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Genome-Wide Association Study (GWAS) Reveals an SNP Associated with Waxy Trait and Development of a Functional Marker for Predicting Waxy Maize (*Zea mays* L. var. ceratina)

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Abstract: Waxy maize (*Zea mays* L. var. ceratina) is a special type of maize characterized by a sticky texture when cooked, due to high amylopectin content in the endosperm. Waxy maize is popular in China and Southeast Asia for fresh consumption. Breeding strategies have been used to improve the quality of waxy maize, including hybrid breeding by crossing super sweet maize and waxy maize. However, the lack of a marker has limited the efficiency of breeding for the waxy trait, especially because the waxy allele is recessive. In this study, we conducted a genome-wide association study (GWAS) in an association panel consisting of 213 inbred lines and recombinant inbred lines (RILs) of field maize and waxy maize to identify loci associated with the waxy kernel phenotype. The genotypic data were 155,768 SNPs derived from the high-density 600 K maize genotyping array for single-nucleotide polymorphisms (SNPs). The GWAS results identified the *qWx9* locus on chromosome 9 (25.06–25.18 Mb) associated with the trait. Based on the most significantly associated SNP (AX-90613979, −log10(P) = 6.8)), which was located on *Wx1*, a MassArray marker was developed and validated in a panel of 139 maize lines containing waxy maize and sweet maize with different amylose content. The newly developed marker had a significant association with amylose content (R^2 value of 0.81, $p < 0.001$) and clearly distinguished between waxy maize and sweet maize lines that had different amylose content. This marker will be useful for maize breeding programs for the waxy trait, as well as for breeding programs for hybrid maize combining the sweetness and waxy traits. The gene-based SNP markers could aid breeders by eliminating the costs and time required to perform lengthy field trials and help to accelerate sweet maize and waxy maize breeding programs.

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1. Introduction

Maize (*Zea mays* L.) is the third most important cereal in the world, after rice and wheat [1]. It is an important resource for food, feed, and biofuel and is the main resource for the starch industry [2,3]. Waxy maize (*Zea mays* L. var. ceratina) is distinguished from normal maize by the texture or starch composition (amylose and amylopectin) in the kernel, because the endosperm of waxy maize contains a large amount of amylopectin and very few or no amylose starch molecules. Waxy starch existing in the endosperm of the grains has also been reported in other cereals, including sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), foxtail millet (*Setaria italica*), barley (*Hordeum vulgare*), and Job's tears (*Coix lacryma-jobi*) [4]. As in other cereals, waxy maize is characterized by its sticky texture when cooked. It is popular in China and Southeast Asia for fresh consumption. Waxy-cereal starch produces pastes with higher viscosity and less rigidity than ordinary starch. Waxy starch, especially waxy cornstarch, can be used as a raw material in the food, textile, adhesive, and paper industries [5–7]. Waxy maize was first discovered in China in 1908 [8] and subsequently disseminated to other parts of Asia [9]. It is believed to have originated from cultivated flint maize and recently diverted from common maize [6].

Waxy maize is unique in that it has a *waxy* mutant (*wx*) in the endosperm that controls the properties of the endosperm. Starch synthesis in the endosperm of maize is mediated by four types of enzymes: (1) ADP-glucose pyrophosphorylase (AGPase, the small subunit encoded by *Brittle-2* (*Bt2*), and the large subunit encoded by *Shrunken2* (*Sh2*)); (2) starch synthase: soluble starch synthase (SS) and granule-bound starch synthase (GBSS, encoded by *Waxy1* or *Wx1*); (3) starch-branching enzyme (BE, encoded by *Amylose extender1* (*Ae1*)); (4) starch debranching enzyme (DBE, encoded by *Sugary1* (*Su1*)) [10]. The *Wx1* locus of *Zea mays* encodes a granule-bound starch synthase (GBSSI, waxy protein) involved in the biosynthesis of amylose [10], and the biosynthesis of amylopectin requires a well-coordinated enzyme complex including SSs, BE, and DBE [11]. The absence of GBSSI in *wx* genotypes leads to the elimination of amylose and the accumulation of amylopectin. On the other hand, the absence of the BE enzyme in *ae1* leads to the accumulation of up to 50% amylose due to lower amylopectin production [11]. A reduction in amylopectin has also been reported in *su* mutants [12]. The starch of the wild-type endosperm is 15–30% amylose and 70–85% amylopectin, whereas the starch of the endosperm of most waxy mutants is almost 100% amylopectin. The *Wx1* locus was mapped on chromosome 9, and this gene is expressed in the endosperm, leaf, and pollen grain [13–15].

Waxy maize is grown as a common vegetable and is a staple food crop in many Asian countries. There are several limiting factors in the production and consumption of waxy maize, such as poor eating quality, poor agronomic performance and yield, and susceptibility to environmental stress [14]. The improvement of waxy maize for quality or nutritional traits and yields is key to increasing the production and consumption of waxy maize. Because waxy maize is controlled by a recessive gene [16], molecular markers can facilitate the selection of the desired genotypes in marker-assisted (MAS) breeding schemes. However, functional markers for the waxy trait are not available. Genome-wide association studies (GWAS) are an effective method for identifying genes or loci underlying traits of interest. GWAS analyses have been conducted to identify loci associated with a variety of traits in many crops [17]. In maize, GWAS has been used to identify QTLs and genes associated with starch granule size [18], kernel starch content [19], kernel composition and flour pasting behavior [20], and amylose content [11]. Molecular markers are being used for the acceleration of plant selection through MAS breeding schemes. Single-nucleotide polymorphism (SNP) markers have risen to the forefront of molecular genetics due to their

prevalence in genomes and appropriateness for high-throughput detection [21]. Significant SNPs identified from GWAS can be converted into readily usable SNP markers [22]. The development of molecular markers to be used in MAS schemes based on the results of GWAS has been demonstrated for panicle architecture and grain traits in rice [23] and inflorescence type and remontancy in bigleaf hydrangea (*Hydrangea macrophylla* L.) [22].

In this study, we performed a GWAS to identify a QTL associated with the waxy/normal kernel trait in a collection of 213 maize inbred lines and recombinant inbred lines (RILs) containing waxy maize and field maize. The high-quality SNP set obtained from the Axiom® Maize 600 K genotyping array (Thermo Fisher Scientific, Waltham, MA, USA) was used for the population study and GWAS analysis. An SNP marker for the waxy maize was developed based on the most strongly associated SNP identified by GWAS. This marker has proven useful for application in breeding programs for waxy maize.

2. Materials and Methods

2.1. Plant Materials and Phenotyping

A total of 213 maize accessions consisting of 42 inbred lines of field maize and 171 inbred lines and recombinant inbred lines (RILs) of waxy maize were used for GWAS analysis (Table S1). Based on the structure of the dried kernels, the kernel trait of these maize accessions was defined as a simple qualitative trait (Figure S1). The coding numbers 0 for normal maize with a flint-like kernel and 1 for waxy maize with an opaque kernel were used for phenotyping. A total of 139 maize accessions, including sweet maize and waxy maize, were used for marker validation (Table S2). The amylose content of these maize accessions was analyzed using immature ears aged 24 days after pollination with two replications using the method previously described [24].

2.2. SNP Array Genotyping and Data Filtering

DNA from the 213 maize lines was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen, CA, USA)) and subsequently genotyped using the Axiom® Maize 600 K Genotyping Array, which contained 616,201 variants (http://www.affymetrix.com/, accessed on 27 August 2022). SNPs with more than 20% missing data and a minor allele frequency (MAF) of less than 0.05 were removed, resulting in 410,575 high-quality SNPs. The resulting SNPs were then further pruned to obtain SNPs with a distance of at least 1 kb between each pair of adjacent SNPs, yielding 155,768 SNPs (Figure S2). These SNPs were used to perform GWAS analysis for the kernel trait (waxy/normal kernels). Another set of 17,467 SNPs was acquired based on linkage disequilibrium (LD) using a variant pruning tool (-indep-pairwise 50 10 0.1) in PLINK [25]. These LD-pruned SNPs were used for population structure analysis using principal component analysis (PCA), phylogenetic tree, and STRUCTURE [26]. The genome coordinates of SNP loci in the study were based on B73_RefGen_V5 (http://maizegdb.org/; accessed on 27 August 2022).

2.3. Analysis of Population Structure and Linkage Disequilibrium (LD) Decay

Based on the 17,467 LD-pruned SNP data, the genetic population structure of 213 maize lines was determined using three different approaches: the phylogenetic tree; principal component analysis (PCA); STRUCTURE. Genetic distance was calculated using Nei's standard distance [27], and the phylogenetic tree was constructed using the CLC Genomics Workbench (Qiagen, Hilden, Germany) based on the unweighted pair group with arithmetic mean (UPGMA) with 1000 bootstrap repeats. PCA was performed using the R package Genome Association and Prediction Integrated Tool (GAPIT) version 3 [28]. STRUCTURE analysis was performed using the Admixture model with correlated allele frequencies and a Bayesian model-based clustering algorithm implemented in STRUCTURE version 2.3.4 [29]. A total of 10 independent replicates were performed for each genetic cluster (K) value (K = 1–10), with a burn-in period of 10,000 and a run length of 10,000 iterations. LnP(D) values were derived for each K and plotted to find the plateau of the ΔK. The final population structure was calculated using Structure Harvester [29]. PopLDdecay was used

to estimate genome-wide LD decay using the pairwise analysis of neighboring SNPs within a chromosome [30].

2.4. Genome-Wide Association Study (GWAS) and Candidate Gene Analysis

GWAS analysis was performed for a simple qualitative trait of kernel structure (waxy (opaque) or normal (flint) phenotypes) in a panel of 213 inbred lines and recombinant inbred lines (RILs), based on 155,768 SNPs with a mixed linear model (MLM) that incorporated three PCs and a kinship matrix using GAPIT version 3 [28]. The significance threshold for SNP–trait associations was the Bonferroni correction of $P \leq 0.05/n$, where n represents the number of SNP markers in the whole genome. For candidate gene analysis, genomic regions within the LD block of significant SNPs (peak SNPs) were selected to identify candidate genes. Gene annotation was based on the B73 reference genome (B73_RefGen_v5). SNP probe sequences of ~150 bp on the Axiom® Maize 600 K genotyping array were used as queries in a BLAST algorithm-based search against the reference genome sequence in MaizeGDB (http://www.maizegdb.org/gbrowse; accessed on 27 August 2022) to identify the positions in the maize genome.

2.5. Development and Validation of SNP Marker

The genomic sequences for the primer design were obtained from the B73 sequence version 5 at Gramene (https://ensembl.gramene.org/; accessed on 27 August 2022). The primer set for the MassArray high-throughput procedure was designed and analyzed according to the MassArray® iPLEX system manufacturer's protocol (https://www.agenabio.com/products/massarray-system; accessed on 27 August 2022). Primers for SNP sites were designed to have a mismatch of 1 bp at the second base closest to the 3′ end of the forward primer. The marker was validated in the 139 maize accessions (Table S2). Linear regression was conducted to determine the association of marker genotypes and amylose content using Jamovi (open-access software, https://www.jamovi.org; accessed on 27 August 2022).

3. Results

3.1. Phenotyping, Genotyping, and Population Study in the Panel of 213 Maize Lines

The phenotypic evaluation of 213 maize lines based on the structure and appearance of dried kernels classified 42 lines as normal maize and the other 171 lines as waxy maize (Figures 1A and S1; Table S1). Based on the 155,768 SNPs, the heterozygosity of maize lines in this panel was found to be in the range of 0.02 to 0.20, with an average of 0.09 (Table S1). The results of the three approaches for population structure determination based on 17,467 LD-pruned SNP data showed two subpopulations in this maize panel (Figure 1B–D). The PCA result showed that the large subpopulation contained all the non-waxy maize lines (field maize) and most of the waxy maize lines, while the small subpopulation contained the remaining 17 waxy maize lines (Figure 1B). We also analyzed the genome-wide LD decays among the 213 maize lines based on the 155,768 SNPs. The results showed that the average LD decay across the entire genome of the 213 maize lines was 60 kb, with a cut-off of $r^2 = 0.2$ (Figure 2).

3.2. Genome-Wide Association Study (GWAS) for Waxy Trait

The SNP-based heritability of the kernel trait in the 213 lines was 90%. The GWAS result identified a significant SNP (AX-90613979) on chromosome 9 that exceeded the Bonferroni threshold ($-\log10P = 6.49$) and was associated with the kernel trait (Figure 3). According to the LD decay ($R^2 > 0.2$), the 120 kb region (25.06–25.18 Mb) flanking the significant SNPs was defined as a QTL, *qWx9* (Table 1). Seven genes were identified within this QTL region (Table S3). Among these, four genes were annotated with a function, including Zm00001eb378140 (*GBSSI* or *Wx1*) (Figure 4). Because the significant SNP (AX-90613979) was located on exon 8 of GBSSI and this gene is known to play a key role in

amylose biosynthesis in the crop endosperm, we considered GBSSI to be the best candidate gene associated with the kernel trait in this study.

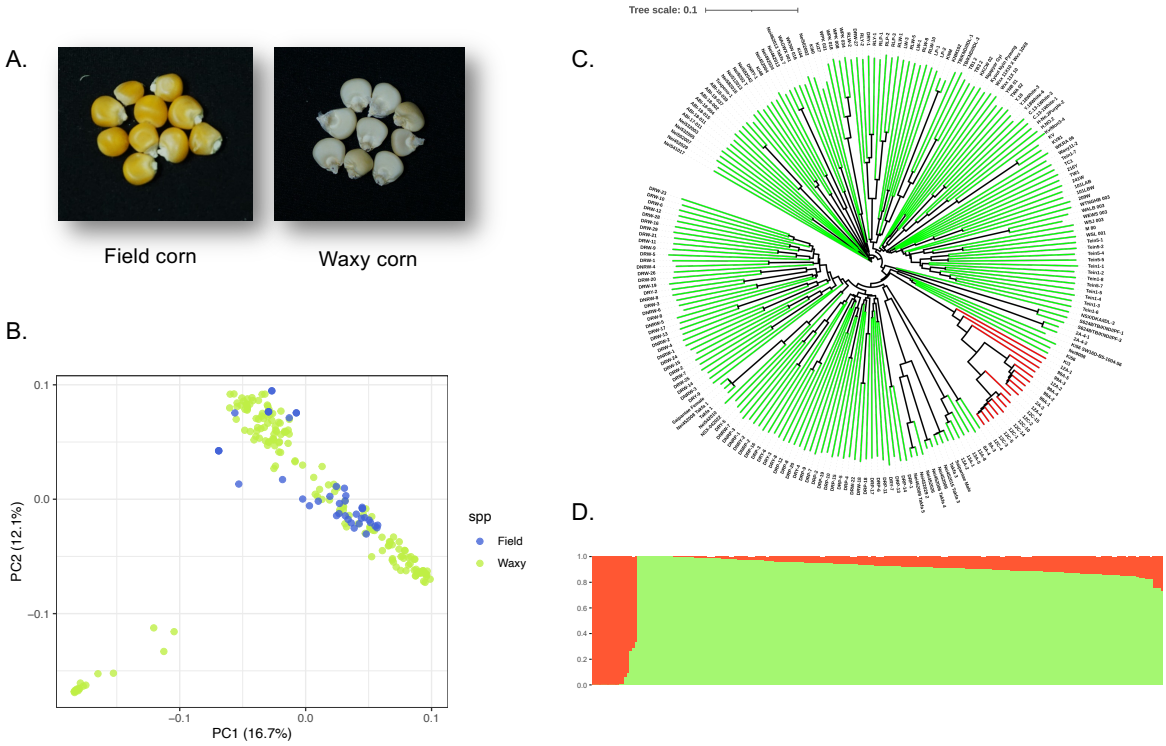

Figure 1. Genetic structure of 213 lines of field maize and waxy maize. (**A**) Appearance of kernel structures that was used as a criterion to assign maize lines as field maize (non-waxy) and waxy maize. (**B**) Two-dimensional plot of principal components (PC1 vs. PC2), (**C**) phylogenetic tree, and (**D**) STRUCTURE based on 17,467 SNP markers. The field maize and sweet maize accessions in (**B**) are shown in blue and green, respectively. The two different subpopulations in (**C**,**D**) are shown in green and red.

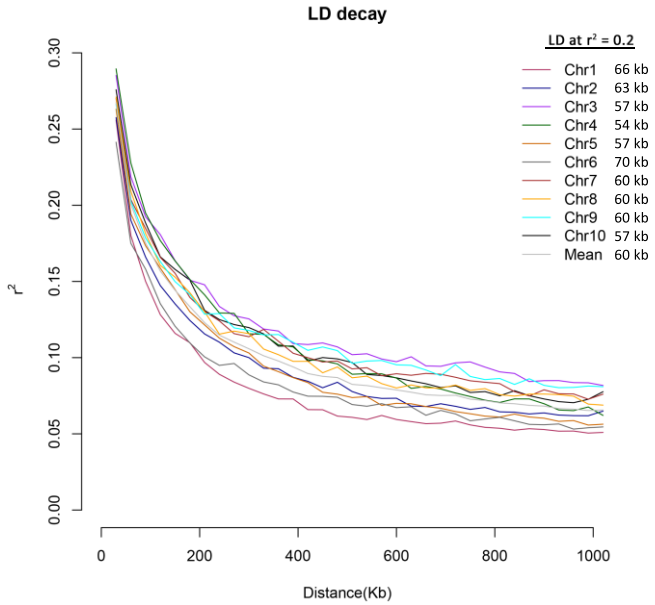

Figure 2. Overall chromosome-wide linkage disequilibrium (LD) decay estimated from 155,768 SNP genotypes of 213 maize lines. Each line plot represents a smoothed r^2 for all marker pairs on each chromosome depending on the distance between marker pairs.

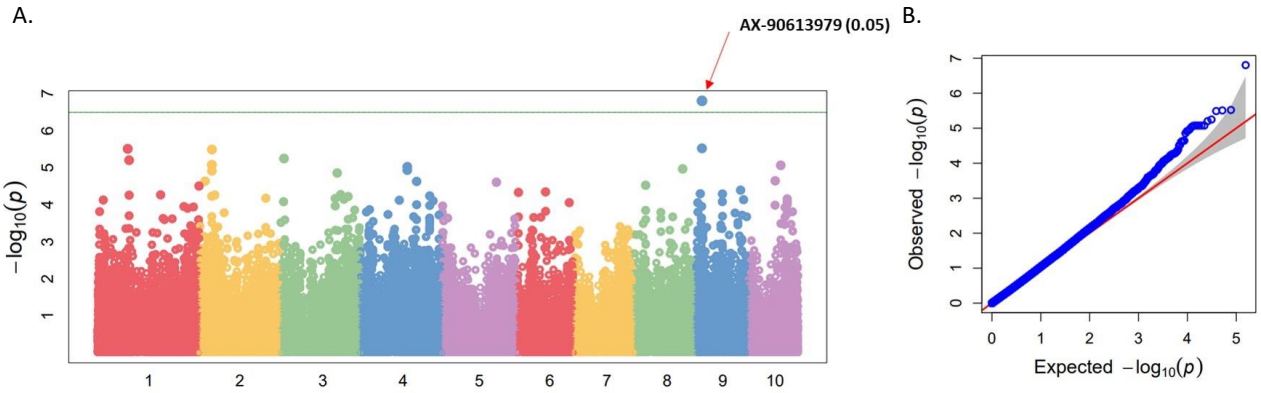

Figure 3. GWAS results for the trait waxy/normal kernel in 213 maize inbred and recombinant inbred lines genotyped with 155,768 SNPs using an MLM model implemented in GAPIT. (**A**) Manhattan plots. Each dot represents one SNP. The numbers on the horizontal axis represent maize chromosome number. The Bonferroni threshold of −log10(P) = 6.49 is represented by a green line on the Manhattan plots. The most associated SNP (AX-90613979) is indicated by a red arrow. (**B**) Quantile–quantile (Q–Q) plots. The plot shows the expected versus observed −log10(P) of each marker (blue dots). The red line is a benchmark for perfect fit to the expected −log10(P). The gray shaded area shows the 95% confidence interval for the Q–Q plot under the null hypothesis of no association between the SNP and the trait.

Table 1. QTL and significant SNPs identified by GWAS. SNP ID, chromosome, positions, −log10(P) values, minor allele frequency (MAF) values, and candidate gene are provided.

QTL	Chr.	Flanking Region (Mb)	Significant SNP	Position (v.5)	−log10(P)	MAF	Candidate Gene
qWx9	9	25.06–25.18	AX-90613979	25,128,753	6.80	0.333	Zm00001eb378140 (*Wx1* or *GBSSI*)

3.3. Development of an SNP Marker Associated with Waxy Trait and Validation among Different Maize Types

Based on the genotypes of SNP AX-90613979 (C/G), the 213 maize lines could be divided into three groups with genotypes CC (n = 134), CG (n = 17), and GG (n = 62). The majority of the waxy maize lines contained the homozygous genotype CC (132 of 171 waxy maize lines), and the majority of the non-waxy maize (field maize) contained the homozygous genotype GG (37 of 42 of the non-waxy maize lines) (Table S1 and Figure S3). There were also a number of waxy maize lines that contained the genotype GG (n = 25) and heterozygous genotype CG (n = 12). We developed a MassArray marker based on SNP AX-90613979 and validated it in a panel of 139 maize lines containing waxy and sweet maize with different levels of amylose in the endosperm (Tables 2 and S2). According to the results of genotyping with the MassArray marker, the three genotypes (CC, CG, and GG) could be clearly distinguished (Figure 5A). The amylose content in these 139 maize lines differed significantly among the different groups of genotypes (Figure 5B). In the maize lines with the CC genotype, the amylose content ranged from 2.34% to 21.64%, while in the lines with the GG genotype, the amylose content ranged from 26.42% to 70.25%. In the maize lines with the heterozygous genotype (CG), the amylose content ranged from 8.20% to 53.96% (Figure 5B). Among the 139 maize lines, the CC genotype was exclusively present in the waxy maize group. In contrast, the GG genotype was found exclusively in the sweet maize group. The heterozygous genotype CG was found in both the waxy maize group (n = 6) and the sweet maize group (n = 3) (Table S2). We also performed marker–trait association analysis in this maize panel to check the efficacy of the marker in predicting maize types according to the variation in amylose content. The result of regression analysis

showed that the MassArray marker had a significant association with amylose content (R^2 value of 0.81, $p < 0.001$) (Table 3).

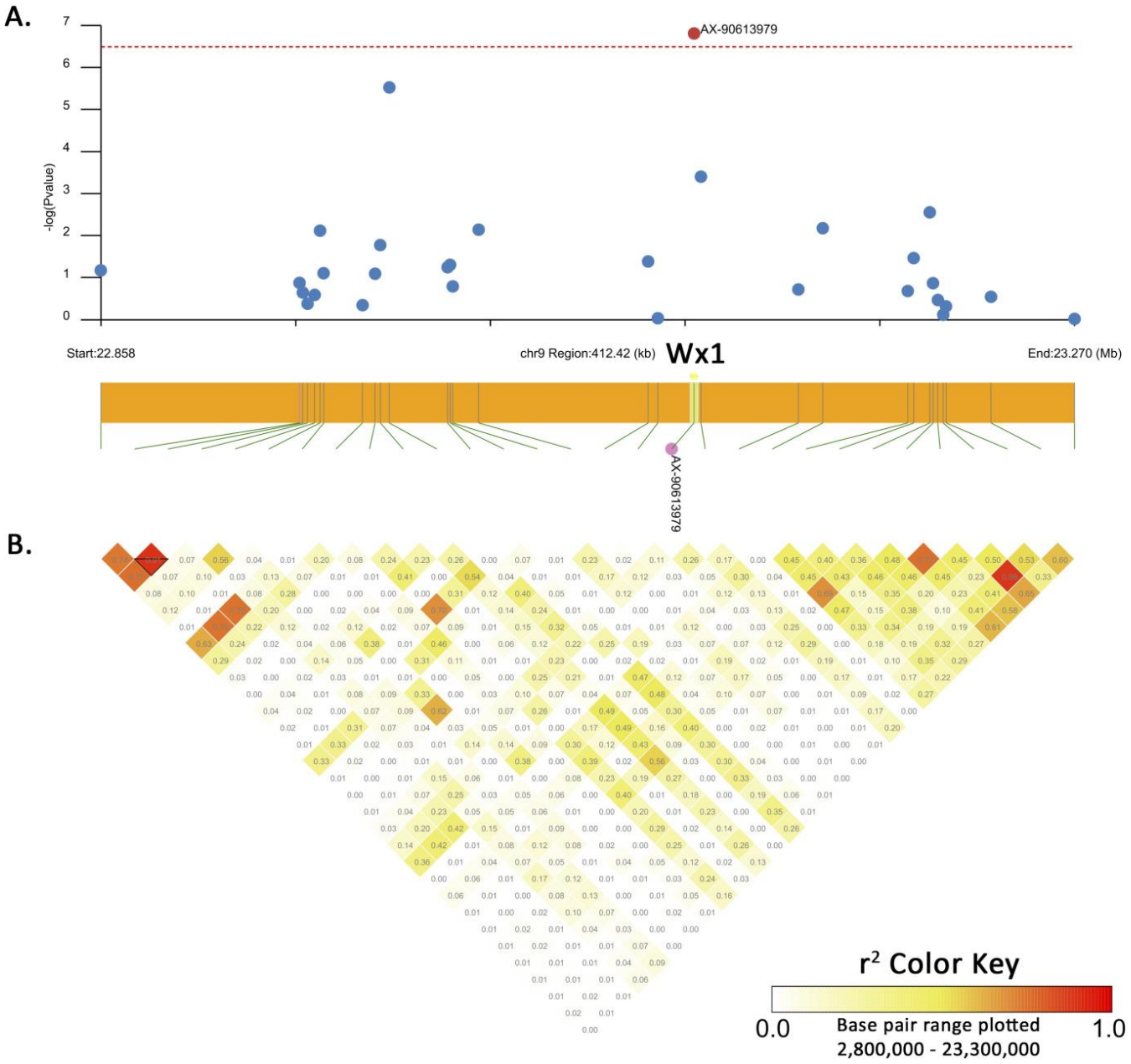

Figure 4. Locus-specific Manhattan plot and LD block analysis for SNPs within the *qWx9* region on maize chromosome 9. (**A**) Manhattan plot with significance line (red). (**B**) Heat map with R^2 estimates.

Table 2. Details of SNP marker for *Wx1* gene used for genotyping by MassArray® platform.

Marker	Allele	Unextended Primer (UEP) Sequence (5′-3′) [a]	UEP Mass (Da)	Call 1	Mass 1 (Da)	Call 2	Mass 2 (Da)
Wx-MassArray (Wx_AX-90613979)	C/G	atctAAGGACGACTTGAATCTCTC	7311.8	C	7559	G	7599

[a] The lowercase letters are the bases added to the 5′ to adjust the mass of UEPs.

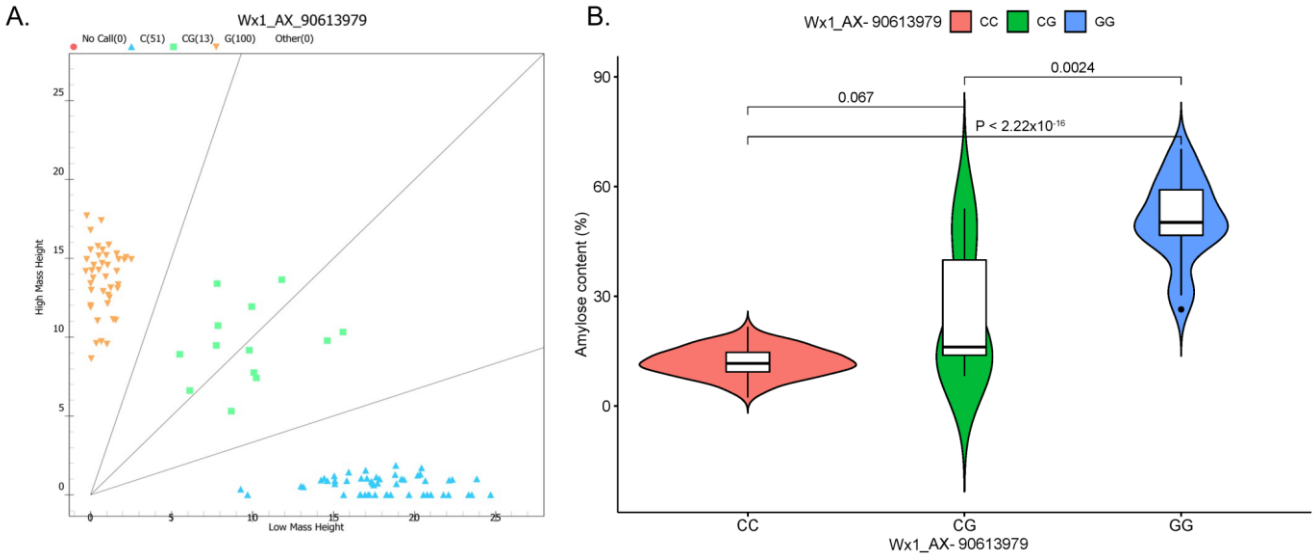

Figure 5. MassArray marker associated with amylose trait developed based on the SNP in *Wx1*. (**A**) Allelic discrimination plot of the marker Wx-MassArray validated in panel of 139 maize lines with different amylose content. Scatter dots with different colors show clustering of homozygous genotype GG (brown), heterozygous genotype CG (green), and homozygous genotype CC (blue). (**B**) Violin plots with box plots showing the average % amylose content of maize lines correlated with the results of allelic discrimination of the MassArray marker. Paired *t*-tests, violin plots, and box plots were obtained using SRplot (http://www.bioinformatics.com.cn; accessed on 27 August 2022).

Table 3. Test of marker–trait association between the Wx-MassArray marker and amylose content in 139 maize lines.

Marker	R	R^2	Adjusted R^2	Overall Model Test			
				F	df1	df2	*p*
Wx-MassArray (*Wx1*_AX-90613979)	0.904	0.817	0.814	304	2	136	<0.001

4. Discussion

In this study, we performed GWAS analysis for the waxy/normal kernel trait in a panel of 213 maize inbred and recombinant inbred lines. The appearance of the kernels in the association panel is clearly distinguishable and can be easily classified as normal (non-waxy) with a flint kernel and waxy with an opaque kernel. Therefore, we simply determined the trait as a qualitative trait for the normal and waxy phenotypes and used it for GWAS analysis. Although GWAS analysis usually targets quantitative traits, several qualitative traits have also been used to perform GWAS and successfully identified genes/QTLs [31]. Recently, we successfully identified a gene associated with sweetness in super sweet maize by also performing GWAS based on a qualitative phenotype of dried kernels [32]. Because of the strong population structure and some degree of individual relatedness in our association panel, we conducted association tests based on a mixed linear model (MLM) that incorporated the PCA and kinship matrices. Accordingly, the GWAS results showed that the SNP AX-90613979 (*qWx9* locus) was significantly associated with the waxy/normal kernel phenotype. This SNP was located on exon 8 of the maize *Wx*, which encodes a granule-bound starch synthase (GBSSI) involved in the biosynthesis of amylose in maize endosperm. Besides the *qWx9* locus, no other loci significantly associated with the trait were identified by GWAS in this maize panel. Because *Wx1* or *GBSSI* is known to be a key gene controlling amylose content in the maize endosperm and no other candidate genes with a relevant function were identified within the *qWx9* region, we speculate that this

gene is likely a potential candidate gene associated with the waxy/normal kernel trait in this panel. The *Wx* locus, along with the other 26 loci, was previously associated with amylose content in maize using GWAS [11]. Since phenotype was determined as a simple qualitative trait in this study, it is reasonable that the *qWx9* was simply identified as a large effect locus.

Mutations at the *Wx* locus eliminate amylose synthesis because granule-bound starch synthase (GBSS) activity is reduced by 5% to 95% [16]. Therefore, the mutations usually result in low-amylose or waxy (amylose-free) mutants. Starch in the normal maize endosperm is approximately 25% amylose and 75% amylopectin [33]. Varieties with the *wxwx* genotype produce kernels whose starch is almost entirely amylopectin and are referred to as waxy (sticky) maize [34]. More than 50 different insertion mutations have been found in the coding sequence of the GBSSI gene. The insertion of *Ac-Ds*, *Spm*, and some other transposons at different positions of the exons of *Wx* altered the protein structure of GBSS and inhibited the synthesis of amylose [35]. Although the SNP on exon 8 identified in this study may not be a functional SNP because it is a synonymous SNP that does not cause an amino acid change, it is possible that this SNP is associated with other functional variants in the gene that have not been genotyped. Therefore, it is likely that this SNP can be used as a functional marker for predicting the waxy trait. As noted in the entire panel of 213 maize lines, most of the maize lines containing the C allele of this SNP are waxy maize, whereas lines containing the G allele could be both normal maize and waxy maize, although the proportion of normal maize with the G allele is greater than that of waxy maize with the same allele. This result suggests that most waxy maize lines contain the C allele, with the exception of those containing the G allele. It is possible that the latter group of waxy maize lines contains a different allelic variation in the *Wx* gene.

Since waxy maize is also consumed directly from the ear, the characteristics of the appearance and eating quality of the ear are of great importance [36]. Strategies to breed waxy maize for improved eating quality have been used, including hybrid maize breeding by crossing super sweet maize and waxy maize [36]. Since both sweet and waxy traits are controlled by recessive genes, i.e., *sh2* and *wx1*, the molecular markers specific to the recessive alleles are important. Recently, we successfully developed an SNP marker for sweetness caused by *sh2* [32]. In this study, we developed a MassArray marker based on the SNP AX-90613979 and validated it in a panel of 139 maize lines containing waxy maize and sweet maize with different amylose content. The newly developed marker clearly distinguished between waxy maize and sweet maize lines that had different ranges of amylose content. The maize lines with genotype CC in this panel are all waxy maize, and those with genotype GG are all sweet maize. However, the maize lines with the heterozygous genotype CG could have different amylose content and were found in both the waxy maize and normal maize groups. Since the phenotypic variance explained (PVE) or R^2 of this marker was found to be as high as 0.81, this marker is highly useful for maize breeding programs for the waxy kernel trait by marker-assisted selection.

5. Conclusions

In this study, we performed GWAS analysis using an MLM model in a panel of 213 maize inbred and near-isogenic lines. We identified an SNP in the *Wx* gene that was most strongly associated with the waxy trait in this maize panel. This SNP was validated to distinguish waxy maize from other maize types, i.e., sweet maize and field maize. An SNP marker developed based on this SNP has been shown to be useful for maize breeding programs for the waxy trait or for breeding programs for hybrid maize combining the sweetness and waxy traits. The gene-based SNP markers could aid breeders by eliminating the costs and time required to perform lengthy field trials and help to accelerate sweet maize and waxy maize breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102289/s1, Figure S1. The physical appearance

of dried kernels of sweet, waxy, and field maizes. (A–D) represent sweet maize with the *sh2*, *bt*, *su*, and *se* backgrounds, respectively. (E) and (F) represent waxy maize and field maize (common maize), respectively. Figure S2. Density and distribution of SNPs throughout ten maize chromosomes. Figure S3. Frequency of the three genotypes of the SNP AX-90613979 in 213 waxy and non-waxy (field) maize lines. Table S1. List of 213 inbred and recombinant inbred lines used for GWAS analysis in this study. Table S2. List of 139 inbred and recombinant inbred lines of waxy maize and sweet maize used for marker validation. Table S3. Annotated genes within the identified QTL on chromosome 9.

Author Contributions: T.T., S.W., S.A., K.S., J.U. and V.R. designed and supervised the research. K.K.Y., K.K., B.T., W.A., S.S., C.K., A.Y., N.C. and V.R. performed the experiments. K.K., B.T., N.N.O. and V.R. analyzed the data. K.K.Y., S.W. and V.R. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article and the Supplementary Materials.

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