

Genetic dissection of starch paste viscosity characteristics in waxy maize revealed by high-density SNPs in a recombinant inbred line population

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Abstract Starch paste viscosity properties are widely used as important indicators for quality estimation in waxy maize. To elucidate the genetic basis of paste viscosity characteristics of waxy maize, seven parameters from the rapid visco analyzer (RVA) profile were analyzed for quantitative trait loci (QTLs) in this study, using a recombinant inbred line population derived from a cross between the inbred lines Tongxi5 and Hengbai522. A high-density linkage map was constructed using 2703 bin markers, covering 1876.20 cM of the whole genome with an average genetic distance of 0.73 cM between adjacent bin markers. Seventy-two QTLs were detected for RVA parameters across 3 years, of which 17 could be identified in 2 years, and 6 identified in all 3 years. Eight QTL clusters were observed to be co-associated with two or more RVA parameters.

Three major QTLs, *qPV4-1*, *qTV4-1*, and *qFV5-2*, which explained over 10% of the phenotypic variation, were stably mapped to the chromosomes 4 or 5 in all years. Based on functional annotations, two genes were considered as potential candidate genes for the identified major QTLs. The QTLs and candidate genes identified in this study will be useful for further understanding of the genetic architecture of starch paste viscosity characteristics in waxy maize, and may facilitate molecular breeding for grain quality improvement in breeding programs, and simultaneously provide a basis for cloning of the genes underlying these QTLs.

Keywords Waxy maize · Starch paste viscosity characteristics · Quantitative trait loci · SNP marker

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Introduction

Waxy maize (*Zea mays* L. var. *ceratina*) was first discovered in China in 1908 (Collins 1909). It is a special type of maize as it has nearly 100% amylopectin in its endosperm (Perera et al. 2001). For the excellent characteristics in terms of starch composition with low hardness and high digestibility, clarity, and viscosity, waxy maize is mainly consumed as a fresh food in Asia and also used in the food, textile, adhesive, and paper industries (Bao et al. 2012; Fan et al. 2008; Hao et al. 2015b; Lu et al. 2013). In the past several decades, genetic improvements have led to steady increases in waxy maize yield (Ding et al. 2006). Waxy maize breeders are now mandated to develop varieties with good grain

quality to meet particular demands of food and other industry target markets.

Starch is the major component in cereal grains, and changes in biochemical characteristics can affect viscosity and gelatinization properties of starch and thus determine the final usage of waxy maize in food, feed, and industry (Cozzolino 2016; Liu et al. 1997; Yang et al. 2014). The starch viscosity profile (RVA profile, as determined by a Rapid Visco Analyzer), referring to the pasting curve generated from cereal flour (Bao et al. 2000; Yang et al. 2014), has been proven to be useful in estimating the quality of cereal crops, including waxy maize (Bao et al. 2000; Ketthaisong et al. 2013; Lu et al. 2013; Yan et al. 2014; Yan et al. 2011). The RVA profile parameters are classic quantitative traits; they are regulated by a complex genetic system involving many starch-related genes and are also influenced by non-genetic and environmental factors (Yan et al. 2014). Due to these quantitatively genetic characteristics, it is difficult for breeders to efficiently accomplish quality improvements by means of conventional approaches (Raihan et al. 2016). Understanding the genetic mechanism of grain quality will allow the genetic manipulation of waxy maize and will be beneficial to breeding programs (Yan et al. 2014).

The advent of maize genomics and development of molecular markers have proved powerful tools for elucidating the genetic basis of waxy maize quality, thus allowing improvements in breeding efficiency. Molecular markers linked to causal genes and/or quantitative trait loci (QTLs) may be useful for marker-assisted selection (MAS) in multi-gene pyramiding (Xu and Crouch 2008). A great number of QTLs for RVA profile parameters have been dissected in diverse crop populations, including rice (Bao et al. 2000; Bao et al. 2002; Hsu et al. 2014; Xu et al. 2015; Yacouba et al. 2013; Yan et al. 2014; Zhang et al. 2013), barley (Wang et al. 2010), and wheat (Liang et al. 2009). In rice, two major QTL clusters, contributing largely to phenotypic variation of several viscosity characteristics, have been consistently mapped to the chromosome 6 region corresponding to the *Wx* (encoding granule-bound starch synthase-I, GBSSI) and *Alk* (functions as starch synthase, SS) loci, in different segregating populations of different cross-combinations across different environments (Bao et al. 2000; Hsu et al. 2014; Wang et al. 2007; Xu et al. 2015; Yacouba et al. 2013; Yan et al. 2014). Starch paste viscosity characteristics are also

controlled by other genes (Xu et al. 2016), such as *pullulanase* (*PUL*) (Kharabian-Masouleh et al. 2012; Yan et al. 2011), soluble starch synthase (*SSS*) genes (Xu et al. 2016; Xu et al. 2013), starch-branching enzyme (*SBE*) genes (Han et al. 2004), debranching enzyme (*DBE*) genes (Yan et al. 2014), and isoamylase (*ISA*) genes (Hsu et al. 2014; Yang et al. 2014). However, there have been few attempts to address the genetic mechanisms of starch paste viscosity characteristics in maize, including waxy maize (Zhang et al. 2010). QTL analysis of starch paste viscosity characteristics of waxy maize will provide a better understanding of the genetic factors influencing grain quality and also aid discovery of valuable markers for MAS (Wang et al. 2010).

Development of high-throughput genotyping technologies in maize has enabled development of new single nucleotide polymorphism (SNP) markers, thus leading to an increase in genetic marker density. With the advantage of low time consumption, low cost, and high throughput, SNPs have been widely applied in the construction of high-density genetic linkage maps to significantly increase the detection power, accuracy, and resolution needed to identify QTLs (Wang et al. 2015). Recently, the “skeleton bin map,” which combines co-segregating SNP markers into one bin and separates adjacent bins based on single recombination events, has been widely used to map QTLs for kernel starch content and other important grain-related traits in maize (Raihan et al. 2016; Wang et al. 2015). In this study, a waxy maize recombinant inbred line (RIL) population, derived from two parents of Tongxi5 and Hengbai522 of *var.* Suyunuo 1, which is currently the most widely grown waxy maize hybrid in China (Xie et al. 1997; Xie et al. 2009), was developed and genotyped using an Affymetrix microarray CGMB50K SNP Array containing 56,000 maize SNPs developed from the B73 reference sequence (Ganal et al. 2011). The starch paste characteristics of the RIL and parental lines were evaluated over 3 years. The objectives were to construct a high-density genetic linkage map of waxy maize using inferred bins from the high-density SNP markers and to dissect the genetic architecture of starch paste viscosity characteristics in waxy maize RIL population. Information on molecular markers tightly linked to QTLs controlling starch paste viscosity characteristics (RVA profiles) identified in this study will facilitate breeding strategies for quality improvements in waxy maize.

Materials and methods

Plant materials and field experiments

A population consisting of 198 waxy maize RILs, produced by single seed descent, was derived from a cross between the inbred lines Tongxi5 and Hengbai522 with similar amylopectin content; these two inbred lines formed the popular commercial hybrid Suyunuo1 (Xie et al. 1997; Xie et al. 2009). The waxy maize inbred lines of Tongxi5 and Hengbai522 differ significantly in starch viscosity, thus providing the means to clarify the genetic basis of the waxy maize grain quality. All 198 RILs and both parents were evaluated in 2013 (designated as environment E1), 2014 (designated as environment E2), and 2015 (designated as environment E3), at the Experimental Farm of Jiangsu Yanjiang Institute of Agricultural Sciences, Nantong, China.

In each environment, field trials were carried out following a randomized complete block design with two replicates (Yan et al. 2014). Each genotype was grown in a single-row plot, 500 cm long and 60 cm apart, with 20 plants per row. Standard agronomic practices were applied to provide adequate nutrition and keep plots disease free. All plants were manually self-pollinated, and mature grains from each RIL were harvested in bulk for phenotypic measurements. Harvested waxy maize grains were ground using a high-speed universal disintegrator (FW-100, Taisite, Tianjin, China) and then sieved through a 100-mesh sieve.

Phenotypic measurements

Pasting properties of the flours (28 g total weight; 10%, w/w, dry basis) were measured using a rapid visco analyzer (RVA, Model 3D, Newport Scientific, Australia) and analyzed using TCW (Thermal Cycle for Windows) software following the method described by Lu and Lu (2012). The sample suspension of each RIL was incubated at 50 °C for 1 min, the temperature increased to 95 °C and maintained for 2.5 min, and finally cooled down to 50 °C and maintained for 1 min. Three primary RVA parameters, peak viscosity (PV), trough viscosity (TV), and final viscosity (FV), were obtained from the pasting curve. Two secondary RVA parameters, breakdown viscosity (BD = PV – TV) and setback viscosity (SB = FV – TV), were calculated from the primary parameters. Pasting time (PT) and pasting temperature (PTP) were also recorded.

Statistical analysis

Statistical analyses of all phenotypic data of the pasting properties across 3 years were performed using SAS 9.13 software (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was performed using the PROC GLM procedure. The broad-sense heritability (H^2) of each RVA parameter was estimated according to the following formula: $H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$, where σ_g^2 denotes genotypic variance components and σ_e^2 denotes error variance components, which were evaluated using PROC VARCOMP. The best linear unbiased predictors (BLUPs) for each line in the tested RILs were evaluated to account for the effects of environmental factors using PROC MIXED. PROC CORR was used to estimate the correlations between the RVA parameters based on the BLUPs.

Genotyping and construction of genetic linkage maps

All RILs and parental inbred lines were genotyped using an Affymetrix microarray CGMB50K SNP Array containing 56,000 maize SNPs developed from the B73 reference sequence (Ganal et al. 2011), at China Golden Marker (Beijing) Biotech, China. The harvested genotypic matrix was corrected using a hidden Markov model approach from the R package MPR⁵⁰ with some modifications, and the co-segregated SNP markers were grouped in bins using a home-made Perl script (Zhang et al. 2016). Chi-squared tests were conducted to test segregation distortion of the bins at the significance level of $P > 0.05$. All called bins were used to construct the genetic linkage map using JoinMap version 4.0 software (Van Ooijen 2006), with the Kosambi mapping function used to calculate the marker genetic distance.

QTL analysis

QTL mapping and estimation of QTL effects on each RVA parameter were performed using QTL IciMapping v4.1 (Lei et al. 2015). The method of inclusive composite interval mapping (ICIM) was applied to detect additive QTLs. For ICIM, the scanning step size was set to 1 cM, and the largest P value for entering variables in stepwise regression of phenotype on marker variables (PIN) was set at 0.001. QTLs were considered significant at a LOD threshold of 2.5 as this is commonly used for QTL mapping in maize (Song et al. 2016). QTLs detected in different environments and explaining more

Table 1 Descriptive statistics, ANOVA, and broad-sense heritability for RVA parameters in the RIL population and parents over 3 years

Traits	Year	RIL population		Parents (mean \pm SD)		G ^a	G \times E ^b	H ^{2c} (%)
		Mean \pm SD	Range	Tongxi5	Hengbai522			
PV (cP)	2013	1633.66 \pm 408.13	520.89–2590.33	1747.44 \pm 14.42	1200.47 \pm 34.25	**	**	79.9
	2014	1876.83 \pm 473.18	623.00–2988.00	1947.00 \pm 59.89	1504.00 \pm 28.57			
	2015	1484.34 \pm 382.46	445.50–2427.50	1743.50 \pm 45.50	1282.50 \pm 12.9			
TV (cP)	2013	1486.33 \pm 385.89	419.01–2486.01	1634.10 \pm 11.46	1096.63 \pm 30.94	**	**	80.5
	2014	1701.11 \pm 449.99	486.00–2846.00	1788.00 \pm 55.72	1381.00 \pm 26.94			
	2015	1356.45 \pm 365.89	373.50–2253.50	1654.00 \pm 46.24	1210.50 \pm 14.88			
BD (cP)	2013	147.33 \pm 79.04	8.29–438.99	113.34 \pm 2.96	103.84 \pm 3.31	**	**	76.2
	2014	175.72 \pm 98.48	11.00–521.00	159.00 \pm 4.17	123.00 \pm 1.63			
	2015	127.89 \pm 78.72	6.00–409.50	89.50 \pm 0.74	72.00 \pm 1.98			
FV (cP)	2013	2080.33 \pm 419.41	920.50–3051.00	2484.50 \pm 97.90	1918.00 \pm 57.84	**	**	80.6
	2014	2476.32 \pm 525.75	1070.05–3678.04	2526.00 \pm 100.83	2094.00 \pm 70.29			
	2015	2215.45 \pm 452.23	990.36–3252.11	2539.14 \pm 14.29	1756.95 \pm 14.07			
SB (cP)	2013	729.11 \pm 168.67	351.98–1401.08	905.04 \pm 25.75	660.32 \pm 16.87	**	**	80.5
	2014	775.20 \pm 200.36	366.05–1462.08	738.00 \pm 45.11	713.00 \pm 43.35			
	2015	723.88 \pm 169.01	361.02–1437.12	830.50 \pm 51.65	707.50 \pm 42.96			
PT (min)	2013	6.71 \pm 0.36	5.70–7.05	6.71 \pm 0.08	7.05 \pm 0.07	**	**	73.5
	2014	6.54 \pm 0.47	5.47–7.00	6.47 \pm 0.25	6.87 \pm 0.22			
	2015	6.75 \pm 0.28	5.77–7.00	6.73 \pm 0.23	7.00 \pm 0.21			
PTP ($^{\circ}$ C)	2013	81.40 \pm 2.87	74.82–92.72	82.52 \pm 1.25	84.51 \pm 1.18	**	**	68.9
	2014	81.30 \pm 2.75	75.95–94.85	80.70 \pm 1.46	82.30 \pm 1.58			
	2015	82.02 \pm 3.45	70.53–91.11	82.79 \pm 1.79	84.87 \pm 1.94			

PV peak viscosity, TV trough viscosity, BD breakdown viscosity, FV final viscosity, SB setback viscosity, PT pasting time, PTP pasting temperature

**Significant at $P < 0.001$

^a Genotype across different environments

^b Genotype \times environment

^c Broad-sense heritability

than 10% of phenotypic variance were considered as major QTLs (Raihan et al. 2016).

Results

Phenotypic variations and correlations

In the 3 years investigated, the variation of pasting properties (RVA profiles) revealed a continuous distribution among the 198 RILs, and significant differences were observed between the two parents for most pasting properties (Table 1). The RVA parameters of PV, TV, BD, FV, and SB for Tongxi5 were significantly higher than those for Hengbai522 in all years, except for PT and PTP. Transgressive

segregation was detected in both directions for all traits in the RIL population. Analysis of variance for all tested RVA parameters revealed that the variances of genotypes (G) and the interactions between genotype and all environmental factors (G \times E) were highly significant at the 0.001 probability level (Table 1), which indicated that all these pasting properties were quantitative traits affected by the environment. The estimated broad-sense heritability (H^2) of RVA parameters in the RIL population ranged from 68.9% (PTP) to 80.6% (FV).

To explore the relationships among pasting properties, pairwise correlation analysis was carried out based on the BLUPs in the RIL population. The RVA parameters were significantly correlated with each other, except for BD and PTP, and SB and PT (Table 2).

Table 2 Correlations among RVA parameters based on BLUPs in all RILs

Traits	PV	TV	BD	FV	SB	PT
TV	0.982**					
BD	0.366**	0.181*				
FV	0.942**	0.930**	0.315**			
SB	0.277**	0.205**	0.429**	0.549**		
PT	-0.737**	-0.702**	-0.374**	-0.564**	0.094	
PTP	-0.389**	-0.410*	-0.001	-0.183**	0.447**	0.481**

PV peak viscosity, TV trough viscosity, BD breakdown viscosity, FV final viscosity, SB setback viscosity, PT pasting time, PTP pasting temperature

*Significant at $P < 0.01$; **significant at $P < 0.001$

Significant positive correlations were observed between any two parameters among PV, TV, BD, FV, and SB. Nevertheless, PT was negatively correlated with PV, TV, BD, and FV, whereas PTP was negatively correlated with PV, TV, and FV, and positively correlated with SB and PT.

Construction of a high-density bin map

The Tongxi5/Hengbai522 RIL population and both parental inbred lines were genotyped using 56,000 SNPs. Following quality control, 12,268 SNPs with precise physical positions based on the B73 reference sequence showed polymorphism between the two parental lines. Based on these polymorphic SNPs, a skeleton bin map consisting of 2703 recombinant bins was constructed for all 198 RILs. The resulting high-density bin map spanned a total length of 1876.20 cM of the whole genome, with an average genetic distance of 0.73 cM between adjacent bin markers (Table 3, Supplementary Fig. S1). The number of bin markers in each chromosome ranged from 189 (chromosome 2) to 352 (chromosome 1), with an average of 270 bin markers per chromosome. The genetic length of the 10 chromosomes ranged from 129.15 cM (chromosome 6) to 273.47 cM (chromosome 2), with an average size of 187.62 cM. There were 16 gaps larger than 5 cM in the bin map, six of which were located on chromosome 2.

Identification of QTLs for pasting properties

Based on the ICIM mapping, a total of 72 QTLs were identified for all seven RVA parameters involved in pasting properties; these were distributed on all 10

chromosomes (Supplementary Table S1). Of these QTLs, 23 were stably identified in at least 2 years (Table 4), but the remaining 49 were only detected in a specific year, indicating that most QTLs identified in the RIL population were affected by environment.

Peak viscosity

Ten QTLs were detected for PV across the 3 years; these were located on chromosomes 1, 2, 3, 4, 5, and 9 (Supplementary Table S1). Of these QTLs, *qPV4-1*, *qPV5-1*, and *qPV5-2* were identified in 2 or more years (Table 4). A major QTL of *qPV4-1*, flanked by PZE-104141018 and PZE-104141424 on chromosome 4, was stably detected in all 3 years and accounted 15.51, 14.43, and 11.82% of phenotypic variation, respectively. The allele from Tongxi5 increased the trait value. The other two QTLs (*qPV5-1* and *qPV5-2*) were detected in 2 years (2013 and 2014); both were located on chromosome 5.

Trough viscosity

For TV, 12 QTLs were identified across 3 years, which were located on chromosomes 2, 3, 4, 5, 6, 7, and 9 (Supplementary Table S1). Among them, three QTLs, *qTV4-1*, *qTV5-2*, and *qTV5-3*, were identified in at least two of the years (Table 4). A major QTL of *qTV4-1* was stably identified in all years, which was located at the interval of PZE-104141018_PZE-104141424 on chromosome 4 and explained 11.87 to 13.33% of phenotypic variation in the 3 years. The favorable allele was from Tongxi5, which could increase the TV value by 192.8 cP on average.

Table 3 Characteristic descriptions for the 10 constructed chromosomes

Chr ID	Total bin	Total distance (cM)	Average distance (cM)	Gap number (>5 cM in length)
Chr1	352	190.15	0.54	0
Chr2	189	273.47	1.45	6
Chr3	346	207.57	0.60	0
Chr4	312	189.20	0.61	1
Chr5	326	219.60	0.67	1
Chr6	231	129.15	0.56	0
Chr7	258	194.08	0.75	1
Chr8	231	171.89	0.74	2
Chr9	252	147.74	0.59	0
Chr10	206	153.35	0.74	5
Total	2703	1876.20	0.73	16

Breakdown viscosity

In total, nine QTLs were detected for BD in the 3 years investigated; these were located on chromosomes 1, 3, 5, 7, 8, and 10 (Supplementary Table S1). Of these QTLs, *qBD1-1*, *qBD3-3*, and *qBD8-1* were detected in 2 or more years (Table 4). Only one stable QTL of *qBD1-1* was identified for BD in all years, which was flanked by PZE-101125948 and SYN25740 on chromosome 1 and accounted for 2.29 to 4.19% of phenotypic variation in different years. The allele from Tongxi5 showed an increasing effect.

Final viscosity

Twelve QTLs were identified for FV across the 3 years; these were located on chromosomes 2, 5, 6, 7, and 9 (Supplementary Table S1). Four of these QTLs for FV (*qFV2-1*, *qFV5-1*, *qFV5-2*, and *qFV5-3*) were identified in 2 or more years (Table 4). Among which, two stable QTLs, *qFV2-1* (in the marker interval of SYN5616_PZE-102161143 on chromosome 2) and *qFV5-2* (a major QTL flanked by PZE-105159228 and PZE-105158503 on chromosome 5), were consistently detected in all years, accounting for 4.41 and 13.95% of phenotypic variation on average, respectively. Furthermore, *qFV2-1* and *qFV5-2* showed oppositely additive effects: the Tongxi5 allele of *qFV2-1* increased FV by a mean value of 139.56 cP, whereas that of *qFV5-2* decreased it by 254.77 cP on average.

Setback viscosity

Seven QTLs were identified for SB across the 3 years, which were located on chromosomes 2, 3, 4, 5, and 7 (Supplementary Table S1). Among them, two QTLs, *qSB2-1* (flanked by SYN5616 and PZE-102161143 on chromosome 2) and *qSB5-1* (flanked by PZE-105041558 and PZE-105041198 on chromosome 5), were persistently detected in two of the years and explained 5.63 and 7.42% of the average phenotypic variation, respectively (Table 4). All favorable alleles of *qSB2-1* and *qSB5-1* were from Tongxi5, which could increase SB by a mean value of 42.69 and 44.19 cP, respectively. No major QTL was identified in all years.

Pasting time

For PT, a total of 14 QTLs were identified across 3 years, distributing on eight chromosomes, the exceptions being chromosomes 2 and 6 (Supplementary Table S1). Of these QTLs, eight were detected in two of the years and explained 0.92 to 2.70% of total phenotypic variance (Table 4). In all years, only one QTL of *qPT8-1* was stably identified and explained only a small percentage of the phenotypic variance, with an average of 0.92%, and was flanked by PZE-108100090 and PZE-108100367 on chromosome 8. The allele from Tongxi5 could increase the trait value by 0.12 min.

Table 4 QTLs detected for RVA parameters in the RIL population in at least 2 years

Traits	QTL ^a	Chr	Marker interval	2013 (E1)			2014 (E2)			2015 (E3)		
				LOD	PVE (%)	Add	LOD	PVE (%)	Add	LOD	PVE (%)	Add
PV	<i>qPV4-1</i>	4	PZE-104141018_PZE-104141424	4.22	15.51	221.32	3.77	14.43	249.16	4.60	11.82	194.93
	<i>qPV5-1</i>	5	PZE-105159228_PZE-105158503	3.83	11.45	-192.06	4.09	12.37	-233.62			
	<i>qPV5-2</i>	5	PZE-105028457_PZE-105027956	4.42	3.62	107.47	3.94	3.25	119.26			
TV	<i>qTV4-1</i>	4	PZE-104141018_PZE-104141424	2.98	11.87	166.45	3.49	13.33	227.97	3.62	11.95	183.99
	<i>qTV5-2</i>	5	PZE-105159228_PZE-105158503	3.28	10.94	-162.24	4.70	14.07	-237.71			
	<i>qTV5-3</i>	5	PZE-105028457_PZE-105027956				3.19	2.62	102.04	3.01	2.06	77.16
BD	<i>qBD1-1</i>	1	PZE-101125948_SYN25740	2.98	2.29	84.79	3.42	2.43	100.23	4.32	4.19	76.12
	<i>qBD3-3</i>	3	PZE-103066568_PZE-103123538	3.53	1.89	-106.12				3.29	2.84	-92.34
	<i>qBD8-1</i>	8	PZE-108002149_PZE-108032584	3.95	2.71	93.84				11.57	7.96	81.96
FV	<i>qFV2-1</i>	2	SYN5616_PZE-102161143	6.33	3.45	129.82	6.44	3.27	150.82	7.52	6.51	138.03
	<i>qFV5-1</i>	5	PZE-105166634_SYN21847	8.00	10.14	-221.17	9.61	11.07	-275.42			
	<i>qFV5-2</i>	5	PZE-105159228_PZE-105158503	6.62	12.07	-245.57	6.59	11.33	-284.10	7.03	18.46	-234.63
	<i>qFV5-3</i>	5	PZE-105156596_PZE-105156438	6.23	4.94	154.46	5.82	4.19	169.40			
SB	<i>qSB2-1</i>	2	SYN5616_PZE-102161143	3.53	7.29	38.76	3.75	3.97	46.61			
	<i>qSB5-1</i>	5	PZE-105041558_PZE-105041198	3.59	8.97	43.01				3.55	5.86	45.37
PT	<i>qPT1-1</i>	1	PZE-101215093_PZE-101214818	2.70	3.01	-0.24	3.63	2.34	-0.33			
	<i>qPT1-2</i>	1	PZE-101201096_PZE-101202983	3.05	2.96	0.24	4.02	2.29	0.32			
	<i>qPT4-1</i>	4	PZE-104027342_PZE-104026739	2.92	3.05	-0.24	3.96	2.35	-0.32			
	<i>qPT7-1</i>	7	SYN22406_SYN32290	3.26	3.05	-0.24	4.00	2.34	-0.33			
	<i>qPT7-2</i>	7	SYN25440_PZE-107005831	2.52	2.58	0.22	3.06	2.09	0.30			
	<i>qPT8-1</i>	8	PZE-108100090_PZE-108100367	3.17	0.70	0.11	3.36	0.55	0.16	2.63	1.50	0.08
	<i>qPT9-1</i>	9	PZE-109084150_PZE-109085111	2.63	2.93	-0.23	3.42	2.27	-0.32			
	<i>qPT9-2</i>	9	SYN39040_PZE-109107920	2.99	2.47	-0.21	3.62	2.05	-0.30			

PV peak viscosity, TV trough viscosity, BD breakdown viscosity, FV final viscosity, SB setback viscosity, PT pasting time, LOD log₁₀ of odds ratio, PVE percentage of phenotypic variance explained by a single QTL, Add additive effect

^aQTLs identified are named with trait abbreviations followed by the chromosome number

Pasting temperature

Eight QTLs were detected for PTP in all years, which were located on chromosomes 1, 2, 5, 6, and 7 (Supplementary Table S1). Among these QTLs, three were identified in 2013, four in 2014, and one in 2015, collectively explaining 16.78, 8.49, and 8.94% of phenotypic variation, respectively. Nevertheless, none was detected in 2 or more years, so these QTLs may have been the result of environmental effects.

Co-localization of QTLs underlying pasting properties

Eight QTLs for RVA parameters were identified as being co-located on the same genome regions of waxy maize (Supplementary Table S1, Table 4). The *qBD3-3* for BD and *qSB3-1* for SB were co-located in the marker

interval of PZE-103066568_PZE-103123538 on chromosome 3, and the QTL for BD (*qBD3-2*) on chromosome 3 coincided with the QTL for PT (*qPT3-1*). Three QTLs for PV, *qPV4-1* (flanked by PZE-104141018 and PZE-104141424 on chromosome 4), *qPV5-2* (flanked by PZE-105028457 and PZE-105027956 on chromosome 5), and *qPV9-1* (flanked by PZE-109094529 and PZE-109096246 on chromosome 9), were also identified for TV (*qTV4-1*, *qTV5-3*, and *qTV9-1*), and the QTL for PV (*qPV5-1*) in the interval of PZE-105159228_PZE-105158503 on chromosome 5 was simultaneously found for TV (*qTV5-2*) and FV (*qFV5-2*). The QTLs for FV (*qFV2-1*) and SB (*qSB2-1*) on chromosome 2 were co-located in the interval of SYN5616_PZE-102161143. A QTL for TV on chromosome 7, *qTV7-1* (flanked by SYN12703 and SYN10108), was also found for FV (*qFV7-3*).

Candidate gene prediction

Candidate genes in the physical intervals of PZE-104141018_PZE-104141424 on chromosome 4 and PZE-105159228_PZE-105158503 on chromosome 5, where the major QTLs of *qPV4-1* and *qTV4-1*, and *qFV5-2* were located, were predicted based on B73 reference genome sequence Version 5b.60 (<http://www.maizegdb.org>). These two genomic intervals of PZE-104141018_PZE-104141424 and PZE-105159228_PZE-105158503 were approximately 475 and 181 kb, encompassed 28 and 11 protein-coding genes, respectively (Supplementary Table S2). According to the maize gene annotation database at MaizeGDB (<http://www.maizegdb.org>), the putative genes within these two major QTL intervals indicated that genes of GRMZM2G146028 on chromosome 4 and GRMZM2G142709 on chromosome 5 were the most likely candidate genes for *qPV4-1* and *qTV4-1*, and *qFV5-2*, respectively. The candidate gene of GRMZM2G146028 encodes an AP2/EREBP family transcription factor (Supplementary Table S2). In rice, an AP2/EREBP family transcription factor of *RSR1* has been identified as a starch biosynthesis regulator underlying the amylopectin structure and consequently determining the physicochemical properties of starch (Fu and Xue 2010). Another candidate gene of GRMZM2G142709 encodes a glucosyltransferase (Supplementary Table S2), which was involved in starch pasting properties in cassava (Thanyasiriwat et al. 2014).

Discussion

Waxy maize is generally reserved for fresh food in Asia, but is also widely used as industry resource for the textile, adhesive, and paper industries. The quality of food and industrial applications represents an important index reflecting the waxy maize grain quality (Yang et al. 2014), and high-quality grain is becoming an increasingly important objective in waxy maize breeding. Starch paste viscosity properties (RVA profile) are widely used as indicators of cereal kernel quality (Bao et al. 2000; Kethaisong et al. 2013; Lu et al. 2013; Yan et al. 2014; Yan et al. 2011; Yang et al. 2014). A few studies have focused on the relationship between starch biosynthesis-related genes and starch paste viscosity properties in maize (non-waxy maize) (Wilson et al. 2004; Yang et al. 2014), but little effort has been made

to elucidate the genetic basis of starch paste viscosity in waxy maize. Waxy maize kernel contains nearly 100% amylopectin. Unfortunately, the genetic information gained from non-waxy maize cannot be applied directly for waxy maize analysis and quality improvement, similarly to glutinous rice vs. non-glutinous rice (Yan et al. 2011). In this study, the genetic basis of starch paste viscosity properties in waxy maize was dissected using high-density QTL mapping. This provided an overview of the genetic mechanism of the starch viscosity profile in waxy maize and may facilitate improvements in the grain quality of waxy maize.

In this study, a RIL population, comprising 198 families derived from a cross between two waxy maize parents with similar amylopectin content, was used to map QTLs for starch viscosity properties using the ICIM method across 3 years. The estimates of broad-sense heritability revealed moderate heritabilities for the tested RVA parameters, with the H^2 value ranging from 68.9% for PTP to 80.6% for FV, which was consistent with the results from previous studies in rice (Wang et al. 2007). The results of linkage analysis indicated that these grain quality traits were genetically complex and significantly influenced by the environment. Seventy-two QTLs were detected for seven RVA parameters, each accounting for 0.55–18.46% of phenotypic variation. Among these QTLs, 17 were identified in two of the years, six were identified in all 3 years. These results indicated that most QTLs for starch viscosity properties in waxy maize might be affected by environmental factors with QTL-by-environment interaction ($G \times E$, QEI) and under the control of multiple genes with minor effects (Hao et al. 2015a; Raihan et al. 2016; Zhou et al. 2016). The QEI may be due to the specific expression of certain genes during the process of adaptation to different ecological environments (Zhou et al. 2016). Due to the QEI, the specific QTL might be strongly expressed in a particular environment, while not or weakly expressed in another environment; or the specific QTL could have opposite effects on the phenotypic variation in different environments (Li et al. 2003). The stable and highly heritable QTLs, which could be expressed in different environments, might be useful for the future MAS for the improvement of grain quality in a wide range of environments in waxy maize breeding programs (Zhou et al. 2016); while particular environmental conditions were important in the expression of grain quality, environment-favorable QTL could be used for MAS within specific target environments (Li et al. 2015;

Shang et al. 2016). In maize, MAS has been successfully used for the improvement of multiple grain quality-related traits, such as provitamin A content (Azmach et al. 2013) and kernel oil content (Hao et al. 2014). In this study, the stable QTLs identified for the RVA parameters with relatively high heritability, including *qPV4-1*, *qTV4-1*, *qBD1-1*, *qFV2-1*, *qFV5-2*, and *qPT8-1*, are likely important QTLs for starch viscosity properties in waxy maize and should be considered priority candidates for MAS in wide range environments in modern waxy maize breeding programs.

In this study, eight QTL clusters, mainly distributed on chromosomes 2, 3, 4, 5, 7, and 9, were observed to be co-associated with two or more RVA parameters, which was coincided with significant correlations among the studied parameters. For example, a QTL cluster in the interval of PZE-104141018_PZE-104141424 on chromosome 4 was responsible for two major QTLs of *qPV4-1* and *qTV4-1*, detected in all years; a major QTL for FV (*qFV5-2*) was in the interval of PZE-105159228_PZE-105158503 on chromosome 5, and the other two QTLs of *qPV5-1* for PV and *qTV5-2* for TV were mapped to the same region; significant positive correlations were observed among these RVA parameters. The co-locations of QTLs for different RVA parameters in the studied population suggest pleiotropy of a single causal gene or close linkage of multiple causal genes (Yan et al. 2014; Zhang et al. 2013). In modern waxy maize breeding schemes for grain quality improvement, MAS of these co-associated genetic loci could simultaneously improve multi-target traits of waxy maize grain quality.

In previous studies, a few gene loci involved in starch biosynthesis were revealed to be related to starch paste viscosity properties in rice, including *Wx*, *Alk*, *PUL*, *ISA*, *SBE*, and *SS* (Han et al. 2004; Xu et al. 2013; Yan et al. 2014; Yan et al. 2011; Zhang et al. 2013). Based on candidate gene association analysis, genes of *isa2*, *ae1*, and *sh2* were identified to be significantly associated with starch viscosity properties in maize (Wilson et al. 2004; Yang et al. 2014). To further address the natural variation of known starch metabolism-related genes and possible molecular mechanisms underlying the detected QTLs for starch paste viscosity properties in this study, their physical positions were compared based on B73 reference genome sequence Version 5b.60. Unexpectedly, no QTL was found in the *wx* locus on chromosome 9, and no other known starch metabolism-related genes co-localized within the QTLs detected in this study.

These results suggest that both parents with similar amylopectin content might have the same *wx* allele, or that some of the genes involved in starch viscosity properties did not show allelic variation between the two parents (Yan et al. 2014; Zhao et al. 2009); therefore, novel molecular mechanisms might account for the QTLs identified in the studied population (Wang et al. 2015) or there may be genotype-by-environment interactions in waxy maize (Liu et al. 2016). The other explanation for this phenomenon might be that other components of waxy maize kernels also influence starch paste viscosity properties, such as protein (the second most abundant component following starch), which significantly affects the physicochemical properties of starch because that gelatinized starch granules are either coated on the peripheral surface of the protein network or trapped in the protein matrix (Lu and Lu 2012).

The quality of genetic maps significantly influences accuracy of QTL mapping. The use of high-density genetic maps enables capture of more recombination events and makes fuller use of the linkage information, thus improving the resolution and accuracy of QTL mappings performed (Chen et al. 2016). In the present study, a high-density genetic map was constructed using 56,000 SNPs, with an average genetic distance of 0.73 cM between adjacent bin markers. Due to the reduction of QTL intervals, candidate genes underlying the mapped locus could be predicted; this will provide valuable information for positional cloning of these QTLs (Zhou et al. 2016). Among three major QTLs detected in this study, the physical distances for *qPV4-1* and *qTV4-1* in the interval of PZE-104141018_PZE-104141424 on chromosome 4 and for *qFV5-2* in the interval of PZE-105159228_PZE-105158503 on chromosome 5 were approximately 475 and 181 kb, respectively. These two genomic intervals encompass 28 and 11 protein-coding genes, respectively. According to the B73 reference genome sequence Version 5b.60 and the gene annotation data available at MaizeGDB, two genes, GRMZM2G146028 (encoding an AP2/EREBP family transcription factor) within the *qPV4-1* and *qTV4-1* interval on chromosome 4 and GRMZM2G142709 (encoding a glucosyltransferase) within the *qFV5-2* interval on chromosome 5, were the most likely candidate genes that might regulate starch pasting properties, as previously reported in rice (Fu and Xue 2010) and in cassava (Thanyasiriwat et al. 2014). These results not only promote further research into the genetic mechanisms of starch viscosity profile in waxy

maize but also provide a basis for MAS of starch pasting properties in waxy maize quality improvement programs. However, their association with starch pasting properties in waxy maize requires further study, and this would involve strategies such as fine mapping of identified QTLs by backcrosses or knock-outs and over-expression of candidate genes in the QTL intervals (Wang et al. 2015).

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