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Genetic Effects of *Indica* Lineage Introgression on Amylopectin Chain Length Distribution in *Japonica* Milled Rice

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Abstract: The fine structure of amylopectin affects rice quality; in particular, the amylopectin chain length distribution (ACLD) in milled rice differs between subspecies of *Oryza sativa* L. However, the correlation between ACLD and quality trait factors, and the genetic basis of ACLD phenotypic variation, are still unknown. Here, the correlations of ACLD with cooking and eating quality and with the rapid viscosity analysis (RVA) index were studied using chromosome segment substitution lines (CSSLs). Clear variations in ACLD were observed in introgression lines: introgression of *indica* segments of chromosome 3 and 7 increased the proportion of amylopectin Fa, and another segment of chromosome 3 reduced the proportion of amylopectin Fb2. A segment of chromosome 11 decreased the proportion of amylopectin Fa but increased that of Fb3. Correlation analysis with the RVA index further showed that the breakdown viscosity (BDV) was negatively correlated with the proportion of amylopectin Fb1, Fb2, and Fb3 chains, and positively correlated with Fa. Consistency viscosity (CSV) values were negatively correlated with the proportion of amylopectin Fb1, Fb2, and Fb3 chains. We thus clarified the quality trait factors determined by variation in ACLD, and provide key information for pyramiding inter-subspecific genetic superiority in molecular design breeding for rice quality.

Keywords: amylopectin chain length distribution; quality trait factors; RVA; *indica* lineage; genetic background

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1. Introduction

Rice is an important grain grown throughout China, where it has occupied the position of primary food crop since ancient times. In recent years, increasing attention has been paid to rice quality. *Indica* varieties generally have greater yield potential, whereas *japonica* varieties have advantages in quality. Therefore, although *indica–japonica* hybrid breeding technology is widely used to improve the yield of *japonica* rice, it causes quality deterioration due to the introgression of the *indica* rice lineage [1,2]. Amylose content and the ratio of amylose and amylopectin are important indicators in evaluating rice quality because they affect characteristics related to cooking and eating [3]. However, the texture and taste of varieties with similar amylose content can differ, which is believed to be the result of differences in amylopectin fine structure [4–6]

Research in recent years has shown that amylose content and amylopectin structure are the main factors affecting rice quality. Amylopectin accounts for 75–80% of starch content, and its configuration directly affects the taste quality and physicochemical properties of starch [7,8]. The fine structure of amylopectin encompasses chain length distribution, degree of branching, and average chain length, and the degree of polymerization (DP) is an important indicator to evaluate chain length distribution [9,10]. Compared with *japonica* rice, *indica* rice has higher amylose content, a lower amylopectin short-chain ($6 \leq DP \leq 11$) allocation ratio, and a higher amylopectin middle chain ($12 \leq DP \leq 24$) allocation ratio [7,11,12]. Studies have found that rice containing amylopectin with a high proportion of short chains and a low proportion of long chains has high peak viscosity (PKV)

and breakdown viscosity (BDV) values, which lead to a soft, sticky texture and better taste quality [13,14]. Li et al. found that rice hardness was positively correlated with the number of ultra-long amylopectin branches and negatively correlated with amylopectin having fewer than 70 branches [15]. Peng et al. found that the initial gelatinization temperature was significantly negatively correlated with the allocation rate of short-chain amylopectin ($6 \leq DP \leq 11$) and positively correlated with the proportion of medium-chain amylopectin ($12 \leq DP \leq 24$) [7].

Previous research showed that the genes encoding starch branching enzyme IIb (*BEIIb*), starch synthase I (*SSSI*), and soluble starch synthase IIIa (*SSSIIa*) are key genes regulating amylopectin chain length distribution (ACLD), but these genes cannot be responsible for differences in ACLD between subspecies [16–18]. In this study, chromosome segment substitution lines (CSSLs) were constructed using the high-quality *japonica* cultivar SN625 as the recipient parent and the high-yield, low-quality *indica* cultivar TN013 as the donor parent; both parental genotypes were Wx^b. A correlation analysis was conducted between ACLD and quality traits in the CSSLs. We identified the genetic basis of ACLD, providing key information for future pyramiding of inter-subspecific genetic superiority in molecular design breeding.

2. Materials and Methods

2.1. Experimental Materials and Design

Variations in eating quality and gelatinization between rice subspecies are hypothesized to be a result of differences in ACLD. Therefore, a single chromosome segment substitution line (CSSL) population was constructed using the *japonica* rice cultivar SN625 as the recurrent parent and the *indica* rice cultivar TN013 as the donor parent; both parents had the Wx^b genotype, corresponding to low amylose content (Figure 1 and Supplementary Table S1). A total of 42 families, including the parents, were included in the CSSL population. The use of CSSLs can prevent the interference of different genetic backgrounds to a great extent, which aided in accurately analyzing the correlation between ACLD and quality trait factors. The samples were grown in a paddy field of the Chinese Academy of Agricultural Sciences in Beijing (116°25′ E, 39°54′ N) in May 2017. Each CSSL was planted in 3 rows that were spaced 30.0 cm apart; there were 10 holes per row, and plants were spaced 13.3 cm apart. Fertilizers were applied as follows: urea, 150 kg/hm^2; potassium sulfate, 75 kg/hm^2; and diammonium phosphate, 150 kg/hm^2. Cultivation methods and field management followed local practices. Three independent biological replicates were performed for each experiment.

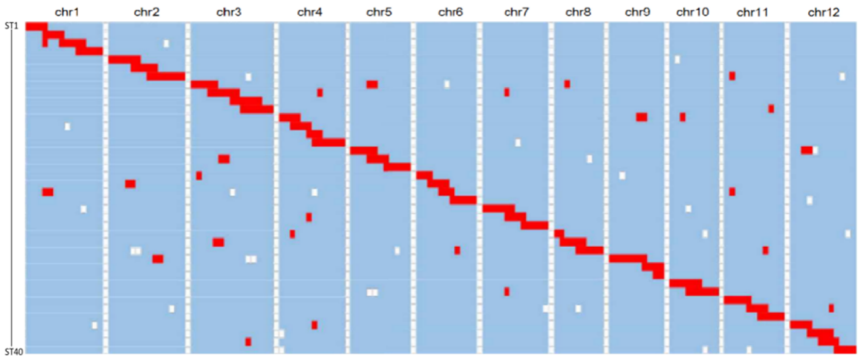

Figure 1. Schematic diagram of consanguinity infiltration of the *indica* lineage in the chromosome segment substitution line (CSSL) population. Red indicates the infiltrated *indica* rice fragment, blue indicates the background *japonica* parent SN625, and white indicates undetected regions.

2.2. Amylopectin Separation and Purification

Refined rice flour (0.15 g) was dispensed into a tube and soaked with anhydrous ethanol, then 5.25 mL of 0.5 mol/L NaOH was added. The mixture was placed in a boiling

water bath for 10 min, after which it was shaken until the solution was clear. Samples were centrifuged at 14,681× *g* for 10 min at 4 °C and the supernatant was neutralized with 1 mol/L HCl until the solution color changed to milk-white. N-butanol:isoamyl alcohol (3:1; 1.5 mL) was added to the tube and the mixture was boiled for 10 min with regular shaking. After cooling to room temperature, samples were incubated at 4 °C for 24 h. Samples were then centrifuged at 4 °C for 20 min at 14,681× *g*, and 1 mL n-butanol:isoamyl alcohol (1:1) was added to the supernatant. The resulting mixture was boiled for 10 min then shaken. Samples were cooled to room temperature and incubated at 4 °C for 48 h. This last centrifugation and boiling step was repeated 2–3 times to purify the sample. The supernatant was concentrated with nitrogen at 35 °C for 3–4 h to reduce the volume of the solution to half of the original volume. Anhydrous ethanol (twice the sample volume) was added and the samples were incubated on ice for 1–2 h. Samples were centrifuged at 14,681× *g* for 20 min at 4 °C and the precipitate was dried in a vacuum for 48 h at 35 °C to obtain amylopectin powder.

2.3. Debranching and Detection of Amylopectin

Purified amylopectin (0.05 g) was dissolved in 5 mL HAc-NaAc buffer (50 mmol/L, pH = 3.5), and the solution was incubated in a boiling water bath for 15 min. After cooling, 25 µL isoamylase (1000 U/µL, 15284, Sigma, St. Louis, MO, USA) was added and oscillated at 40 °C for 48 h. The mixture was then boiled for 15 min to inactivate the enzyme. Samples were centrifuged at 14,681× *g* and 4 °C for 10 min, followed by ultrasonic degassing of the supernatant for 10 min, then filtering with a 0.22-µm cellulose ester microporous membrane. The first 6 drops of the filtrate were discarded, and the remaining filtrate was transferred to a sample bottle and stored at 4 °C. The samples were analyzed with an ICS-3000 ion chromatograph (Dionex, Sunnyvale, CA, USA) with a CarboPac PA20 (3 mm × 150 mm) analytical column and a CarboPac PA20 (3 mm × 30 mm) guard column. The detection conditions were as follows: injection volume, 25 µL; column temperature, 30 °C. The mobile phase was 0.1 mol/L NaOH(A), 0.1 mol/L NaOH + 1 mol/L NaAc(B), and H_2O (C) with a 0.5 mL/min flow. The reference electrode was Ag/AgCl and the standard four-potential waveform was used for detection.

2.4. Determination of Rice Cooking and Eating Quality

Milled rice (30 g) was placed into a stainless steel tin and washed until the water ran clear. Water (42 g) was added at a weight ratio of 5:7, then samples were covered with paper and soaked for 30 min. Rice samples were steamed for 30 min and stewed for 10 min. The steamed rice was then stirred gently, taking care not to damage the rice grain structure, and kept cool in the fume hood for 20 min while covered with paper. An iron cover was then used while samples were cooled at room temperature for 1.5 h. Finally, 8 g of each sample was pressed into a rice cake. Appearance, viscosity, hardness, balance, and taste values of the rice cakes were measured with a rice taste meter (STA-1A, Japan Joshi Society).

2.5. Determination of Rice Starch Viscosity

The starch viscosity was measured with 3-D Rapid Viscosity Analyzer (Newport Scientific, Australia) and analyzed using Tew software (Thermal Cycle for Windows). Measurements were conducted following the standard method of the American Association of Cereal Chemists (AACC) Code of Practice (199561-02). Refined rice flour samples (3 g) with 14% water content were used, and 25.00 mL distilled water was added to the flour samples. Viscosity values are defined in Rapid Visco Units (RVU).

2.6. Determination of Amylose Content

The amylose content of rice was determined as described in the National Standard (GB/T 15683-2008) [19].

2.7. High-Throughput Sequencing

The genomic DNA of each sample was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method. 1.5 μg of genomic DNA from each sample was used to construct a library. The insert size of each library was about 350 bp. Whole-genome paired-end reads were sequenced using Illumina platforms (HiSeq2000/HiSeq2500/HiSeq X Ten) after confirming the quality of libraries. Clean reads were obtained by filtering the raw sequencing data, then the whole-genome sorted BAM file was obtained using BWA ver0.7.18 [20] and Samtools ver1.12 [21].

2.8. Statistical Analysis

GraphPad Prism 6.02 was used for variance analysis and histogram drawing, IBM SPSS Statistics 22 was used for data correlation analysis, and the corrplot package of Rv4.1.0 was used to draw the correlation heatmap [22]. The sequence data for 12 quality-related genes were retrieved from https://www.ricedata.cn/gene/, accessed date: 28 October 2021. Based on the physical location of each gene in the reference genome, the sections with parental genotyping and a read number greater than 5 were considered credible, and these data were used to compare the genotypes between parents.

3. Results

3.1. Genotyping of Amylopectin Synthesis-Related Genes in Parent Lines

Many genes are known to affect amylopectin synthesis and control its structure, the most typical of which are genes encoding soluble starch synthase (SSS), starch branching enzyme (SBE), and starch debranching enzyme (DBE). We performed whole-genome sequencing of the two parent lines (SN625 and TN013) with Next Generation Sequencing (NGS) to determine their genotypes for these genes. The sequencing data showed polymorphisms in seven genes, including *SSI*, *SSIIb*, *SSIIc*, *SSIIIa*, *BEIIb*, *ISA*, and *GIF1*, between the two parents (Table 1, Supplementary Tables S2–S8). However, the CSSLs with *indica* introgression segments that overlapped with those seven genes did not show significant differences in ACLD compared to SN625. Therefore, the genes with a natural variation that regulate ACLD differences between the two parents were still unknown.

Table 1. Gene information related to amylopectin synthesis between two parents.

Gene Names	Annotation	Position	CSSLs with Introgression	Polymorphism between Parents
SSI	starch synthase I	chr06:3079296...3086808	ST21	yes
SSIIa	soluble starch synthase 2–3	chr06:6748398...6753302	ST21	no
SSIIb	starch synthase	chr02:19355790...19367127	ST7 ST8	yes
SSIIc	starch synthase	chr10:15673243...15681075	ST35	yes
SSIIIa(FLO5)	starch synthase III	chr08:5352105...5363276	ST29	yes
BEI	1,4-alpha-glucan-branching enzyme	chr06:30897378...30905803	NA	no
BEIIa	1,4-alpha-glucan-branching enzyme 2	chr04:20240211...20243460	ST16	no
BEIIb	1,4-alpha-glucan-branching enzyme	chr02:19355790...19367127	ST7 ST8	yes
ISA(su1)	Alpha amylase	chr08:25893657...25900576	ST30	yes
PUL	Starch debranching enzyme	chr04:4408357...4418889	ST14	no
Pho1	alpha-glucan phosphorylase isozyme	chr03:31332033...31339163	ST13	no
GIF1	glycosyl hydrolases	chr04:20422171...20426921	ST16	yes

3.2. Amylose Content in CSSLs

Amylose content has a key impact on rice quality and is an important index used to measure rice quality. We, therefore, measured amylose content in CSSLs and the parent lines. Results showed that there was no significant difference in amylose content between

indica and *japonica* rice parents. Furthermore, the average amylose content of the CSSL population was 15.55 ± 0.89. This was comparable to the low amylose content found in *japonica* strains and showed a small coefficient of variation (Table 2). These results indicated that the quality differences in rice were not affected by amylose content.

Table 2. Amylose content in chromosome segment substitution lines and their parents.

Characters	SN265	TN013	Mean ± SD	Variation Coefficient
AC	16.09 a	17.23 a	15.55 ± 0.89	5.72%

The significant differences were analyzed with one-way ANOVA analysis ($p < 0.05$). The values with the same lowercase letters indicate no significant difference.

3.3. Determination of the Chromosome Segments Regulating ACLD in CSSLs

The proportion of amylopectin Fa chains (DP = 6–12) in the *japonica* parent SN625 was higher than that of the *indica* parent TN013. In addition, the introgression of the *indica* lineage led to increasing proportions of Fa chains (DP = 6–12) in many CSSLs compared with SN625. The introgression of 17318506...30301498 on chromosome 3 and 18576999...28940485 on chromosome 7 resulted in a significant increase in the proportion of Fa chains (DP = 6–12) in lines ST12 and ST27 compared with the *japonica* parent, whereas the introgression of 18077883...2810589 on chromosome 11 caused a significant decrease in the proportion of Fa chains (DP = 6–12) in ST38 (Figure 2a). The proportion of Fb1 chains (DP = 13–24) was significantly lower in SN625 than in TN013; the proportion of amylopectin Fb1 chains (DP = 13–24) in many CSSLs also increased compared with parent SN625 (Figure 2b). The proportion of amylopectin Fb2 chains (DP = 25–36) was also lower in SN625 than in TN013. Moreover, the proportion of Fb2 chains in many CSSLs was higher than in SN625. In contrast, the proportion of amylopectin Fb2 chains in ST11 was significantly lower than in SN625 due to the introgression of the 9789579...20785166 *indica* rice segment on chromosome 3 (Figure 2c). The proportion of amylopectin Fb3 chains (DP > 36) was lower in SN625 than in TN013. Furthermore, the Fb3 proportion in many CSSLs increased compared to SN625; for example, the proportion of Fb3 in ST38 was significantly higher than in SN625 as a result of the introgression of 18077883...28105891 *indica* rice fragments on chromosome 11 (Figure 2d).

3.4. Identification of Chromosome Segments Regulating Taste Quality Factors and RVA in CSSLs

Introgression of the *indica* lineage resulted in a significant decrease in rice appearance of four CSSLs compared to *japonica* rice SN625, and a significant increase in the appearance of 12 lines (Table 3). It also caused a significant decrease in rice hardness in five families, with ST1 reaching a significant level, and a significant increase in the viscosity of 10 families compared with SN625. Introgression of the *indica* lineage also resulted in significantly lower balance and taste values of the lines ST7, ST11, and ST37 compared with SN625 but caused a significant increase in the balance and taste values of multiple other families. Introgression of the *indica* lineage had an effect on each index of rapid viscosity analysis (RVA), leading to very significant increases or decreases of PKV, hot paste viscosity (HPV), and cool paste viscosity (CPV) in many families. However, BDV was less affected by the introgression of *indica* rice; only the BDV of lines ST6, ST28, ST32, and ST34 were reduced. Influenced by the lineage of *indica* rice, pasting temperature (PaT) increased or decreased to varying degrees in different lines. In addition, introgression of the Chr07:18576999...28940485 fragments resulted in a very significant increase in the proportion of Fa chains in ST27 compared with SN625, whereas the proportions of Fb1, Fb2, and Fb3 chains decreased; the taste value of ST27 was significantly higher than SN625, indicating that a gene in that interval affected the eating quality of rice by regulating ACLD. Identification of a specific gene in that region requires additional study.

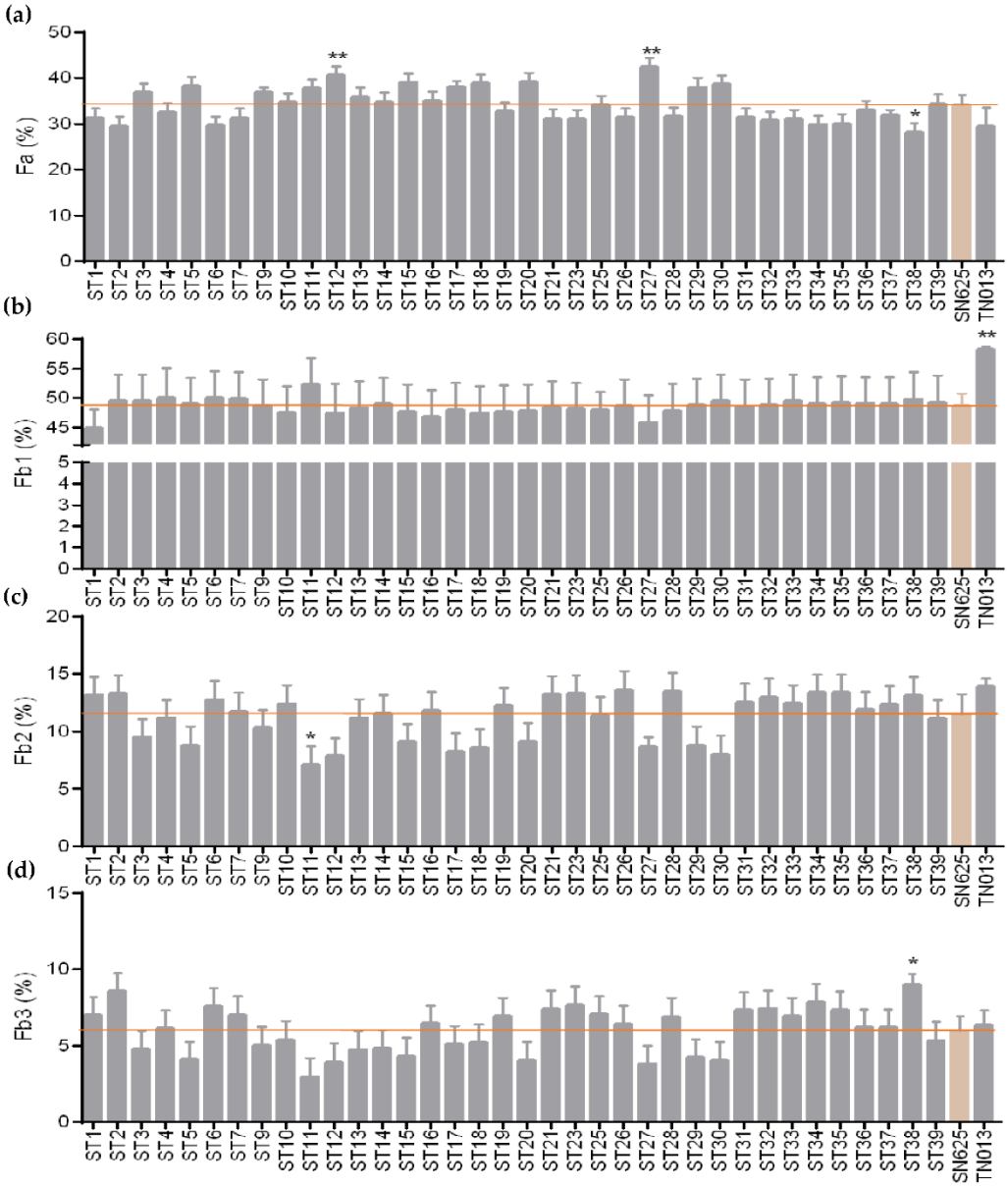

Figure 2. Distribution of amylopectin chain length in chromosome segment substitution lines (CSSLs). The proportion of amylopectin (**a**) Fa chains, where the degree of polymerization (DP) is 6–12, (**b**) Fb1 chains (DP = 13–24), (**c**) Fb2 chains (DP 25–36), and (**d**) Fb3 chains (DP > 36) in CSSLs. The orange line indicates the ACLD of SN625. * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA analysis of variance for CSSL lines compared to *japonica* parent; n = 3).

Table 3. Expression of taste quality factors and rapid viscosity analysis (RVA) in chromosome segment substitution line population.

Infiltration Fragment	CSSLs with Introgression	App	Hardness	Viscosity	Balance Degree	Taste Value	PKV	HPV	BDV	CPV	CSV	PaT
Chr1:301947...2169253	ST1	↑ **	↓ **	↑ **	↑ **	↑ **						
Chr1:2169222...10837608	ST2	↑ **		↑ **	↑ **	↑ **						
Chr1:10837575...21900536	ST3	↑ **			↑ **	↑ **	↑ *	↑ **		↑ **		
Chr1:21900509...35465528	ST4									↑ **		
Chr1:32331665...42916633	ST5	↑ **			↑ *	↑ **	↑ **					
Chr2:124679...8980638	ST6						↓ **		↓ **	↓ *		
Chr2:8980638...20076203	ST7	↓ **			↓ **	↓ **						
Chr2:29331976...35450234	ST9									↓ **		
Chr3:390594...12297803	ST10									↑ **	↑ **	
Chr3:9912382...21193876	ST11	↓ **	↓ *		↓ **	↓ **	↓ **	↓ **		↓ **		
Chr3:17228486...30221231	ST12						↓ **	↓ **		↓ **		↓ **
Chr3:26547397...35948544	ST13						↑ **	↑ *		↑ **		↑ *
Chr4:167824...5624490	ST14	↑ **		↑ **	↑ **	↑ **						
Chr4:5624467...20041204	ST15	↑ *			↑ *	↑ **						
Chr4:24065173...35393320	ST17						↑ **	↑ **		↑ **		↑ **
Chr5:6949503...19913036	ST19	↑ **	↓ *	↑ **	↑ **	↑ **	↑ **	↑ **		↑ **	↓ **	↑ *
Chr5:19912953...29323354	ST20	↑ **		↑ **	↑ **	↑ **	↑ **	↑ **		↑ **		
Chr6:220065...7422860	ST21	↑ **		↑ **			↑ **	↑ **		↑ **		
Chr6:15361500...25612939	ST23						↑ *	↑ **		↑ **		↓ *
Chr7:494948...16146549	ST25	↓ **										↓ **
Chr7:5762230...18340360	ST26	↑ **			↑ **	↑ **	↑ **	↑ **		↑ **		
Chr7:18340334...28986034	ST27					↑ **					↑ **	↓ **
Chr8:119337...3733832	ST28						↑ **	↑ **	↓ *	↑ **		

Table 3. *Cont.*

Infiltration Fragment	CSSLs with Introgression	App	Hardness	Viscosity	Balance Degree	Taste Value	PKV	HPV	BDV	CPV	CSV	PaT
Chr8:3733809...21575917	ST29	↑ **	↓ *	↑ **	↑ **	↑ **	↑ **	↑ **		↑ **		↑ **
Chr8:18814405...27386064	ST30	↑ **	↓ *	↑ **	↑ **	↑ **	↑ **	↑ **		↑ **		↑ **
Chr8:26315198...28231436	ST31	↑ **		↑ **	↑ **	↑ **	↑ **	↑ **		↑ **		
Chr9:99249...16620834	ST32						↑ **	↑ **	↓ **	↑ **		
Chr10:33968...11221582	ST34								↓ *	↑ **		
Chr10:11221543...22561956	ST35						↑ **	↑ **		↑ **		↑ **
Chr11:305169...8024755	ST36							↑ **		↑ **		
Chr11:8024726...19439119	ST37	↓ **			↓ **	↓ **	↑ **	↑ **		↑ **		↑ **
Chr11:17076271...28322996	ST38			↑ *				↑ **		↑ *	↓ *	
Chr12:976022...7425080	ST39	↑ **			↑ *	↑ **						

* and ** indicate significant differences compared with the *japonica* parent. App, appearance; PKV, peak viscosity; HPV, hot paste viscosity; BDV, breakdown viscosity; CPV, cool paste viscosity; CSV, consistency viscosity; PaT, pasting temperature. * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA analysis).

3.5. Correlation Analysis of ACLDs in Rice

The proportion of amylopectin Fa chains in each line was significantly negatively correlated with the proportions of Fb1, Fb2, and Fb3 chains. In contrast, there were significant positive correlations between the proportions of amylopectin Fb1 chains and Fb2 chains and of Fb2 chains and Fb3 chains. Furthermore, the proportions of Fa and Fb1 chains showed a typical normal distribution in the CSSLs, which indicated that the Fa and Fb1 chain proportion was subject to complex genetic regulation. This suggested that the introgression of the *indica* lineage into the *japonica* genome had a slight impact on the proportion of amylopectin Fa and Fb1 chains and induced larger changes in the proportion of amylopectin Fb2 and Fb3 chains (Figure 3).

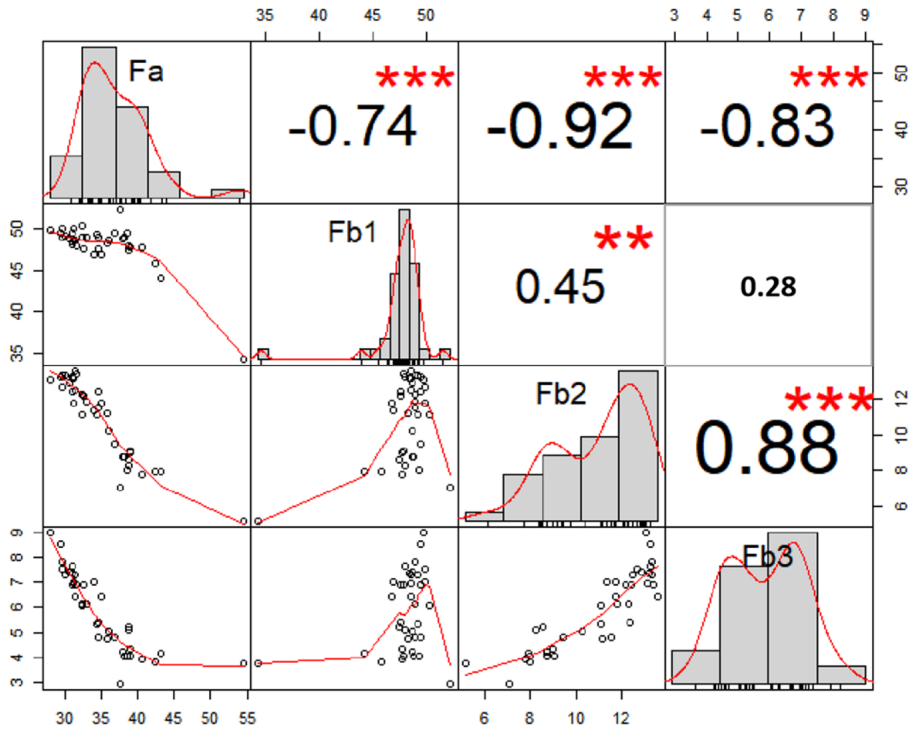

Figure 3. Correlation between amylopectin chain lengths in chromosome segment substitution lines. ** $p < 0.01$, *** $p < 0.001$. Error bars show the standard error of the mean.

3.6. Correlation Analysis between ACLD and Quality Trait Factors in CSSLs

Appearance, viscosity, hardness, balance, and taste values obtained from the rice taste meter can replace qualitative human sensory evaluations to reflect rice-eating quality more consistently and accurately [23]. Establishing the relationship between ACLD and those specific indicators can demonstrate how ACLD affects eating quality. We found that the appearance, viscosity, balance degree, and taste values of rice were negatively correlated with the proportion of amylopectin Fa chains and positively correlated with the proportion of Fb1 and Fb2 chains. In addition, rice viscosity was positively correlated with the proportion of amylopectin Fb3 chains. Rice hardness was negatively correlated with the proportion of amylopectin Fb1 chains and positively correlated with the proportion of Fb3 chains, but the correlation was not significant (Figure 4).

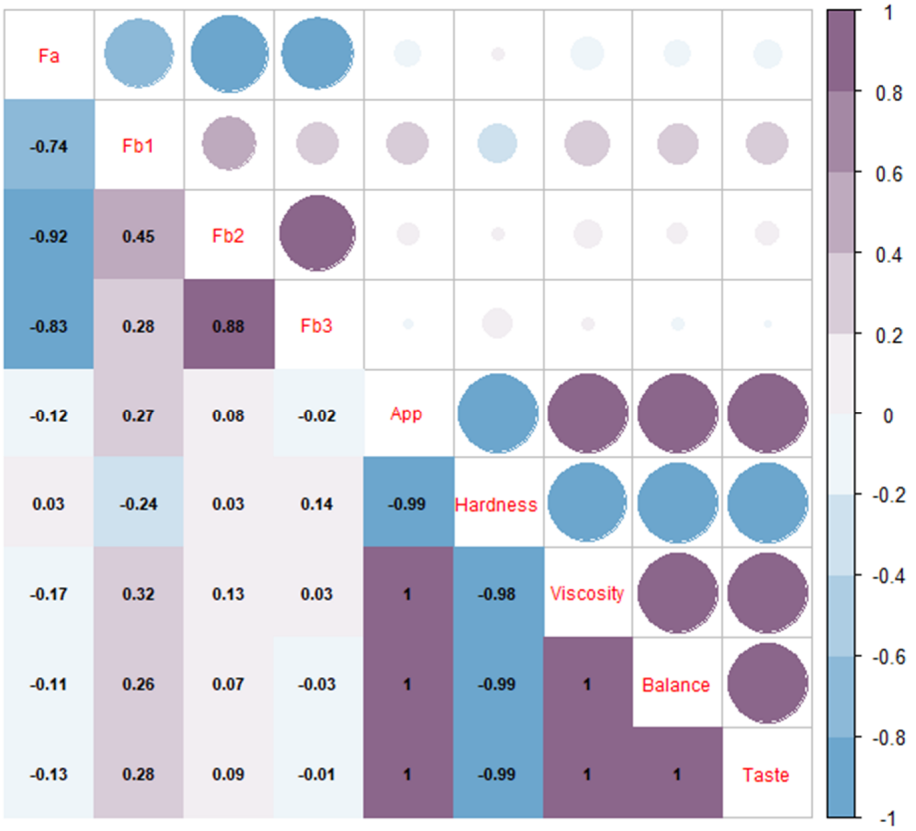

Figure 4. Correlation of amylopectin length distribution with eating and cooking qualities in chromosome segment substitution lines. App, appearance.

3.7. Correlation between ACLD and RVA Eigenvalues

The RVA index, which reflects the gelatinization process of starch, is an important parameter to evaluate the cooking and eating quality of rice [24]. PKV reflects the water absorption and expansion ability of starch; HPV reflects the tolerance of starch to high temperatures; BDV reflects the thermal stability of starch; CPV reflects the softness and hardness of rice at room temperature; consistency viscosity (CSV) reflects the retrogradation characteristics of rice, and; PaT reflects the water demand during cooking and the length of cooking time [25,26]. Previous studies have shown that ACLD is closely related to the gelatinization process and that ACLD differs between subspecies. It is, therefore, necessary to establish the relationship between ACLD and RVA. HPV and CPV were negatively correlated with Fa chain distribution and positively correlated with the proportion of amylopectin Fb2 and Fb3 chains, but the correlation was not significant. BDV was positively correlated with the proportion of Fa chains and negatively correlated with the proportion of the other three chain types; the correlation with Fb3 chains was significant. CSV was also positively correlated with the proportion of amylopectin Fa chains and negatively correlated with the proportion of the other three chains. PaT was negatively correlated with the proportion of Fa chains and positively correlated with the proportion of Fb1 and Fb2 chains (Figure 5).

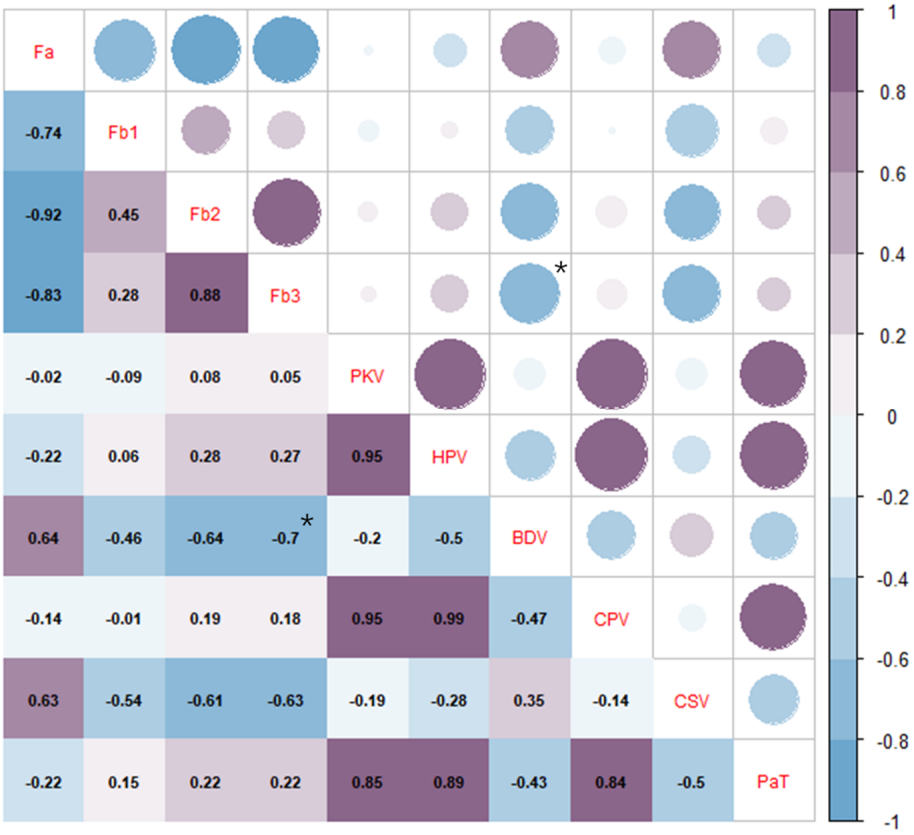

Figure 5. Correlation of amylopectin length distribution with rapid viscosity analysis eigenvalues in chromosome segment substitution lines. PKV, peak viscosity; HPV, hot paste viscosity; BDV, breakdown viscosity; CPV, cool paste viscosity; CSV, consistency viscosity; PaT, pasting temperature. * $p < 0.05$ (bilateral correlation).

4. Discussion

4.1. Genetic Factors Affecting ACLD

Amylopectin synthesis is affected by the *Wx* gene. Research has shown great differences in the amylopectin structure of rice containing five different *Wx* alleles: *Wx*, Wx^t, Wx^{g1}, Wx^{g2}, and Wx^{g3}. The proportion of short A chains (DP = 6–12) of waxy (*Wx*) and Wx^{g3} genotypes is significantly higher than that of other *Wx* genotypes, and the proportion of short A chains is lowest in the Wx^{g2} genotype. The proportion of amylopectin short B1 chains (DP = 13–24) in rice with different *Wx* alleles is as follows: $Wx = Wx^t > Wx^{g1} = Wx^{g2} > Wx^{g3}$. The relative influence order of the five *Wx* alleles on the number of ultra-long B3 + (DP $\geq$ 37) chains is: $Wx^{g1} = Wx^{g2} = Wx^{g3} > Wx^t > Wx$ [27]. Sartbayeva found that the proportion of DP 7–9 chains was higher in Wx^b than in Wx^a rice, whereas the proportions of DP 10–13 and DP 6–13 chains were lower in Wx^b than in Wx^a rice [28]. In this study, ACLD and amylose content analysis was based on CSSLs derived from Wx^b-type parents, allowing identification of novel, non-*Wx* factors affecting ACLD.

Many other genes have been identified as participants in determining ACLD. These genes can be roughly divided into three categories: soluble starch synthases (SSSs), starch branching enzymes (SBEs), and starch debranching enzymes (DBEs). *SSI* encodes SSS enzyme that preferentially catalyzes the synthesis of short chains (DP = 6–15); *SSIIa* extends short chains (DP $\leq$ 11) to medium chains (DP = 13–25) in *indica* rice while keeping the proportion of long chains (DP $\geq$ 29) unchanged. *SSIIIa* affects the synthesis of amylopectin chains with DPs of 9–15 and 22–29; moreover, the proportion of chains with DPs of 6–8, 16–20, and $\geq$30 decreased in an *SSIIIa* mutant. *SSIV* is rarely studied in rice and has an unclear function [17,18,29–32]. *BEI* is an SBE enzyme that preferentially synthesizes B1 chains. *BEIIa* is mainly used to synthesize short chains (DP = 6–11) [28,33–35]. *BEIIb*

plays an important role in the synthesis of Fa chains. *ISA* is a DBE enzyme that first catalyzes the synthesis of short chains (DP $\leq$ 12) in rice, and *PUL* also affects the formation of short chains. Studies have shown that *pho1* mainly plays a role in the synthesis of chains with DPs $\leq$ 11. *pho1* may also play an important role in the initiation of glucose formation through the synthesis of DP glucan primers [29,36–38]. However, we here found that CSSLs having *indica* introgression segments that overlapped with the seven genes discussed above did not show significant differences in ACLD compared to the *japonica* parent line SN625 (Supplementary Tables S2–S8). Although prior studies have shown that these seven genes affect ACLD, their functions have primarily been studied in mutants and single subspecies, meaning there is no relevant research showing whether the different haplotypes of each gene affect ACLD [29,30,33,39–41]. In the present study, although these seven genes showed polymorphisms between the parent lines, those differences did not lead to significant variations in ACLD based on the genetic analysis. The genes containing natural variations that regulate ACLD differences between subspecies are therefore still unknown. Previous studies explored the pattern in different varieties that were greatly confounded by differences in genetic backgrounds. Here, CSSLs were used to accurately analyze the genetic contributions of ACLD to quality trait factors due to the consistent genetic background.

4.2. Genetic Factors Affecting Rice Eating Quality

The eating quality of rice is an extremely complex trait, the key indicators of which are gel consistency (GC), gelatinization temperature (GT), and RVA [42]. In recent years, many genes and QTLs associated with eating quality have been identified. Among the identified genes, *ALK* (*SSIIa*, *SSII-3*) primarily affects eating quality through changes in GT, whereas *AGPiso*, *SBE3*(*BEIIb*), *SSIV-2*, and *ISA* affect both GC and GT. In addition, starch synthesis-related genes also have some minor effects on rice eating quality [43–45]. *PUL* and *SSIIa* play important roles in affecting PKV, HPV, BDV, CPV, CSV, and PaT; *SBE1* (*BE1*) primarily affects HPV and CPV, and *SBE3* (*BEIIb*) affects PKV and CSV [46,47]. In the present study, there were no polymorphisms in *ALK* (*SSIIa*, *SSII-3*), *PUL*, or *SBE1* (*BE1*) between the two parent lines, and changes in eating quality and RVA values of the CSSL population were therefore not related to the effects of these genes (Supplementary Table S9). However, there were polymorphisms in *AGPiso*, *SBE3* (*BEIIb*), *SSIV-2*, and *ISA* between the two parent lines, indicating that these genes may be responsible for changes in eating quality and RVA indicators in the CSSL population (Supplementary Tables S3, S6, S10 and S11). The *indica* alleles for *AGPiso*, *SBE3* (*BEIIb*), *SSIV-2*, and *ISA* were present in CSSLs ST4, ST7, ST20, and ST30. The eating quality of ST4 was not significantly different from that of the *japonica* parent SN625; in ST7, eating quality was significantly lower, and in ST20 and ST30, eating quality was significantly higher than in SN625. The introgression of the above genes resulted in a very significant increase of CPV in ST4 compared with SN625. Furthermore, PKV, HPV, and CPV were also significantly higher in ST20 and ST30, as was PaT in ST30 (Table 3). This indicated that the four genes with polymorphisms identified—except *AGPiso*—did affect the eating quality of CSSLs. RVA-related QTLs were mapped to each chromosome in the CSSLs. Yang et al. identified five GT-related QTLs and one GC-related QTL, of which qGT6-1 and qGC6 overlapped with *ALK*, and qGC6 overlapped with the ST21-introgressed segment in this study [41,48,49]. Zhang et al. identified 10 RVA-related QTLs on chromosomes 2, 5, 7, and 8; qPKV3, qTV6, and qCPV6 overlapped with the ST21 introgression, and qPaT3 overlapped with the ST12 introgression. In addition, introgressed segments in ST11, ST12, ST19, and ST32 coincided with the RVA-related QTLs identified by Shao Xin et al. [50,51]. This demonstrates the stability of these coincident QTLs, which should be the focus of a future study.

4.3. Effects of ACLD on Rice Eating Quality

ACLD is a key factor determining the amylopectin structure of rice, which affects rice quality [52,53]. It has generally been shown that the fine structure of amylopectin, such as

chain length distribution, can affect the gelatinization process of starch. Cai YX found that the branch chain length of amylopectin affects starch expansion, gelatinization, rupture degree of starch grains, and recovery of viscosity after cooling. A higher proportion of long chains in amylopectin increases the possibility that they will form a double helix structure or a complex with lipids and proteins, inhibiting starch expansion and reducing PKV. A high number of long chains also decreases the breaking of the amylopectin structure, helping to maintain the structure of the gelatinized starch particle. Therefore, HKV will be high, which reduces the BDV and the cooking and eating quality. In contrast, a high proportion of short chains is conducive to starch gelatinization, high PKV and BDV, and improvement of rice taste [24]. Consistent with these general trends, introgression of the indica Chr07:18576999...28940485 segments in ST27 resulted in a significant increase in the proportion of Fa chains, a decrease in the proportions of Fb1, Fb2, and Fb3 chains, and a higher taste value compared with SN625. These findings point to that segment of Chr07 as a priority for follow-up research. Through the study of high-quality Chinese japonica rice in the lower reaches of the Yangtze River and Koshihikari, Lee et al. found that the proportion of amylopectin long chains is negatively correlated with PKV and BDV due to insufficient gelatinization of starch grains, which affects rice taste. Consistent with those findings, CAI YX found that a high proportion of amylopectin Fa chains (DP = 6–12) is accompanied by increased BDV [54]. We here found that the proportion of amylopectin short chains had no effect on the PKV, and that Fb1, Fb2, and Fb3 chains were negatively correlated with rice appearance, viscosity, balance, and taste value, and positively correlated with hardness. This may be due to the consistent genetic background of the CSSLs, effectively eliminating the interference of other genetic noise. The genetic basis of inter-subspecific ACLD variation revealed in this study is different from that of intraspecific ACLD variation.

4.4. Correlation between ACLD and RVA

Many previous studies have reported a correlation between ACLD and RVA viscosity characteristics in rice [55]. Peng XS found that the proportion of DP 6–11 chains in amylopectin showed a significant negative correlation with the initial pasting temperature, and the proportion of DP 12–24 chains was significantly positively correlated with the initial pasting temperature. He XP came to the same conclusion in studying different types of rice varieties [7,56]. Li DL found that the proportion of amylopectin short-chain Fa is significantly positively correlated with PKV and BDV, consistent with the results of CAI YX, and the proportion of Fa/Fb3 in amylopectin is significantly positively correlated with PKV and BDV [54]. Zhou HY found that the ratio of A chains to B chains is positively correlated with PKV and BDV and negatively correlated with the setback value (SBV) [57]. PKV is produced through the increase of friction between starch particles during the process of water absorption and expansion, and therefore reflects the water absorption and expansion ability of starch. BDV represents the thermal stability of starch granules; a greater BDV corresponds to lower thermal stability and easier gelatinization. High values of these two indicators represent superior rice quality [58]. This demonstrates that the content of amylopectin short chains could significantly promote rice quality. Mar et al. found that the proportion of DP 6–11 chains was significantly positively correlated with the PKV and negatively correlated with PaT; the proportion of DP 13–24 chains was significantly negatively correlated with PKV and positively correlated with maximum PaT [59]. In this study, we found that the proportion of amylopectin Fa chains (DP = 6–12) was negatively correlated with PaT and positively correlated with BDV and that the proportion of Fb3 chains (DP > 36) was significantly negatively correlated with BDV, all of which is consistent with the prior reports discussed above. Therefore, we speculate that the influence of Fa and Fb3 chains on quality trait factors is lower than the influence of Fb1 and Fb2 chains. Fb1 may be the key cause of amylopectin fine structure variation among inter-subspecies and further affect the eating quality of rice. The *indica* rice lineage may change the eating quality of *japonica* rice by affecting the proportion of Fb1 and Fb2 chains or other fine structure indexes of amylopectin, such as branching degree and average chain length. We also

found that the proportion of amylopectin Fa chains (DP = 6–12) was not correlated with PKV, but was positively correlated with CSV. This finding was in contrast to previously published results, possibly due to differences in the genetic background or environmental growth conditions.

5. Conclusions

In this study, we found that introgression of the *indica* rice lineage in the *japonica* lineage led to significant changes in the proportions of amylopectin Fa, Fb2, and Fb3 chains in some families of CSSLs. However, CSSLs containing *indica* introgression segments that overlapped with the seven interspecific polymorphic genes related to ACLD did not show significant differences in ACLD compared to the parent *japonica* line SN625. Correlation analysis showed that the proportion of Fb2 chains was positively correlated with appearance, viscosity, balance, and taste values of rice, and negatively correlated with hardness. The proportion of amylopectin short chains was positively correlated with BDV, negatively correlated with CSV, and the proportion of medium and long chains was negatively correlated with BDV and positively correlated with CSV. Introgression of the *indica* lineage caused significant changes in quality trait factors and RVA in multiple CSSLs, many of which overlapped with QTLs for these traits mapped in previous studies. In the CSSL ST27, introgression of the *indica* Chr07:18576999...28940485 segments resulted in a very significant increase in the ratio of Fa chains, a decrease in the ratio of Fb1, Fb2, and Fb3 chains, and an increase in taste value. This region should therefore be prioritized for future study to locate the related genes that regulate the ratio of Fa chains and taste quality. This study provides both a specific key region for future study and a theoretical basis for molecular design breeding to improve rice quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12040472/s1: Table S1: Introgression interval and marker information of the CSSLs. Tables S2–S8: Polymorphisms in seven amylopectin synthesis-related genes between the *indica* and *japonica* parental lines. Table S9: Gene information related to quality trait factors and RVA between the two parent lines. Table S10: Different sites information of *AGPiso* between the two parent lines. Table S11: Different sites information of *SSIV-2* between the two parent lines.

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