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The relationship between starch synthesis enzyme activity, gene expression, and amylopectin fine structure in waxy maize

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Abstract

Two waxy maize hybrids were selected to explore the relationship between amylopectin fine structure and gene expression of starch synthesis-related enzymes. High-performance anion-exchange chromatograph analysis showed that waxy maize had the highest content of amylopectin B1 chain (DP 13–24) and the secondary proportion of A chain (DP 6–12) while the low proportions of B2 (DP 25–36) and B3 chains (DP ≥ 37). SBE activity was positively correlated with the proportion of A chain and negatively correlated with the proportion of B3 chain and average chain length, but DBE activity showed the almost opposite relation. AGPase and SSS activities were not found to be significant with chain-length distribution. SS*I* preferentially generated short A chain, which would be elongated further by *SSIIa to* produce long B1 chain that was continued to extend by *SSIIIa* to produce longer B2 and B3 chains. *SBEI* was more likely responsible for the synthesis of B2 and B3 chains, while *SBEIIb* played a specific role in the formation of A chain. *ISA2* regulated amylopectin structure through the removal of A and B1 chains and the formation of B2 and B3 chains.

Keywords Waxy maize · Amylopectin · Fine structure · Gene expression

Introduction

Starch, the most significant carbohydrate reserve in maize grain, has comprehensive industrial applications due to its advantages such as stable supply, low price, wide source, and renewability (Chen et al. 2015). Starch mainly consists of the D-glucose polymers, amylose, and amylopectin. Amylose is an essentially linear polymer with 1,4-linked α-D-glucose chains. Amylopectin is a highly branched glucan chain composed of α-D-glucose units, with α-1,6-glycosidic bonds between branches and α-1,4-glycosidic bonds within branches in a clustered manner (Hizukuri 1986). The amylopectin could be debranched by isoamylase and/or pullulanase to obtain the unit chain-length distribution (Bertoft 2004). The unit chains are classified into A chain (without carrying any other chains), B chain (carrying other chains via α-1,6-linkage), and C chain (carrying the only reducing residue in a single amylopectin molecule) (Peat et al. 1952). Through high-performance size-exclusion chromatography (HPSEC) analysis, Hizukuri (1986) suggested that the B chains could be fractionated into B1, B2, and B3 chains which involve in one, two, and three clusters, respectively. Using high-performance anion-exchange chromatography (HPAEC) analysis, Hanashiro et al. (1996) reported that chain-length distributions were fractionated into fa, degree of polymerization (DP) 6–12; fb1, DP 13–24; fb2, DP 25–36; and fb3, DP ≥ 37. The unit chain-length distribution of amylopectin is the key determinant of starch properties (Li et al. 2020; Zhu 2018).

Starch is synthesized via concerted reactions catalyzed by several enzymes, including adenosine diphosphate-glucose (ADP-Glc) pyrophosphorylase (AGPase), soluble starch synthase (SSS), granule-bound starch synthase (GBSS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) (Jeon et al. 2010; Nakamura et al. 2022).

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AGPase mainly catalyzes the synthesis of ADP-Glc and pyrophosphate (PPi) from glucose-1-phosphate (Glc-1-P) and adenosine triphosphate (ATP) (Jeon et al. 2010). GBSS and SSS are involved in the synthesis of amylose and amylopectin, respectively. SBE can cut the α-1,4-glycosidic bond to introduce α-1,6-glucosidic linkages into α-polyglucans, and DBE can directly hydrolyze α-1,6-glucosidic linkages of α-polyglucans (Nakamura 2002). The coordinated action of these starch-metabolizing enzymes, which are regulated by their corresponding genes, is closed related to the extension of the glucan chain, thereby affecting the structure of starch (Huang et al. 2021; Li and Gilbert 2016), and ultimately changing the physicochemical properties and processing quality of starch (Tappiban et al. 2022; Wang et al. 2014; Yu et al. 2019).

In maize, there are three isoforms of SS, namely, SSI, SSII, and SSIII. SSI is responsible for extending shorter A and B_1 chains to a certain length, and then, SSII and SSIII control further glucan extension (Commuri and Keeling 2001). SSI, SSIIa, and SSIII specifically contribute to the synthesis of very short chains, $A+B_1$ chains, and long B_1 and B_2 chains of amylopectin, respectively (Nakamura 2002). A high GBSSI expression is responsible for the shorter amylose chains in high amylose maize lines (Zhong et al. 2020). SBEI mainly generates longer chains with $DP \geq 16$ by branching lesser branched polyglucans, and SBEII produces shorter chains with $DP \leq 12$ by branching highly branched polyglucans (Jeon et al. 2010). There exist two classes of DBE, isoamylase (ISA) and pullulanase (PUL). ISA plays a key role in highly ordered structure of amylopectin through the editing of excessively branched chains or the removal of improper branches formed by SBEs (James et al. 1995; Jeon et al. 2010). PUL may supplement the function of ISA in starch metabolism (Dinges et al. 2003).

A better understanding of starch biosynthesis–fine structure relationship is essential for controlling and producing the predictable starch in crop plant. Waxy maize is a special type of maize controlled by a recessive *waxy* gene mutation in common maize (Wu et al. 2022). In common maize, the *Waxy* gene expresses GBSSI protein for amylose synthesis, which makes the amylose-to-amylopectin ratio from 15:85 to 35:65 in common maize starch (Varghese et al. 2022). In waxy maize, *Waxy* mutation causes the GBSSI protein to be inactive, and the synthesis of amylose is blocked, which results in almost 100% amylopectin in waxy maize starch. Waxy maize has huge popularity with a wide market prospect owing to its unique taste, smooth viscosity, fragrant smell, and convenient consumption after simple steaming and cooking (Ketthaisong et al. 2014). It has been regarded as the "new nutritional health food," "green vegetable," and "longevity food" (Simla et al. 2016). Waxy maize starch has different properties with common maize starch, such as easy digestion, good stability, high viscosity, and low retrogradation (Hsieh et al. 2019; Yang et al. 2016). As such, waxy maize is endowed with special utilization and high economic value. However, there are still lots of uncertainties in the understanding of the formation of amylopectin fine structure in waxy maize. In this study, two waxy maize hybrids were used to determine amylopectin fine structure, starch synthesis enzyme activity, and gene expression during grain filling, and the amylopectin fine structure–starch biosynthesis relationships were analyzed. The study is expected to provide a reference for controlling the formation of amylopectin fine structure to improve grain quality in waxy maize. Further, the same analysis pipeline could be applied in parallel with non-waxy maize hybrids to investigate how gene expression and enzyme activity function in wild-type case.

Materials and methods

Plant materials

Maize varieties Suyunuo2 (SYN2) and Jingkenuo2000 (JKN2000), the very popular waxy maize hybrid varieties in China, were selected as materials. The female and male parents of SYN2 are Tong361 (derived from Baiye4 × Tongxi5) and 366 (derived from Huotangbai42 × Hengbai522). The female and male parents of JKN2000 are Jingnuo6 (derived from Zhongnuo1) and BN2 (derived from Zinuo3). According to National Crop Variety Approval Committee of China, the amylopectin contents in the total starch of SYN 2 and JKN 2000 are 99.63% and 100.00%, respectively, which meet the standard of NY/T524-2002 waxy maize issued by Ministry of Agriculture of China. Seeds were planted on June 3, 2018, in the experimental farm of Qingdao Agricultural University, Shandong, China. Each hybrid had three plots as replicates. The trial included six plots with an area of 18 m^2 (3.6 m × 5 m). The plants were thinned to 60,000 plants/ha at the six-leaf stage based on 0.6-m row spacing and 0.28-m plant spacing.

Plant sampling

Ears were bagged before silking to avoid cross pollination, and the plants were self-pollinated at anthesis. Six ears were sampled at each 10, 15, 20, and 25 days after pollination (DAP). The grains in the middle of the ear were taken and mixed to divide into two parts. One portion was stored in a refrigerator at − 20 °C for starch isolation. The other portion was frozen in liquid nitrogen and stored in a refrigerator at − 80 °C for starch biosynthesis analysis.

Starch isolation

Starch was isolated according to the method of Lu and Lu (2012) with minor modifications. Fresh grains (10 g) were rinsed with distilled water and ground with a blender. The homogenate was passed into a beaker through 200-mesh screen. The filtrate was kept to stand for 4 h. After that, the sedimentation was shifted to 50-mL centrifuge tube and centrifuged at 1046×*g* for 10 min. The supernatant was discarded, and the upper faint yellow layer was scraped. The white layer was resuspended with distilled water and stirred for 10 min before centrifugation. The isolation step was repeated three times with distilled water suspension and then about three times with ethanol suspension until the supernatant was colorless. Finally, the isolated starch was dried at 40 °C and collected with a 200-mesh sieve.

Determination of chain-length distribution (CLD)

Oligosaccharides standards from DP 4 to DP 7 and starch were taken 5 mg as standard products preparation and sample pretreatment, respectively. The sample was suspended in 5-mL double-distilled water and heated in a boiling water for 60 min. After that, 2.5-mL blending sample was added into 125-µL sodium acetate, 5-µL sodium azide, and 5-µL isoamylase to prepare a 95% sample solution, and then were kept at 38 °C for 24 h. Take 600-µL mixture to the centrifuge tube and dry with nitrogen blowing at room temperature. The mixture was dissolved in 600-µL mobile phase and centrifuged at 5866×*g* for 5 min. The supernatant was injected onto an ICS-5000 ion chromatography system (Thermo Fisher Scientific, Waltham, MA, USA). An electrochemical detector and Dionex™ CarboPac™ PA10 (250 × 4 mm, 10 µm) ion column were used. Mobile phase A was 200-mM NaOH, and mobile phase B was 200-mM NaOH and 200-mM NaAC. The flow rate control was 0.3 mL/min, and column temperature was 30 °C. The total area was the sum of the areas from DP 6 to DP 76. The relative areas named as CLDs were the ratios of the corresponding area to the total area. Average chain length (ACL) was calculated based on the value of DP and its relative area.

Enzyme isolation and assays

The frozen grains (1 g) were homogenized with a pestle in an ice-cold mortar that contained 9-mL PBS phosphate buffer solution (pH 7.2–7.4). The homogenate was transferred to 2-mL centrifuge tube and centrifuged at 1844×*g* at 4 °C for 15 min. The supernatant was used as enzyme preparation. The activities of AGPase, SSS, SBE, and DBE were determined through the enzyme-linked immunosorbent assay kit. The linear regression equation of the standard curve was obtained with the concentration and OD value of the standard substance. The OD value of the sample was substituted into the equation to calculate the sample concentration.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated according to the instructions in the Total RNA Isolation Kit (Sangon, Shanghai, China). The first strands of cDNA were synthesized using FastQuant RT Kit (Tiangen, Beijing, China). Eight genes, *SSI*, *SSIIa*, *SSIIc*, *SSIIIa*, *SBEI*, *SBEIIb*, *ISA2*, and *PUL*, were selected for qRT-PCR analysis (Chen et al. 2011). Their primers are listed in Table 1. The *ZmActin* (ID: XM_008656735.2) gene in maize was used as reference gene. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate the relative expression level.

Statistical analysis

CLD determination, enzyme assay, and qRT-PCR analysis were performed in triplicates. Mean values were reported. Data were subjected to analysis of variance using SPSS software v20.0 (SPSS Inc, USA). Means were compared using LSD test, and differences were regarded as significance at $p < 0.05$. Pearson correlation analysis was obtained using SPSS v20.0.

Results

CLD of amylopectin

Generally, debranched amylopectin CLD data can be divided into four fractions, A (DP 6–12), B1 (13–24), B2 (DP 25–36), and B3 (DP ≥ 37) (Hanashiro et al. 1996). According to this classification, the proportion of every fraction was calculated (Table 2). Waxy maize amylopectin comprised a primary proportion of DP 13–24 (48.31–48.80% for SYN2 and 48.72–50.49% for JKN2000) and a secondary proportion of DP 6–12 (22.08–23.74% for SYN2 and 23.01–23.82% for JKN2000) while a low proportion of DP 25–36 (15.48–15.99% for SYN2 and 14.77–15.58% for JKN2000) and DP ≥ 37 (12.30–13.33% for SYN2 and 11.59–12.37% for JKN2000) (Table 2). During the period of grain development, DP 6–12 percentage was higher at 10 DAP than at 15 DAP, 20 DAP, and 25 DAP, and was not significantly different among the later three stages for both hybrids. DP 13–24 percentage was lower at 10 DAP and 15 DAP than at 20 DAP and 25 DAP for both hybrids. Both DP 25–36 and DP ≥ 37 percentages did not appear a significant difference with grain filling

Table 1 Primers used for quantitative real-time PCR (qRT-PCR)

Gene name	Gene symbol	Primer orientation	Primer sequence 5′–3′	Product size (pb)
ZmSSI	GRMZM2G129451	Reverse	AGGACAACAACACAGGTAATAATC	197
		Forward	GTCTGCTTTGGCTGCCTTG	
ZmSSIIa	GRMZM2G348551	Reverse	GTTCACTTCTAGGTCCTGTCCTGC	198
		Forward	ATCGTGGTGGCTGCTGAATG	
ZmSSIIc	GRMZM2G126988	Reverse	CTCCAGCAACATCCCCAA	267
		Forward	GTTCAGAAGACGACGGCAC	
ZmSSIIIa	GRMZM2G141399	Reverse	GGCTCGTTCCTTGTCATTGTC	244
		Forward	TGTCAACCTGGCGAATAAGC	
ZmSBEI	GRMZM2G088753	Reverse	GCCTCCTTGTCTTCTTTGCTAC	216
		Forward	TGAAGGGGTGCCAGGG	
ZmSBEIIb	GRMZM2G032628	Reverse	CACTGGAGCATAGACGACACAT	174
		Forward	CGAAAGCCTGGGGTGTAT	
ZmPUL	GRMZM2G158043	Reverse	CTGTCCTTTTCGGCACGGT	227
		Forward	CGAAGATGCACGAAATGATAGG	
ZmISA2	GRMZM2G090905	Reverse	CGACCACATACCCCGCAT	173
		Forward	CTCGCCCTCTTCCTCCTG	
ZmActin	XM_008656735.2	Reverse	CCCCCACTGAGGACAACG	190
		Forward	GCTACGAGATGCCTGATGGTC	

Table 2 CLD during grain filling in two waxy maize hybrids

Hybrid	DAP (d)	CLD (%)				A/(B1 + B2 + B3)	ACL (DP)
		A (DP 6–12)	B1 (DP 13–24)	B2 (DP 25–36)	B3 (DP ≥ 37)		
SYN2	10	23.74 ± 0.04^{a}	48.48 ± 0.32^{cd}	15.48 ± 0.08^{ab}	12.30 ± 0.19^{bc}	0.311 ± 0.00^{ab}	21.37 ± 0.02^{c}
	15	22.37 ± 0.18^{d}	48.31 ± 0.28^{d}	15.99 ± 0.18^{a}	13.33 ± 0.29^{a}	0.288 ± 0.00^{d}	21.71 ± 0.03^{b}
	20	22.11 ± 0.11^{d}	48.75 ± 0.11^{c}	15.95 ± 0.31^{a}	13.19 ± 0.34^{ab}	0.284 ± 0.00^{d}	21.88 ± 0.03^{a}
	25	22.08 ± 0.05^{d}	48.80 ± 0.17^{c}	15.91 ± 0.09^{a}	13.21 ± 0.26^{ab}	0.283 ± 0.00^{d}	21.87 ± 0.02^{a}
JKN2000	10	23.82 ± 0.03^{a}	48.73 ± 0.04^{c}	15.46 ± 0.32^{ab}	11.99 ± 0.67^{c}	0.313 ± 0.00^{a}	20.86 ± 0.04^{f}
	15	23.33 ± 0.52^{bc}	48.72 ± 0.02^{c}	15.58 ± 0.41^{ab}	12.37 ± 0.28^{abc}	0.304 ± 0.01^{bc}	21.16 ± 0.10^{e}
	20	23.65 ± 0.03^{ab}	49.88 ± 0.07^{b}	14.88 ± 0.85^{b}	11.59 ± 0.94^{c}	0.310 ± 0.01^{ab}	21.23 ± 0.02^{de}
	25	23.01 ± 0.22^{c}	50.49 ± 0.37^{a}	14.77 ± 0.85^{b}	11.73 ± 0.98^{c}	0.299 ± 0.00^{c}	21.25 ± 0.04^{d}

All data are averages. Mean values in the same column followed by different letters are significantly different at $p < 0.05$

CLD chain-length distribution and *ACL* average chain length

for both hybrids. Here, A was considered as short chain and B1, B2, and B3 as long chain. The short/long chain-length ratio, namely, A/(B1 + B2 + B3), had the highest value (0.311 for SYN2 and 0.313 for JKN2000) at 10 DAP and insignificant difference among 15 DAP, 20 DAP, and 25 DAP. ACL value was the lowest at 10 DAP, and then increased at 15 DAP and 20 DAP, and kept stable at 25 DAP for both hybrids. Comparison of two hybrids showed that JKN2000 had higher proportion of DP 6–12 and DP 13–24 with exception of 10 DAP, and lower proportion of DP 25–36 and DP ≥ 37 with exception of 10 DAP and 20 DAP. This caused that JKN2000 had higher short/long chain-length ratio, but lower ACL than SYN2.

Activities of enzymes involved in starch synthesis and their correlation with CLD

The activities of starch synthetic enzymes of two waxy maize hybrids increased initially and decreased thereafter with grain development (Fig. 1). The maximum emerged at 15 DAP. Compared with 10 DAP, the activities of AGPase, SSS, SBE, and DBE at 15 DAP were increased by 18.1%, 28.8%, 23.4%, and 8.1% for SYN2, and 15.4%, 16.8%, 2.5%, and 6.9% for JKN2000, respectively. The subsequent decrease appeared at 20 DAP and 25 DAP. Compared with 10 DAP, the activities of AGPase, SSS, SBE, and DBE at 20 DAP and 25 DAP were decreased by 20.5% and 21.4%, 16.6% and 16.2%, 17.6% and 22.6%,

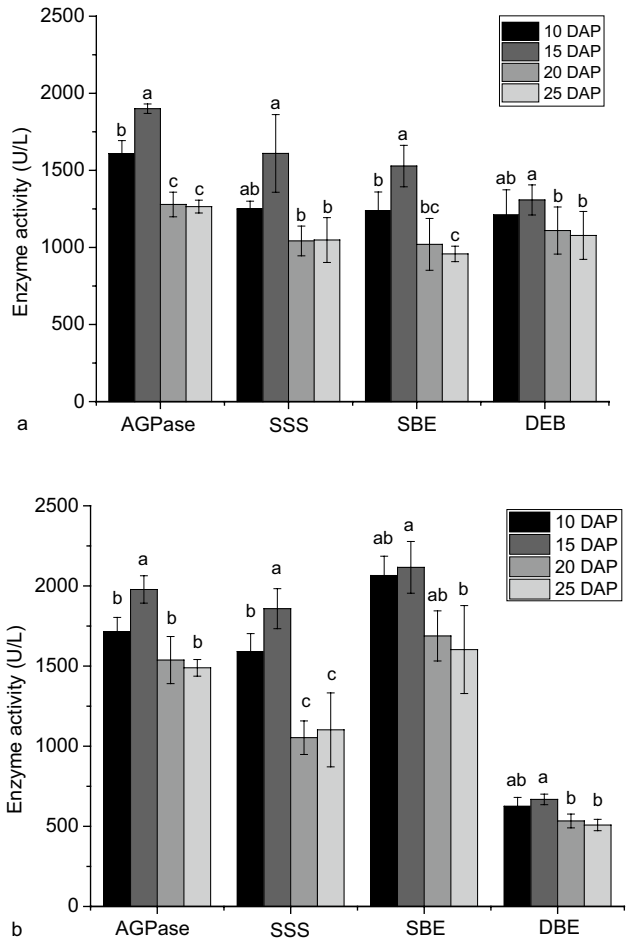

Fig. 1 Activities of AGPase, SSS, SBE, and DBE during grain filling in two waxy maize hybrids of **a** SYN2 and **b** JKN2000. Note: Different letters within a column indicate significant difference among mean values ($p < 0.05$)

and 8.3% and 10.9% for SYN2, and 10.3% and 13.2%, 33.8% and 30.7%, 18.3% and 22.4%, and 14.6% and 18.7% for JKN2000, respectively. The activities of AGPase, SSS, and SBE were higher in JKN2000 than SYN2, and DBE activity was higher in SYN2 than JKN2000.

SBE activity was positively correlated with DP 6–12 proportion ($r = 0.669$*) and negatively correlated with DP ≥ 37 proportion ($r = -0.588$*) and ACL ($r = -0.866$**). DBE activity was negatively correlated with the proportions of DP 6–12 ($r = -0.545$*) and DP 13–24 ($r = -0.747$*) and positively correlated with the proportions of DP 25–36 ($r = 0.807$**) and DP ≥ 37 ($r = 0.843$**) and ACL ($r = 0.746$*) (Table 3). The relationships between AGPase and SSS activities and CLD were not found to be significant.

Table 3 Correlation analysis between enzyme activities and CLD

	CLD				ACL
	DP 6–12	DP 13–24	DP 25–36	DP ≥ 37	
AGPase	0.438	−0.305	0.012	−0.140	−0.511
SSS	0.295	−0.485	0.250	0.046	−0.443
SBE	0.669*	0.145	−0.409	−0.588*	−0.866**
DBE	−0.545*	−0.747*	0.807**	0.843**	0.746*

CLD chain-length distribution and *ACL* average chain length

*Significant at $p < 0.05$ level

**Significant at $p < 0.01$ level

Expression pattern of genes involved in starch synthesis and their correlation with CLD

In the two waxy maize hybrids, the expression levels of eight genes (*SSI*, *SSIIa*, *SSIIc*, *SSIIIa*, *SBEI*, *SBEIIb*, *ISA2*, and *PUL*) were detected. The expression pattern of four genes (*SSI*, *SSIIa*, *SSIIc*, and *SSIIIa*) encoding SSS was different at different grain filling stages. The relative expression of *SSI* and *SSIIIa* showed a high level in the early grain filling stage and then a low level at 20 DAP and 25 DAP. Compared with *SSI* and *SSIIIa*, *SSIIa* appeared an opposite performance, namely, a low level at 10 DAP and 15 DAP and a high level at 20 DAP and 25 DAP. *SSIIc* showed a low expression level compared to *SSI*, *SSIIa*, and *SSIIIa* and an insignificant change with grain development. The relative expression of *SBEI* and *SBEIIb* encoding SBE maintained high levels, and increased initially and decreased from 20 DAP. The relative expression of *ISA2* and *PUL* encoding DBE showed insignificant differences during grain development. The relative expression levels of *SSI* and *SBEIIb* were higher for JKN2000 than SYN2, and those of *SSIIIa*, *SBEI*, and *ISA2* were higher for SYN2 than JKN2000.

There was significant correlation between the gene expression related to starch synthesis (except for *SSIIc* and *PUL*) and the amylopectin CLD (Table 4). The relative expression level of *SSI* was positively correlated with the proportion of DP 6–12 ($r = 0.546$*) and negatively correlated with ACL ($r = -0.616$*). The relative expression level of *SSIIa* was positively correlated with the proportion of DP 13–24 ($r = 0.716$*). *SSIIIa* and *SBEI* expression showed the same relation with CLD. Their levels were negatively correlated with the proportion of DP 13–24 ($r = -0.822$**, $r = -0.842$**) and positively correlated with the proportions of DP 25–36 ($r = 0.627$*, $r = 0.672$*) and DP ≥ 37 ($r = 0.527$*, $r = 0.528$*). The relative expression level of *SBEIIb* was positively correlated with the proportion of DP 6–12 and negatively correlated with ACL. *ISA2* expression was negatively correlated with the proportions of DP 6–12 ($r = -0.797$*) and DP 13–24 ($r = -0.585$*) and positively

Table 4 Correlation analysis between gene expression and CLD

	CLD				ACL
	DP 6–12	DP 13–24	DP 25–36	DP ≥ 37	
SSI	0.546*	−0.515	0.160	−0.125	−0.616*
SSIIa	−0.384	0.716*	−0.370	−0.122	0.363
SSIIc	0.358	−0.106	−0.095	−0.198	−0.507
SSIIIa	−0.055	−0.822**	0.627*	0.527*	0.191
SBEI	0.064	−0.842**	0.672*	0.528*	0.127
SBEIIb	0.679*	−0.096	−0.236	−0.453	−0.795*
ISA2	−0.797*	−0.585*	0.858**	0.899**	0.853**
PUL	0.022	−0.438	0.368	0.204	0.109

CLD chain-length distribution and *ACL* average chain length

*Significant at $p < 0.05$ level

**Significant at $p < 0.01$ level

correlated with the proportions of DP 25–36 ($r = 0.858$**) and DP ≥ 37 ($r = 0.899$**) and ACL ($r = 0.853$**).

Discussion

Contribution of soluble starch synthases to amylopectin fine structure

Starch synthase catalyzes the chain-elongation reaction of α-1,4-glucosidic linkage by transferring a glucose moiety from ADP-glucose to the non-reducing end of the linkage in plants. In this study, there was not a significant relationship between SSS activity and CLD. Plants possess at least three isoforms of SSS, namely, SSI, SSII, and SSIII (Nakamura 2002). Characterization of genetic mutants of these isoforms could provide the basis for assigning preferential functions for individual isoform in the amylopectin synthesis.

The chain-length distribution pattern of the polyglucans synthesized by recombinant maize SS and BE isoforms in *Escherichia coli* supports that SSI isoform preferentially synthesizes short chains (DP 6–15) (Guan and Keeling 1998). Further investigation of the chain-length specificities in maize endosperm has suggested that SSI enzyme prefers the shortest chains as substrates during amylopectin synthesis (Commuri and Keeling 2001). Gene-mapping analyses show that that SSIIa plays a distinct role in the elongation of short chains within clusters (A + B1 chains) of amylopectin in rice (Umemoto et al. 2002). The maize *sugary2* mutants lacking SSIIa activity appear a significant increase in the abundance of DP 6–11 chains and a decrease in the percentage of DP 13–20 chains (Zhang et al. 2004). The specific contributions of SSII and SSIII isoforms to the structure of amylopectin in potato tubers were extensively studied using antisense technology (Edwards et al. 1999). Their data show that depletion of long chains (DP 25–35) in amylopectin

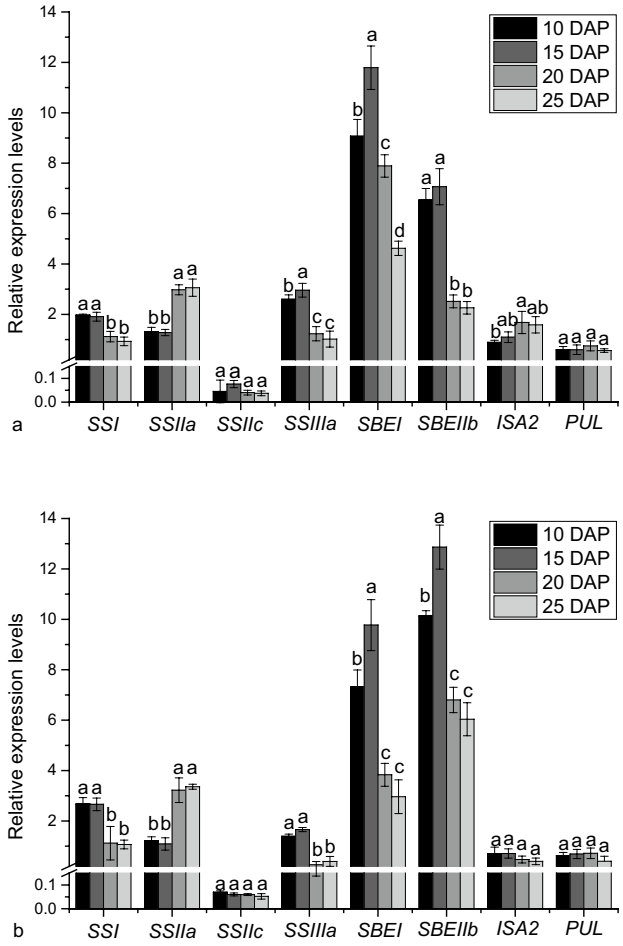

Fig. 2 Relative expression levels of genes of starch biosynthetic enzymes during grain filling in two waxy maize hybrids of **a** SYN2 and **b** JKN2000. *Note*: Different letters within a column indicate significant difference among mean values ($p < 0.05$)

is more pronounced under reduced SSIII activity rather than reduced SSII activity, suggesting that SSIII preferentially synthesizes long chains as compared with SSII. The lesion of *dul* coding SSIIIa in maize decreased the proportions of the relatively long B chains of amylopectin (Wang et al. 1993). In this study, *SSI*, *SSIIa*, and *SSIIIa* expression levels were positively correlated with the chains of DP 6–12, DP 13–24, and DP 25–36 and DP ≥ 37, respectively. *SSIIc* expression level was least abundant and accounted for 1.2–13.1% of the other three *SS* genes in waxy maize grain (Fig. 2). Furthermore, no significant relation was found between *SSIIc* expression and CLD. It is more likely that *SSIIc* plays an insignificant role in the amylopectin fine structure, and *SSI*, *SSIIa*, and *SSIIIa* cooperate to construct various lengths of chains ranging from short to long chains. Although further experiments are needed to identify the role of each SS isoform in amylopectin biosynthesis, the present evidence suggests that *SSI*, *SSIIa*, and *SSIIIa* specifically are

responsible for the construction of short A chain, long B1 chain, and longer B2 and B3 chains of amylopectin, respectively. Namely, SS*I* chooses shorter branches as substrate to produce short A chain, which will be elongated further by *SSIIa to* produce long B1 chain that is continued to extend by *SSIIIa* to produce longer B2 and B3 chains.

Contribution of starch branching enzymes to amylopectin fine structure

Starch branching enzymes create new glucan branches by cleaving the α-1,4-linkage in polyglucans and reattaching the chain via an α-1,6-linkage. Plants have two isoforms of SBE, namely, SBEI and SBEII. In this study, SBE activity had a positive correlation with DP 6–12 proportion and a negative correlation with DP ≥ 37 proportion. Further analysis should be required to understand the distinct roles of SBEI and SBEII in preference for α-1,4-chain lengths.

In vitro experiments of *Escherichia coli* suggest that maize SBEI isoform preferentially generates longer chains whereas SBEII isoform produces shorter chains, and that the minimum chain length required for SBEI is likely DP 16 while that for SBEII is DP 12 (Guan et al. 1997). In rice SBEIIb-deficient mutant (*amylose-extender*, *ae*), the structure of amylopectin in the endosperm was altered by the greatest decrease in chains with DP 8–12, indicating that SBEIIb plays a specific role in the formation of A chain (Nishi et al. 2001). Maize *ae* mutant exhibits the increased proportion of long B chain and ACL (Wang et al. 1993). Further analyses for rice *ae* mutant line demonstrate that SBEIIb preferentially generates short chains of DP 6 and 7, with only a limited increase in DP 8–10 (Sawada et al. 2009). According to the correlation analysis in this study, *SBEI* was more likely responsible for the synthesis of DP ≥ 25, while *SBEIIb* played a specific role in the formation of DP 6–12. Further experiments are needed to characterize the more precise role of each SBE isoform in the amylopectin synthesis.

Contribution of starch debranching enzymes to amylopectin fine structure

Starch debranching enzymes directly hydrolyze α-1,6-glucosidic linkages of polyglucans. There are two types of DBE, isoamylase (ISA) and pullulanase (PUL). Maize ISA1-deficient mutant (sugary, *su1*), which had a reduction in ISA activity, accumulated the highly branched glucopolysaccharide phytoglycogen (James et al. 1995) and resulted in the high degrees of branching (Wang et al. 1993). In vitro, the unrestricted action of ISA generates linear glucans. Mutational analysis of the PUL-type DBE of maize indicated that PUL may supplement the function of ISA (Dinges et al. 2003). In this study, DBE activity had a negative correlation with DP 6–12 and DP 13–24 proportions, and a positive correlation with DP 25–36 and DP ≥ 37 proportions, which was consistent with *ISA2*. There was no significant correlation between *PUL* expression and CLD. The present results showed that the reduced *ISA2* expression level resulted in the increased short chain while *PUL* appeared an insignificant role in CLD formation. It is hypothesized that ISA plays a more important role to hydrolyze the a-1,6-glucosidic linkages of polyglucans. PUL may function in accordance with ISA to promptly clear various lengths of short chains in the process of amylopectin synthesis.

Conclusion

The amylopectin fine structure and the expression levels of the starch biosynthetic genes were investigated in two waxy maize hybrids, and the potential contributions of the genes to amylopectin structure were evaluated. CLD data showed that waxy maize had the highest proportion of amylopectin B1 chain (DP 13–24), the secondary proportion of A chain (DP 6–12), and the low proportions of B2 (DP 25–36) and B3 (DP ≥ 37). SS*I* contributed to the formation of short A chain, which will be elongated by *SSIIa* to generate long B1 chain that was continued to extend by *SSIIIa* to synthesize longer B2 and B3 chains. *SBEI* preferentially produced longer B2 and B3 chains, while *SBEIIb* synthesized short A chain. *ISA2* played an important role for the removal of A and B1 chains and the formation of B2 and B3 chains.

Author contributions HZ contributed to the conceptualization, methodology, funding acquisition, writing—original draft, and writing—review and editing. FQ and GX contributed to the investigation and formal analysis. SG and YY contributed to the text polishing. MW contributed to the design of this study, the software, and the modification of the manuscript. JC and FJ contributed to the supervision and funding acquisition.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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