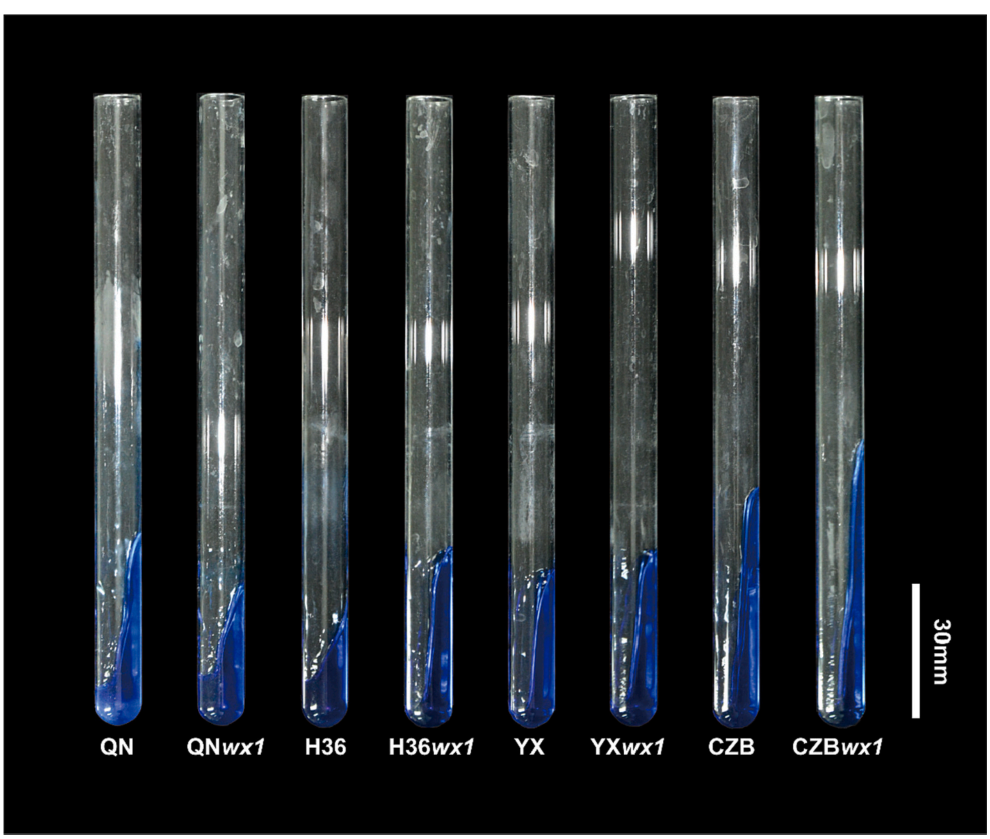

Fig. 3. Gel consistency of wx mutant starches and their corresponding WTs.

and their corresponding WTs, and the pasting properties (PKV, peak viscosity; HPV, hot paste viscosity; CPV, cool paste viscosity; BDV, breakdown viscosity; SBV, setback viscosity) of the starches are presented in Table 1. All *wx* mutant starches had lower pasting properties than their corresponding WT. The HPV among the different *wx* mutant starches ranged from 943 centipoise (cp) to 1359 cp, with the highest in QN*wx1* and the lowest in CZB*wx1*. The CPV in *wx* mutant starch samples ranged from 1171 cp to 1753 cp. The decrease of CPV in *wx* mutant starches was due to a significant reduction in AC (Arocas et al., 2009). The SBV showed that the starch pastes had a tendency to retrograde, while the SBV of waxy starch was often low. This was due to the lack of amylose in waxy starch leading to starch pastes which were not easy to retrograde (Kong, Kasapis, & Bao, 2015). In this study, the SBV of *wx* mutant starches showed negative values ranging from −1014 cp to −1277 cp, similar to waxy rice starches (Wang & Wang, 2002). Peak time is the time for viscosity to reach a peak. The peak time of wx^a mutants, and that of wx^b mutants, were quite similar, and wx^b mutants were lower than wx^a mutants. A high AC leads to a long peak time (Ren et al., 2017).

3.8. Thermal properties

The GT (onset, T_o; peak T_p; and conclusion, T_c) and enthalpy of gelatinization (ΔH) of the starches from four *wx* mutants and their corresponding WT lines are shown in Table 2. The GT of starches can be divided into three classes: low (T_p ranging from 66.6 to 70.5 °C), intermediate (T_p ranging from 73.5 to 77.6 °C), and high (T_p ranging from 79.7 to 82.5 °C) (Guo et al., 2019). For QN and H36 (*wx* mutant and WT), their starches had high GT. For YX and CZB (*wx* mutant and WT), their starches had low GT. The starches of all *wx* mutants had a gelatinization endotherm ranging from 66.07 to 85.86 °C and the ΔH ranged from 12.85 J/g to 15.48 J/g. This observation indicated that compared with WT starches, the starches of *wx* mutants displayed higher GT, enthalpy, and GT range (T_c–T_o). The ΔH indicates the overall degree of crystallinity (Tester & Morrison, 1990). Therefore, *wx* mutant starches were gelatinized longer than their corresponding WTs due to waxy rice starches had higher crystallinity. The results agree with X-ray and FTIR (Figs. S2 and S3). AC is considered to have an influence on GT, and increasing AC significantly increased starch pasting temperature (Jane et al., 1999). We also found that *wx* mutants (wx^b) with a lower-level AC (AC < 1 %) lead to the lower GT compared with *wx* mutants (wx^a) with AC > 1 %. However, it was previously reported that the GT of starch is also associated with chain length distributions of amylopectin (Sevenou et al., 2002). In the current study, we observed *wx* mutant starch had a similar GT to its corresponding WT, and this was because they had the similar ACR values (Fu et al., 2022).

3.9. Structural analysis

Figs. 5 and S5 shows the HPAEC-PAD chromatograms of the four isoamylase debranched *wx* mutant starches and their corresponding WTs, and the chain length distributions of the starches are presented in Table 2. The results indicated that the chain length distributions of wx^a mutant starches, and that of wx^b mutant starches (Fig. 5A, B), were quite similar, determined using HPAEC-PAD. We observed that the chain length proportion of DP6–11 (degree of chain polymerization) in wx^b mutant starches was higher than in wx^a, but that of DP ≥ 25 in wx^a mutant starches was higher than in wx^b, which means that rice varieties with the Wx^b allele will generate *wx* mutant starches containing more short-chains than Wx^a after *Wx* gene editing (Fig. 5C). GT was negatively correlated with the amount of short-chains (DP6–12) and positively correlated with long chains (DP ≥ 25) (Park et al., 2007). Therefore, wx^a mutant starches had the higher GT than wx^b mutant starches. Several studies have suggested that incorporation of the Wx^b allele into rice varieties will produce a higher percentage of short-chain amylopectin, and rice starches with Wx^b allele had a higher percentage of short A chains and a lower percentage of B1 chains than that of rice starches with Wx^a allele (Fan et al., 2017; Inouchi et al., 2005; Umemoto et al., 1999; Umemoto et al., 2002). Similar results were found in wx^b, and wx^a mutants, that is, the percentage of short chains wx^b mutant starches was also higher than wx^a, and the average chain length of wx^a mutants was higher than wx^b mutants (Table 2).

Compared with HPAEC-PAD, not only the variations in the percentage of starch short chains, but also long chains (DP > 70) were observed in HPGPC chromatograms. Fig. 6 shows the HPGPC–RID chromatograms of the four debranched *wx* mutant starches. The debranched CZB*wx1* starch exhibited two peaks (F1 and F2), and H36*wx1*, QN*wx1* and YX*wx1* showed three peaks. The shorter chains eluted first (F1), while the longer chains were subsequently eluted (F3). We observed that CZB*wx1*, with more intermediate molecular weight branched chains of starch (F2) and no or less amylose (F3), had the highest gel consistency and softest viscosity (Table 1). A previous study found that some low-amylose rice varieties showed softer gel consistency and lower viscosity (Zhang et al., 2019). Current research has shown that the ratio of amylose to amylopectin, the molecular weight/distribution of amylose and amylopectin, the degree of branching and the length of amylopectin affect swelling capacity and solubility (Chung et al., 2011; Precha Atsawanan et al., 2018; Wani et al., 2012; Yang

Table 2
The gelatinization temperature and distribution of amylopectin chain-length of *wx* mutant starches and corroding their WT.

Cultivar	Gene type	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_c-T_o (°C)	DP6–12 (%)	DP13–24 (%)	DP25–70 (%)	ACR	AFST
QN	Wx^a	74.32 ± 0.29^{a}	79.90 ± 0.11^{a}	83.01 ± 0.47^{a}	12.90 ± 0.10^{a}	8.69 ± 0.22^{a}	28.41 ± 0.07^{a}	57.13 ± 0.04^{a}	14.45 ± 0.10^{a}	0.16 ± 0.01^{a}	H
QN*wx1*	wx^a	74.91 ± 0.17^{b}	79.79 ± 0.47^{a}	85.87 ± 0.43^{b}	15.48 ± 0.29^{b}	10.97 ± 0.26^{b}	21.01 ± 0.12^{b}	50.12 ± 0.06^{b}	28.88 ± 0.07^{b}	0.14 ± 0.01^{b}	
H36	Wx^a	73.88 ± 0.23^{c}	80.08 ± 0.16^{a}	82.94 ± 0.14^{a}	11.04 ± 0.15^{c}	9.06 ± 0.11^{a}	27.02 ± 0.11^{c}	55.23 ± 0.04^{c}	17.75 ± 0.07^{c}	0.15 ± 0.01^{a}	H
H36*wx1*	wx^a	75.05 ± 0.17^{b}	79.74 ± 0.33^{a}	85.86 ± 0.16^{b}	15.17 ± 0.17^{b}	10.81 ± 0.05^{b}	21.26 ± 0.06^{d}	48.98 ± 0.10^{d}	29.76 ± 0.04^{d}	0.15 ± 0.01^{ab}	
YX	Wx^b	66.94 ± 0.09^{d}	70.32 ± 0.18^{b}	75.36 ± 0.22^{c}	10.86 ± 0.36^{c}	8.42 ± 0.14^{a}	35.19 ± 0.13^{e}	50.93 ± 0.11^{e}	13.88 ± 0.03^{e}	0.23 ± 0.01^{c}	L
YX*wx1*	wx^b	66.07 ± 0.11^{e}	70.25 ± 0.22^{b}	77.59 ± 0.51^{d}	12.85 ± 0.15^{a}	11.52 ± 0.50^{b}	26.92 ± 0.09^{c}	45.38 ± 0.09^{f}	27.69 ± 0.18^{f}	0.21 ± 0.01^{d}	
CZB	Wx^b	67.02 ± 0.10^{d}	70.56 ± 0.12^{b}	75.75 ± 0.23^{c}	11.69 ± 0.30^{d}	8.73 ± 0.16^{a}	26.51 ± 0.09^{f}	44.92 ± 0.11^{g}	28.57 ± 0.04^{g}	0.21 ± 0.01^{d}	L
CZB*wx1*	wx^b	66.35 ± 0.17^{e}	70.44 ± 0.13^{b}	77.21 ± 0.45^{d}	13.19 ± 0.15^{a}	10.86 ± 0.58^{b}	27.74 ± 0.17^{g}	45.32 ± 0.11^{f}	26.94 ± 0.08^{h}	0.22 ± 0.01^{cd}	

To, Tp, Tc, ΔH (J/g), ACR, AFST, I and H = onset, peak, and final gelatinization temperature, gelatinization enthalpy, amylopectin chain ratio, amylopectin fine structure type, high gelatinization temperature type, and low gelatinization temperature type, respectively.
Values in the same column with the same letters do not differ significantly ($p < 0.05$).

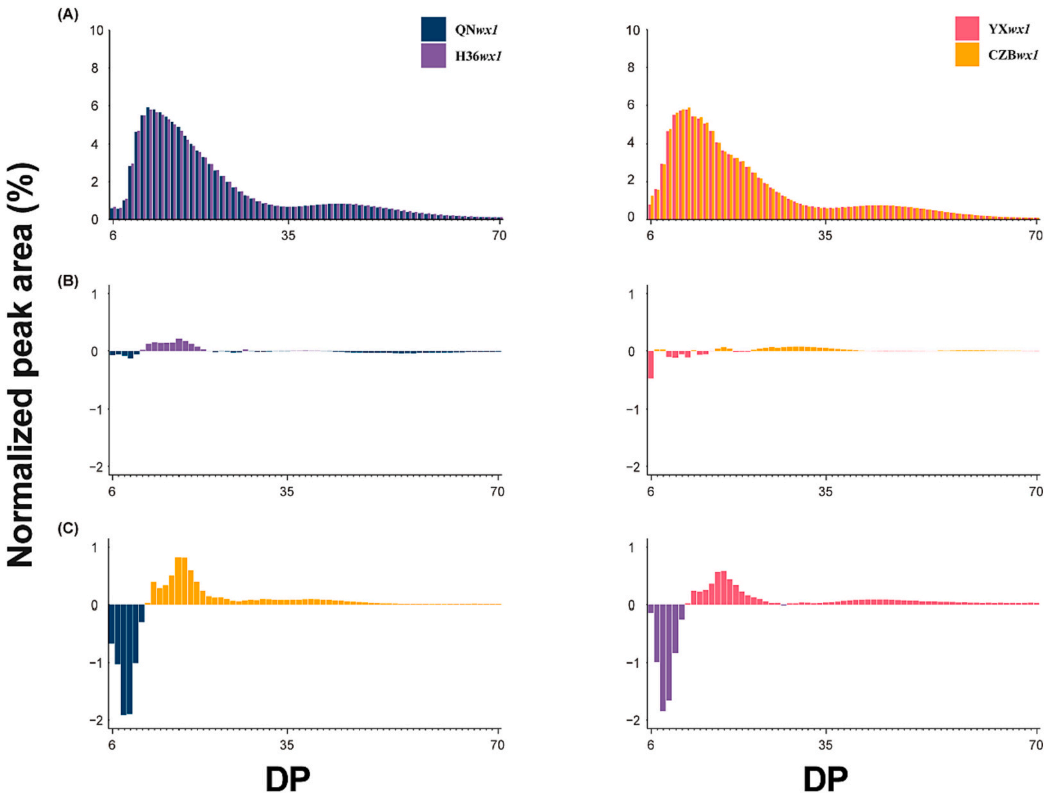

Fig. 5. HPAEC-PAD chromatograms of enzymatic debranched *wx* mutant starches. (A) The difference in the chain-length distribution of amylopectin between *wx* mutants with the same *Wx* allele. (B) Comparison of percentage values of amylopectin chain length between *wx* mutants with the same *Wx* allele. From left to right: QN*wx1* – H36*wx1* and YX*wx1* – CZB*wx1*. (C) Comparison of percentage values of amylopectin chain length between wx^a mutants and wx^b mutants. From left to right: QN*wx1* – CZB*wx1* and H36*wx1* – YX*wx1*.

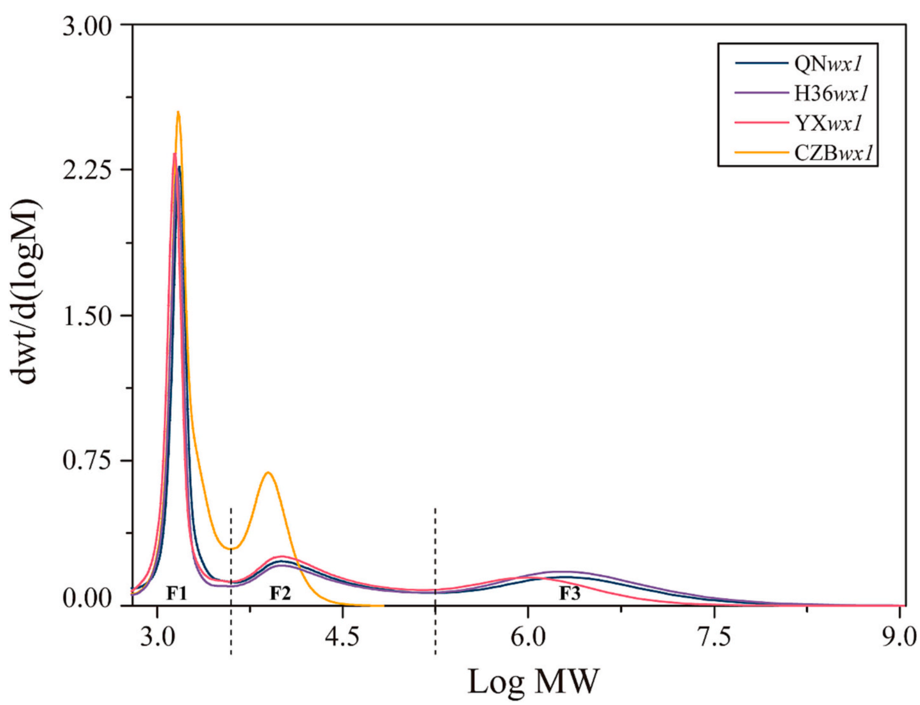

Fig. 6. HPGPC chromatograms of enzymatic debranched *wx* mutant starches.

et al., 2020). As a consequence, this may be the reason for the difference in solubility and gel consistency between CZB*wx1* and the other three varieties.

Based on the ACR value and the range of GT, the type of amylopectin fine structure can be classified into one of two types: LGT-type (GT < 72 °C, ACR > 0.18) and HGT-type (GT > 77 °C, ACR < 0.18) (Fu et al., 2022). The ACR value is a significant factor affecting physicochemical properties of rice starches, is negatively correlated with GT, as the range of GT is directly determined by the ACR value (Nakamura, 2002). In this study, the amylopectin fine structure type of wx^a mutants were HGT-type, and that of wx^b mutants were LGT-type. More importantly, our results demonstrated that the amylopectin fine structure of *wx* mutant starches was the same type as their corresponding WT lines (Table 2), indicating that the production of waxy rice starch with a well-defined amylopectin fine structure, a well-defined GT, and different physicochemical properties is possible.

3.10. Selection of waxy rice starch

From these study results, we can highlight several points to produce waxy rice starch. First, WT with the *Wx*^{b} allele or low initial AC should be selected for gene editing by CRISPR/Cas9 if a waxy rice variety with a lower AC, or vice versa is required (Huang et al., 2020; Pérez et al., 2019; Zhang et al., 2018). Second, according to the GT, we can predict the type of amylopectin fine structure, and the percentage of the A and B1 chains. Therefore, it is easy to produce waxy rice starch with a well-defined GT and a well-defined amylopectin fine structure (Fu et al., 2022). Moreover, we found that the *Wx*^{b} allele will generate *wx*^{b} mutant starches with more short-chains and less long-chains than *wx*^{a} mutant starches; thus, the production of waxy rice starches with target chain length distributions of amylopectin is possible. Finally, we can also generate waxy rice starches with different water solubility, gel consistency and pasting properties using CRISPR/Cas9 to edit the *Wx* gene, which means they will have different applications, and the reasons for this discrepancy require further study.

4. Conclusion

In the current study, four waxy rice starches were obtained by *Wx* gene editing. The *wx*^{b} mutant starches had lower AC than *wx*^{a} mutants, and the WT with low initial AC will generate *wx* mutant starch with lower AC. The *wx*^{b} mutants would generate starches with more short-chains, and less long-chains than *wx*^{a} mutant starches, leading to the GT of *wx*^{b} mutant starches being lower than that of *wx*^{a} mutant starches. Both the *wx* mutants and their corresponding WTs had similar amylopectin fine structure and GT types, suggesting that waxy rice starch with well-defined GT and amylopectin fine structure could be selected for production programs. Moreover, CZB*wx1* had higher water solubility and gel consistency than H36*wx1*, QN*wx1* and YX*wx1*, due to its unique amylopectin fine structure, but the cause of this discrepancy is unclear. Therefore, further studies are necessary to analyze the relationship between the physicochemical properties of the *wx* mutants and their corresponding WTs with a view to producing waxy rice starch with other well-defined physicochemical properties.

CRediT authorship contribution statement

Y.F., and J.Z. designed the strategy. Y.H., T.L., C.L., B.Z. X.Z., Z.Z. and T.Y. completed part of the experiments. Y.F., Z.Z. and J.Z. organized the Figures and article modification. Y.F., Z.Z., P.L. and J.Z. analyzed data, and wrote the paper. All authors commented on the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Data availability

Data will be made available on request.

Acknowledgements

We thank the Sichuan Science and Technology Program (2022ZDZX0016, 2021YFN0043, 2020ZHFP0017, and 2020YFN0006), Xinglin Scholar Research Promotion Project of Chengdu University of Traditional Chinese Medicine (MPRC2022036), and Chengdu Science and Technology Program (2021-YF05-02115-SN) for supporting this work. The authors thank Sanshu Biotechnology for their technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.120595.

References

Arocas, A., Sanz, T., & Fiszman, S. M. (2009). Clean label starches as thickeners in white sauces. Shearing, heating and freeze/thaw stability. *Food Hydrocolloids, 23*(8), 2031–2037. https://doi.org/10.1016/j.foodhyd.2009.04.006

Bao, J., Corke, H., & Sun, M. (2004). Genetic diversity in the physicochemical properties of waxy rice (Oryza sativa L) starch. *Journal of the Science of Food & Agriculture, 84* (11), 1299–1306. https://doi.org/10.1002/jsfa.1750

Bao, J., Xiao, P., Hiratsuka, M., Sun, M., & Umemoto, T. (2009). Granule-bound SSIIa protein content and its relationship with amylopectin structure and gelatinization temperature of Rice starch. *Starch - Stärke, 61*(8), 431–437. https://doi.org/10.1002/star.200800115

Bean, M. M., Esser, C. A., & Nishita, K. D. (1984). Some physicochemical and food application characteristics of California waxy rice varieties. *Cereal Chemistry, 61*(6), 475–480. https://www.cerealsgrains.org/publications/cc/backissues/1984/Documents/chem61_475.pdf.

Cagampang, G. B., Perez, C. M., & Juliano, B. O. (1973). A gel consistency test for eating quality of rice. *Journal of the Science of Food & Agriculture, 24*(12), 1589–1594. https://doi.org/10.1002/jsfa.2740241214

Chung, H. J., Liu, Q. A., Lee, L., & Wei, D. Z. (2011). Relationship between the structure, physicochemical properties and in vitro digestibility of rice starches with different amylose contents. *Food Hydrocolloids, 25*(5), 968–975. https://doi.org/10.1016/j.foodhyd.2010.09.011

Fan, M., Wang, X., Sun, J., Zhang, Q., Xu, Z., & Xu, Q. (2017). Effect of indica pedigree on eating and cooking quality in rice backcross inbred lines of indica and japonica crosses. *Breeding Science, 67*(5), 450–458. https://doi.org/10.1270/jsbbs.16191

Fu, Y., Luo, T., Hua, Y., Yan, X., Liu, X., Liu, Y., Zhang, B., Liu, R., Zhu, Z., & Zhu, J. (2022). Assessment of the characteristics of waxy Rice mutants generated by CRISPR/Cas9 [Original Research]. *Frontiers in Plant Science, 13*. https://doi.org/10.3389/fpls.2022.881964

Gani, A., Wani, S. M., Masoodi, F. A., & Salim, R. (2013). Characterization of rice starches extracted from Indian cultivars. *Food Science and Technology International, 19*(2), 143–152. https://doi.org/10.1177/1082013212442189

Guo, L., Li, J., Gui, Y., Zhu, Y., & Cui, B. (2019). Improving waxy rice starch functionality through branching enzyme and glucoamylase: Role of amylose as a viable substrate. *Carbohydrate Polymers, 230*, Article 115712. https://doi.org/10.1016/j.carbpol.2019.115712

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research, 147*(2), 342–347. https://doi.org/10.1016/s0008-6215(00)90643-8

Huang, L., Li, Q., Zhang, C., Chu, R., Gu, Z., Tan, H., Zhao, D., Fan, X., & Liu, Q. (2020). Creating novel wx alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system. *Plant Biotechnology Journal, 18*(11), 2164–2166. https://doi.org/10.1111/pbi.13391

Huang, Y.-C., & Lai, H.-M. (2014). Characteristics of the starch fine structure and pasting properties of waxy rice during storage. *Food Chemistry, 152*, 432–439. https://doi.org/10.1016/j.foodchem.2013.11.144

Inouchi, N., Hibiu, H., Li, T., Horibata, T., Fuwa, H., & Itani, T. (2005). Structure and properties of endosperm starches from cultivated Rice of Asia and other countries. *Journal of Applied Glycoscience, 52*(3), 239–246. https://doi.org/10.5458/jag.52.239

Jane, J., Chen, Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S., Radosavljevic, M., & Kasemsuwan, T. (1999). Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry Journal, 76*(5), 629–637. https://doi.org/10.1094/cchem.1999.76.5.629

Juliano, B. O. (1998). Varietal impact on rice quality, 214-216, 218-222 *Cereal Foods World, 43*(4), 207–211 https://eurekamag.com/research/003/331/003331373.php.

Kong, X., Kasapis, S., & Bao, J. (2015). Viscoelastic properties of starches and flours from two novel rice mutants induced by gamma irradiation. *LWT - Food Science and Technology, 60*(1), 578–582. https://doi.org/10.1016/j.lwt.2014.08.034

Kong, X., Zhu, P., Sui, Z., & Bao, J. (2015). Physicochemical properties of starches from diverse rice cultivars varying in apparent amylose content and gelatinisation temperature combinations. *Food Chemistry, 172*, 433–440. https://doi.org/10.1016/j.foodchem.2014.09.085

Lee, J. S., Kumar, R. N., Rozman, H. D., & Azemi, B. M. N. (2005). Pasting, swelling and solubility properties of UV initiated starch-graft-poly(AA). *Food Chemistry, 91*(2), 203–211. https://doi.org/10.1016/j.foodchem.2003.08.032

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., & Liu, Y. G. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant, 8*(8), 1274–1284. https://doi.org/10.1016/j.molp.2015.04.007

Man, J., Yang, Y., Huang, J., Zhang, C., Chen, Y., Wang, Y., Gu, M., Liu, Q., & Wei, C. (2013). Effect of simultaneous inhibition of starch branching enzymes I and IIb on the crystalline structure of Rice starches with different amylose contents. *Journal of Agricultural and Food Chemistry, 61*(41), 9930–9937. https://doi.org/10.1021/jf4030773

Nakamura, Y. (2002). Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: Rice endosperm as a model tissue. *Plant & Cell Physiology, 43*(7), 718–725. https://doi.org/10.1093/pcp/pcf091

Nakamura, Y., Sakurai, A., Inaba, Y., Kimura, K., Iwasawa, N., & Nagamine, T. (2002). The fine structure of amylopectin in endosperm from asian cultivated Rice can be largely classified into two classes. *Starch - Stärke, 54*(3–4), 117–131. https://doi.org/10.1002/1521-379X(200204)54:3/4<117::AID-STAR117>3.0.CO;2-2

Ong, M. H., & Blanshard, J. M. V. (1995). Texture determinants in cooked, parboiled rice. I: Rice starch amylose and the fine stucture of amylopectin. *Journal of Cereal Science, 21*(3), 251–260. https://doi.org/10.1006/jcrs.1995.0028

Park, I. M., Ibáñez, A. M., Fang, Z., & Shoemaker, C. F. (2007). Gelatinization and pasting properties of waxy and non-waxy rice starches. *Starch - Stärke, 59*(8), 388–396. https://doi.org/10.1002/star.200600570

Patindol, J., Gu, X., & Wang, Y. J. (2009). Chemometric analysis of the gelatinization and pasting properties of long-grain rice starches in relation to fine structure. *Starch - Stärke, 61*(1), 3–11. https://doi.org/10.1002/star.200800022

Pérez, L., Soto, E., Farré, G., Juanos, J., Villorbina, G., Bassie, L., Medina, V., Serrato, A. J., Sahrawy, M., Rojas, J. A., Romagosa, I., Muñoz, P., Zhu, C., & Christou, P. (2019). CRISPR/Cas9 mutations in the rice Waxy/GBSSI gene induce allele-specific and zygosity-dependent feedback effects on endosperm starch biosynthesis. *Plant Cell Reports, 38*(3), 417–433. https://doi.org/10.1007/s00299-019-02388-z

Precha Atsawanan, S., Puncha Arnon, S., Wandee, Y., Uttapap, D., Puttanlek, C., & Rungsardthong, V. (2018). Physicochemical properties of partially debranched waxy rice starch. *Food Hydrocolloids, 79*, 71–80. https://doi.org/10.1016/j.foodhyd.2017.12.014

Puchongkavarin, H., Varavinit, S., & Bergthaller, W. (2005). Comparative study of pilot scale Rice starch production by an alkaline and an enzymatic process. *Starch - Stärke, 57*(3–4), 134–144. https://doi.org/10.1002/star.200400279

Ren, J., Li, Y., Li, C., Gu, Z., Cheng, L., Hong, Y., & Li, Z. (2017). Pasting and thermal properties of waxy corn starch modified by 1,4-alpha-glucan branching enzyme. *International Journal of Biological Macromolecules, 97*, 679–687. https://doi.org/10.1016/j.ijbiomac.2017.01.087

Sano, Y. (1984). Differential regulation of waxy gene expression in rice endosperm. *Theoretical and Applied Genetics, 68*(5), 467–473. https://doi.org/10.1007/BF00254822

Sevenou, O., Hill, S. E., Farhat, I. A., & Mitchell, J. R. (2002). Organisation of the external region of the starch granule as determined by infrared spectroscopy. *International Journal of Biological Macromolecules, 31*(1), 79–85. https://doi.org/10.1016/S0141-8130(02)00067-3

Shi, M.-M., & Gao, Q.-Y. (2011). Physicochemical properties, structure and in vitro digestion of resistant starch from waxy rice starch. *Carbohydrate Polymers, 84*(3), 1151–1157. https://doi.org/10.1016/j.carbpol.2011.01.004

Shufen, C., Yicong, C., Baobing, F., Guiai, J., Zhonghua, S., Ju, L., Shaoqing, T., Jianlong, W., Peisong, H., & Xiangjin, W. (2019). Editing of Rice isoamylase gene ISA1 provides insights into its function in starch formation. *Rice Science, 26*(2), 77–87. https://doi.org/10.1016/j.rsci.2018.07.001

Singh, H., Lin, J. H., Huang, W. H., & Chang, Y. H. (2012). Influence of amylopectin structure on rheological and retrogradation properties of waxy rice starches. *Journal of Cereal Science, 56*(2), 367–373. https://doi.org/10.1016/j.jcs.2012.04.007

Singh, N., Kaur, L., Sandhu, K. S., Kaur, J., & Nishinari, K. (2006). Relationships between physicochemical, morphological, thermal, rheological properties of rice starches. *Food Hydrocolloids, 20*(4), 532–542. https://doi.org/10.1016/j.foodhyd.2005.05.003

Singh, N., Nakaura, Y., Inouchi, N., & Nishinari, K. (2007). Fine structure, thermal and viscoelastic properties of starches separated from indica Rice cultivars. *Starch - Stärke, 59*(1), 10–20. https://doi.org/10.1002/star.200600527

Singh Sodhi, N., & Singh, N. (2003). Morphological, thermal and rheological properties of starches separated from rice cultivars grown in India. *Food Chemistry, 80*(1), 99–108. https://doi.org/10.1016/S0308-8146(02)00246-7

Song, Y., & Jane, J. (2000). Characterization of barley starches of waxy, normal, and high amylose varieties. *Carbohydrate Polymers, 41*(4), 365–377. https://doi.org/10.1016/S0144-8617(99)00098-3

Tester, R. F., & Morrison, W. R. (1990). Swelling and gelatinization of cereal starches. I. Effects of amylopectin, amylose, and lipids. *Cereal Chemistry, 67*, 551–557. https://www.cerealsgrains.org/publications/cc/backissues/1990/documents/67_551.pdf.

Umemoto, T., Nakamura, Y., & Ishikura, N. (1995). Activity of starch synthase and the amylose content in rice endosperm. *Phytochemistry, 40*(6), 1613–1616. https://doi.org/10.1016/0031-9422(95)00380-P

Umemoto, T., Terashima, K., Nakamura, Y., & Satoh, H. (1999). Differences in amylopectin structure between two Rice varieties in relation to the effects of temperature during grain-filling. *Starch - Stärke, 51*(2–3), 58–62. https://doi.org/10.1002/(sici)1521-379x(199903)51:2<58::Aid-star58>3.0.Co;2-j

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. *Theoretical and Applied Genetics, 104*(1), 1–8. https://doi.org/10.1007/s001220200000

Van Soest, J. J. G., Tournois, H., de Wit, D., & Vliegenthart, J. F. G. (1995). Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy. *Carbohydrate Research, 279*, 201–214. https://doi.org/10.1016/0008-6215(95)00270-7

Vandeputte, G. E., Derycke, V., Geeroms, J., & Delcour, J. A. (2003). Rice starches. II. Structural aspects provide insight into swelling and pasting properties. *Journal of Cereal Science, 38*(1), 53–59. https://doi.org/10.1016/S0733-5210(02)00141-8

Vandeputte, G. E., Vermeylen, R., Geeroms, J., & Delcour, J. A. (2003). Rice starches. III. Structural aspects provide insight in amylopectin retrogradation properties and gel texture. *Journal of Cereal Science, 38*(1), 61–68. https://doi.org/10.1016/S0733-5210(02)00142-X

Wang, Y.-J., & Wang, L. (2002). Structures of four waxy Rice starches in relation to thermal, pasting, and textural properties. *Cereal Chemistry, 79*(2), 252–256. https://doi.org/10.1094/CCHEM.2002.79.2.252

Wani, A. A., Singh, P., Shah, M. A., Schweiggert-Weisz, U., Gul, K., & Wani, I. A. (2012). doi:10.1111/j.1541-4337.2012.00193.x *Comprehensive Reviews in Food Science and Food Safety, 11*(5), 417–436. https://doi.org/10.1111/j.1541-4337.2012.00193.x.

Yang, T., Tan, X., Huang, S., Pan, X., Shi, Q., Zeng, Y., Zhang, J., & Zeng, Y. (2020). Effects of experimental warming on physicochemical properties of indica rice starch in a double rice cropping system. *Food Chemistry, 310*, Article 125981. https://doi.org/10.1016/j.foodchem.2019.125981

Zeng, D., Liu, T., Ma, X., Wang, B., Zheng, Z., Zhang, Y., Xie, X., Yang, B., Zhao, Z., Zhu, Q., & Liu, Y.-G. (2020). Quantitative regulation of waxy expression by CRISPR/Cas9-based promoter and 5'UTR-intron editing improves grain quality in rice. *Plant Biotechnology Journal, 18*(12), 2385–2387. https://doi.org/10.1111/pbi.13427

Zeng, F., Chen, F., Kong, F., Gao, Q., Aadil, R. M., & Yu, S. (2015). Structure and digestibility of debranched and repeatedly crystallized waxy rice starch. *Food Chemistry, 187*, 348–353. https://doi.org/10.1016/j.foodchem.2015.04.033

Zhang, C., Zhou, L., Zhu, Z., Lu, H., Zhou, X., Qian, Y., Li, Q., Lu, Y., Gu, M., & Liu, Q. (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. *Journal of Agricultural and Food Chemistry, 64*(20), 4048–4057. https://doi.org/10.1021/acs.jafc.6b00083

Zhang, C., Zhu, J., Chen, S., Fan, X., Li, Q., Lu, Y., Wang, M., Yu, H., Yi, C., Tang, S., Gu, M., & Liu, Q. (2019). Wx(lv), the ancestral allele of Rice waxy gene. *Molecular Plant, 12*(8), 1157–1166. https://doi.org/10.1016/j.molp.2019.05.011

Zhang, J., Zhang, H., Botella, J. R., & Zhu, J.-K. (2018). Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the waxy gene in elite rice varieties. *Journal of Integrative Plant Biology, 60*(5), 369–375. https://doi.org/10.1111/jipb.12620

Zhu, Z., Xie, C., Li, W., Hang, F., Li, K., Shi, C., & Doherty, W. O. S. (2020). Nutritional and antioxidant properties of non-centrifugal cane sugar derived from membrane clarified juice. *LWT - Food Science and Technology, 131*, Article 109717. https://doi.org/10.1016/j.lwt.2020.109717