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A metabolic roadmap of waxy corn flavor

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ABSTRACT

As well as being a popular vegetable crop worldwide, waxy corn represents an important amylopectin source, but little is known about its breeding history and flavor characteristics. In this study, through comparative-omic analyses between 318 diverse waxy corn and 507 representative field corn inbred lines we revealed that many metabolic pathways and genes exhibited selection characteristics during the breeding history of waxy corn, contributing to the divergence between waxy and field corn. We showed that waxy corn is not only altered in its glutinous property but also its sweetness, aroma, and palatability are all significantly affected. A substantial proportion (43%) of flavor-related metabolites have pleiotropic effects, affecting both flavor and yield characteristics, and 27% of these metabolites are related to antagonistic outcomes on yield and flavor. Furthermore, through multiple concrete examples, we demonstrated how yield and quality are coordinately or antagonistically regulated at the genetic level. In particular, some sweet molecules, such as DIMBOA and raffinose, which do not participate in the starch biosynthesis pathway, were identified as potential targets for breeding a new type of "sweet-waxy" corn. Taken together, our findings shed light on the historical selection of waxy corn and demonstrate the genetic and metabolic basis of waxy corn flavor, collectively providing valuable resources and knowledge for future crop breeding for improved nutritional quality.

Keywords: waxy corn, breeding, multi-omics, flavor, mGWAS, sweet-waxy corn

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INTRODUCTION

Waxy corn (*Zea mays* L. *sinensis Kulesh*), also known as sticky corn or glutinous corn, is a specialized cultivated type of maize highly valued for its unique characteristics and culinary uses. It is an important crop globally, serving as a significant source of amylopectin, a highly branched type of starch with distinct properties. Waxy corn differs from field corn in terms of

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its genetics, appearance, and most notably its texture and flavor. It is generally believed that waxy corn is derived from field corn caused by the mutation of the *Waxy* gene. Compared to conventional corn varieties, waxy corn contains a higher proportion of amylopectin, resulting in a sticky gel-like consistency when cooked (Nelson and Rines, 1962; Yang, 2008). This characteristic makes it particularly suitable for culinary applications, such as making traditional Asian dishes like mochi, sticky rice, and glutinous desserts (Yang et al., 2008; Sa et al., 2015). The content of vitamins, proteins, lysine, sugar, and fat in waxy corn is much higher than in field corn (Li et al., 2022; Dang et al., 2023). Additionally, waxy corn is used to produce food additives thickeners and as a raw material for the starch industry. The earliest record of waxy corn was found in an ancient Chinese book, ''Three Rural Issues,'' written during the Qing dynasty sometime before 1760. Southwest China is a recognized center of origin for waxy corn (Zeng, 1987), with abundant waxy corn germplasm still available in this region. Since the 20th century, breeding and industrial production of waxy corn have developed rapidly, and it is now widely planted in Southeast Asia, North America, and Europe. China has now become the largest producer and consumer of waxy corn in the world, with a current planting area of about 800,000 hm^2 (Shi et al., 2019).

Despite its widespread popularity and economic significance, the breeding history and flavor characteristics of waxy corn remain unclear. Moreover, the flavor quality of modern commercial waxy corn is relatively uneven. Understanding the genetic and metabolic basis of its flavor, as well as the historical selection processes involved in its cultivation, could therefore, provide valuable insights for breeders and researchers aiming to improve its quality and adaptability. In the past few decades, population multi-omics analysis has been used to reveal the genetic architecture of complex quantitative traits such as crop growth and development, nutritional quality formation, and stress physiology response in field corn, which provide an important reference for the dissection of waxy corn quality improvement. At the molecular level, some metabolic compounds in waxy corn kernels may be key factors that directly interact with human taste and smell receptors, affecting flavor. Metabolomics combined with genomic and transcriptomic data may help to elucidate the genetic basis of waxy corn flavor. For this purpose, whole-genome resequencing was performed on 318 waxy elite inbred lines from a global collection, and a high-density genomic variation map of the waxy population was constructed. Integrative omics analyses, comparative genetic analysis with 507 field corn lines (Chen et al., 2022), and consumer tasting experiments were used to deeply explore the genetic basis of waxy corn flavor-related traits.

RESULTS

A high-density genetic variation map of waxy corn

The waxy population employed for the present study consists of 318 waxy corn inbred lines from a global collection (Supplemental Table 1). The population was resequenced with an average depth of 10× coverage per line. A total 5.7 Tb of data was obtained (Supplemental Table 2). Following a standard data pipeline (Supplemental Figure 1), 21,744,459 high-quality single-nucleotide polymorphisms (SNPs) were identified. To better understand the evolutionary basis of waxy corn and its genetic difference to field corn, the waxy corn population SNPs were integrated with datasets from 507 inbred lines from a variety of temperate, subtropical, and tropical field corn germplasm (detailed in methods). SNPs from both populations were combined and re-called, and all sites with low quality and high deletion rates were filtered out through a rigorous pipeline (detailed in methods). Finally, a high-density variation map of the waxy and field corn populations with 31,175,750 SNPs was obtained for comparative analysis.

Population genetics with a focus on the *Waxy* gene reveals the origin of waxy corn

The mixed-model population structure analysis of the integrated panel indicated that waxy corn and field corn populations exhibit relatively independent genetic characteristics. When subdividing the temperate and tropical subgroups within field corn, it was observed that the genetic distance between waxy corn and temperate lines is less than between waxy corn and tropical lines (Figure 1A). This suggests that waxy corn has a distinct genetic composition and may have undergone specific breeding processes separate from field corn. These findings were further supported by principal-component analysis (PCA) and phylogenetic tree analysis (Figure 1B and Supplemental Figure 2). Additionally, a few ''mixed'' waxy corn lines are likely attributed to recent genetic interactions with field corn lines, as indicated by their pedigree information (Supplemental Table 1). The average linkage disequilibrium (LD) decay distance for the waxy corn panel was approximately 600 kb (for $r^2 = 0.1$), which is considerably longer than the LD decay distance of approximately 180 kb observed in the field corn panel (Figure 1C). Moreover, the waxy corn panel exhibited lower nucleotide diversity (π = 9.80E−04) compared to the field corn panel (π = 1.3E−03) (Figure 1D). These results highlight the distinct genetic structure and reduced genetic diversity within waxy corn, indicating its unique breeding history compared to field corn.

The *Waxy* gene is one of the important genes studied in the evolution and improvement of waxy corn. The recessive mutant of the *Waxy* gene suppresses the action of GBSS-I (granule-bound starch synthase I) that utilizes ADP-glucose in the synthesis of amylopectin (Bao et al., 2012; Zhang et al., 2013), causing the main difference between waxy and field corn. In other words, the mutation type of the *Waxy* gene in waxy corn is very complex, including insertions, deletions, and repetitive sequences, which are difficult to accurately capture through genome-wide resequencing data. To better understand this most differentiated gene, full-length genomic sequences of 165 randomly selected materials were sequenced to identify potential mutations of the *Waxy* gene in our waxy corn population (Supplemental Table 3). Results indicated that nucleotide variation of *Waxy* loci in waxy corn lines was diverse but conserved in two field corn inbred lines (Supplemental Figure 3A). A total of 70 different variations were detected, including 39 SNPs and 31 insertion–deletion (InDel) structural variants (Supplemental Table 4 and Supplemental Figure 3B), which covered almost all previously reported mutants (Hossain et al.,

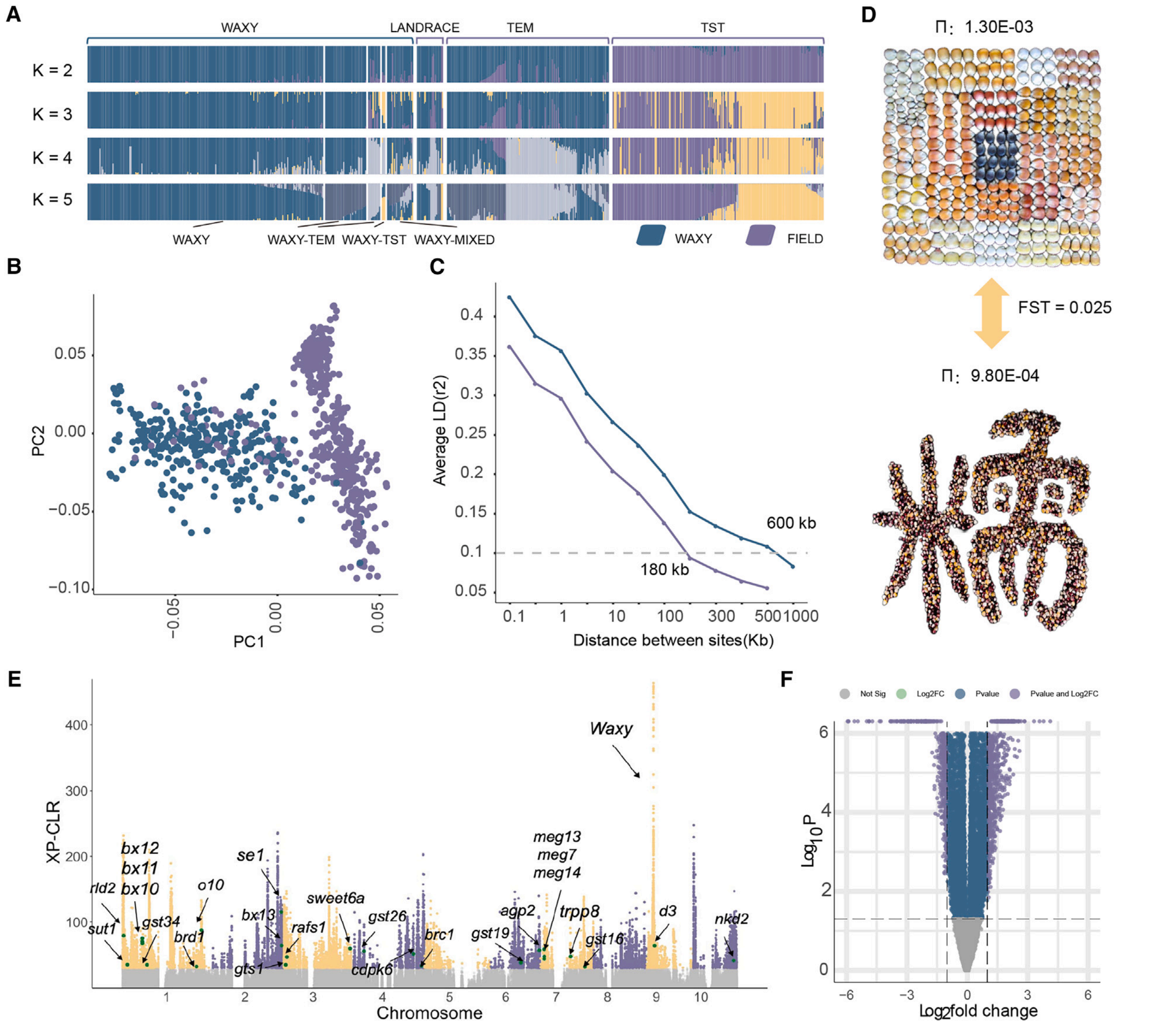

Figure 1. Population genetics and divergence of waxy and field corn populations.

(A) Population structure and inferred group differentiation process of waxy and field corn by Admixture. WAXY, waxy corn; LANDRACE, landraces; TEM, temperate; TST, tropical and subtropical.

(B) Principal-component (PC) analysis of waxy and field corn, the first two components showing that two populations were identifiable, namely waxy corn (dark-blue dots) and field corn (light-blue dots).

(C) LD decay–distance analysis. Genome-wide averaged distance of LD decayed to r^2 = 0.1 for waxy and field corn populations.

(D) Kernels (dry) with a rich diversity of a subset of individuals from the waxy and field corn population, the waxy kernels form the Chinese word "waxy" (糯). Nucleotide diversity (π) and population divergence (FST) across the two panels are indicated.

(E) Results of XP-CLR score between waxy corn (318) and field corn (507) population for detection of selection signatures. Each dot represents cross-population composite likelihood ratio values in the non-overlapping 2-kb genomic region. The purple and yellow dots represent the top 5% of selection candidates. Known genes are marked.

(F) Differentially expressed genes were detected between waxy and field corn populations. Blue dots: $p < 0.01$; purple dots: log fold change(MLE) >2, odds ratio < -2, and $p < 0.01$.

2019). Based on these sequences, a phylogenetic tree was constructed by the neighbor-joining method. These accessions can be roughly divided into two haplotypes through multiple sequence alignment analysis of *Waxy* sequences (Supplemental Figure 3C). According to the tree, all inbred lines formed two branches. Most waxy corn inbred lines are closely clustered in one branch. Another branch contained only 11 waxy corn lines and the field corn B73, indicating that several glutinous varieties may have been developed from non-glutinous domesticated corn. Further combined with the population structure analysis (Supplemental Figure 3D), the waxy corn inbred lines in our population may have various origins or genetic improvement events in the process of breeding: (1) most waxy corn displays the unique germplasm of this population during the process of genetic improvement, clearly distinct from field corn, and genomic-level composition analysis also supports this hypothesis (n = 109; Supplemental Figure 3E1); (2) a few waxy corns have *Waxy* gene sequences similar to those of field corn, possibly due to the recent mutation of the *Waxy* gene that made field corn waxy (n = 10; Supplemental Figure 3E2); and (3) another group of waxy corn has a genomic background similar to that of field corn, indicating that breeders utilized waxy materials as donors for selection and hybridization with field corn in recent breeding programs (n = 46; Supplemental Figure 3E3).

Genomic and transcriptional divergence between waxy and field corn

To further uncover the corresponding differential genome fragments between waxy and field corn, a cross-population composite likelihood ratio method (XP-CLR) was employed to assess the divergence between waxy corn lines and the field corn reference panel. About 39M regions in the top 5% of the XP-CLR values were identified, and 4462 genes within these regions, or strongly in LD with them, were candidates of presumed selection (Supplemental Table 5 and Figure 1E). Among these candidates, the *Waxy* gene was the most significant differentiation locus, responsible for regulating the change of starch content in waxy corn and directly influencing its viscosity characteristics. Apart from the *Waxy* gene, 22 other genes in the starch biosynthesis pathway also exhibited strong signals of selection (Supplemental Figure 3F), indicating that the entire pathway has been affected during the waxy maize breeding process. Several other well-known genes also showed strong signals of selection, some of which are involved in sugar metabolism, such as *raf1* in the raffinose biosynthesis pathway and *trpp8* in the sucrose biosynthesis pathway. Additionally, numerous genes related to sugar or amino acid transport were identified, including *sut1*, *sweet6a*, *gst16*, *gst19*, and *gst34*. Moreover, many genes known to be involved in kernel development, such as *meg13*, *meg7*, *meg14*, *o10*, *nkd2*, and *d3*, were also identified (Supplemental Table 6).

The differences between waxy and field corn are present not only at the genomic level but also at the transcriptional level. To elucidate these differences, RNA sequencing (RNA-seq) analysis was performed on kernels of 230 waxy corn lines after 17 days of pollination. RNA-seq data from 368 inbred lines of the field corn population were obtained through a literature survey (Fu et al., 2013). By comparing the gene-expression datasets, a total of 3365 genes were found to have different expression levels between the two panels (Supplemental Table 7 and Figure 1F). Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology enrichment analysis of these differentially expressed genes (DEGs) (Supplemental Figure 4A) revealed enrichment in terpenoid and polyketide metabolism (sesquiterpenoid and triterpenoid biosynthesis, as well as carotenoid biosynthesis), amino acid metabolism (arginine biosynthesis, seleno-amino acid metabolism), lipid metabolism (biosynthesis of unsaturated fatty acids), carbohydrate metabolism (fructose and mannose metabolism and the pentose phosphate pathway), as well as biosynthesis of other secondary metabolites (biosynthesis of flavonoids, stilbenoids, diarylheptanoids, and gingerol). Comparative analysis of expression quantitative trait loci (eQTLs) between the two populations also revealed enrichment of certain differential KEGG ontologies in carbohydrate and amino acid metabolism pathways (Supplemental Figure 4B).

These findings indicate that the discrepancy between waxy and field corn is not solely limited to the glutinous traits and that several genes involved in diverse primary and secondary metabolic pathways shape the differences between waxy and field corn.

Metabolomics helps to reveal the genetic basis of waxy corn flavor

As the primary source of vegetable corn, improving the flavor-related characteristics of waxy corn is particularly important. At the molecular level, some metabolic compounds in waxy corn kernels may be key factors that directly affect taste and smell and may be the key factors determining the flavor perception of waxy corn. We evaluated the flavor of waxy corn through two experiments. First, an expert tasting group conducted initial tasting and scoring. From this, 79 samples with evenly distributed scores were selected for a taste test involving 100 consumers to obtain taste ratings (Supplemental Figure 5 and Supplemental Table 8). Second, over 1600 metabolites from waxy corn kernels were quantitatively analyzed via gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) metabolite profiling (Supplemental Tables 9 and 10). The key starch properties of waxy corn grains were also determined, including amylopectin content, amylose content, and seven characteristic measures related to starch viscosity (Supplemental Tables 11 and 12). It was found that glutinous properties did not significantly affect the flavor of waxy corn, and that during breeding many important substances have been selected and improved, resulting in the flavor of waxy corn being considerably better than that of field corn.

Subsequently, a correlation analysis between taste ratings and these metabolites and starch-related traits was conducted. A total of 84 annotated metabolites were significantly correlated with scores for overall consumer preferences and flavor intensity (Figure 2A), including sugars and sugar derivatives, amino and organic acids, and some select secondary metabolites. Combined with the high-density genetic variation map of waxy corn, a genome-wide association study (GWAS) of the identified flavor-related metabolites was performed, whereby a large number of candidate genes was uncovered: a total of 513 candidate genes were identified for these 84 flavor-associated metabolites (Figure 2B and Supplemental Table 14). Additional correlation analysis of gene expression and flavor scores were also conducted. Furthermore, gene ontology enrichment analysis and KEGG pathway annotation were performed with the 2842 genes significantly correlated at the gene-expression level (Figure 2C and Supplemental Table S15). Significant enrichments were found in several glycosylation-related pathways as well as in the starch and sucrose metabolism and phenylpropanoid biosynthesis pathway. Some 72 annotated genes in sugar-related metabolic pathways were identified that correlated with waxy corn flavor by their expression levels. Furthermore, 21 genes in the phenylpropanoid pathway were significantly negatively correlated with flavor scores (Supplemental Figure 6). Moreover, in the phenylpropanoid biosynthetic pathway, various precursors were also significantly associated with the sensory evaluation of waxy corn in this study (Supplemental Figure 6). Overall, literature review and analysis suggest that these flavor-associated metabolites and

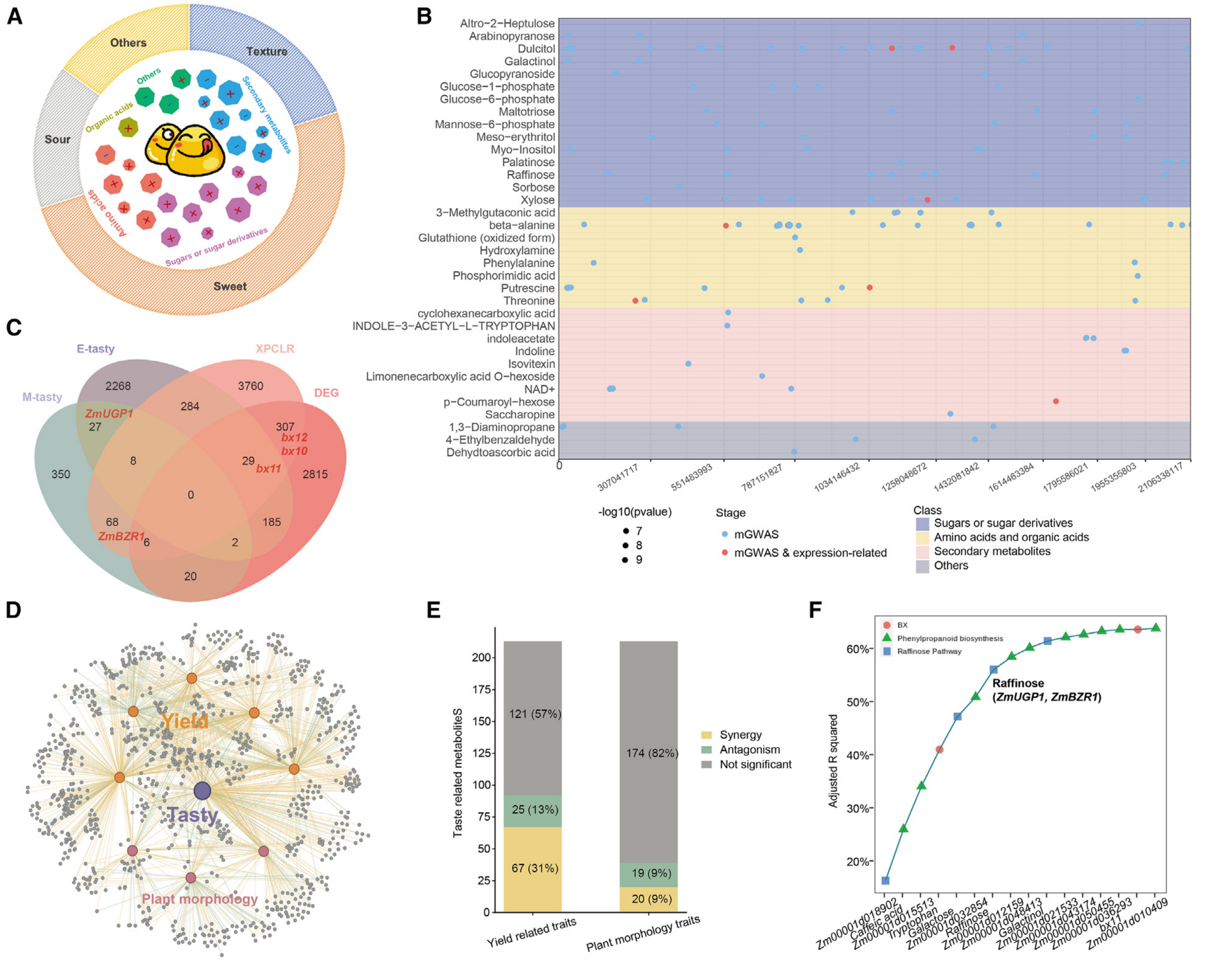

Figure 2. The genetic roadmap of waxy corn flavor.

(A) Schematic diagram of key metabolites affecting waxy corn flavor, including sugars and sugar derivatives, amino acids and organic acids, secondary metabolites, and others.

(B) Whole-genome-wide expression association map of 84 annotated flavor-related metabolites. Blue dots represent mGWAS candidate genes; red dots represent genes identified by mGWAS and expression association analyses.

(C) Venn plot of selective genes identified in the selective sweeps of the XP-CLR analysis and differentially expressed genes (DEGs) between waxy and field corn, and flavor-related genes including genes identified in flavor-related metabolite GWASs and expression-flavor-related genes.

(D) Network of significant metabolites related to yield, taste, and plant morphology traits of the waxy corn population. Orange dots represent yield-related traits (100-grain weight, ear length, ear row number, cob weight, and cob width); purple dots represent taste score; pink dots represent plant morphology traits (plant height, ear height, and number of tassel branches); gray dots represent metabolites significantly associated with these traits ($p \leq 0.05$). The yellow line represents a significant positive correlation, the green line represents a significant negative correlation, and the thickness reflects the degree of correlation.

(E) Relationship between 213 flavor-related metabolites with yield-related and plant morphology traits.

(F) Regression analyses of flavor scores with genes in raffinose, benzoxazinoid (BX), and phenylpropanoid pathways.

expression-flavor-associated genes may affect waxy corn flavor by altering sweetness, sourness, color, texture, or other aspects (Figure 2A; see details in the discussion). These metabolites and loci collectively constitute a comprehensive flavor-related metabolic genetic roadmap of waxy corn. Taking our proposed key pathways and validated gene cases as examples, modifications to these key metabolites and loci are expected to explain 64.5% of the population variation of flavor in waxy corn (Figure 2F). Combined with the selection analysis of the waxy and field corn populations, many overlaps between these waxy corn flavor-related genes and divergence genes were found (Figure 2C). Notably, 18% of the flavor-related genes located in microbiome GWASs (mGWASs) were identified as genes subject to selection in XP-CLR analysis, and their proportion was significantly enriched (p = 4.736E−5). This suggests that during the improvement process of waxy corn, the flavor quality was also subtly changed. Furthermore, the relationship between yield-related traits and flavor-associated metabolites was investigated. Our findings revealed that 92 flavor-associated metabolites (43%) influenced yield and quality. Notably, 25 (27%) of these metabolites exhibit antagonistic effects on yield and flavor traits, which provides information about the trade-off between yield and

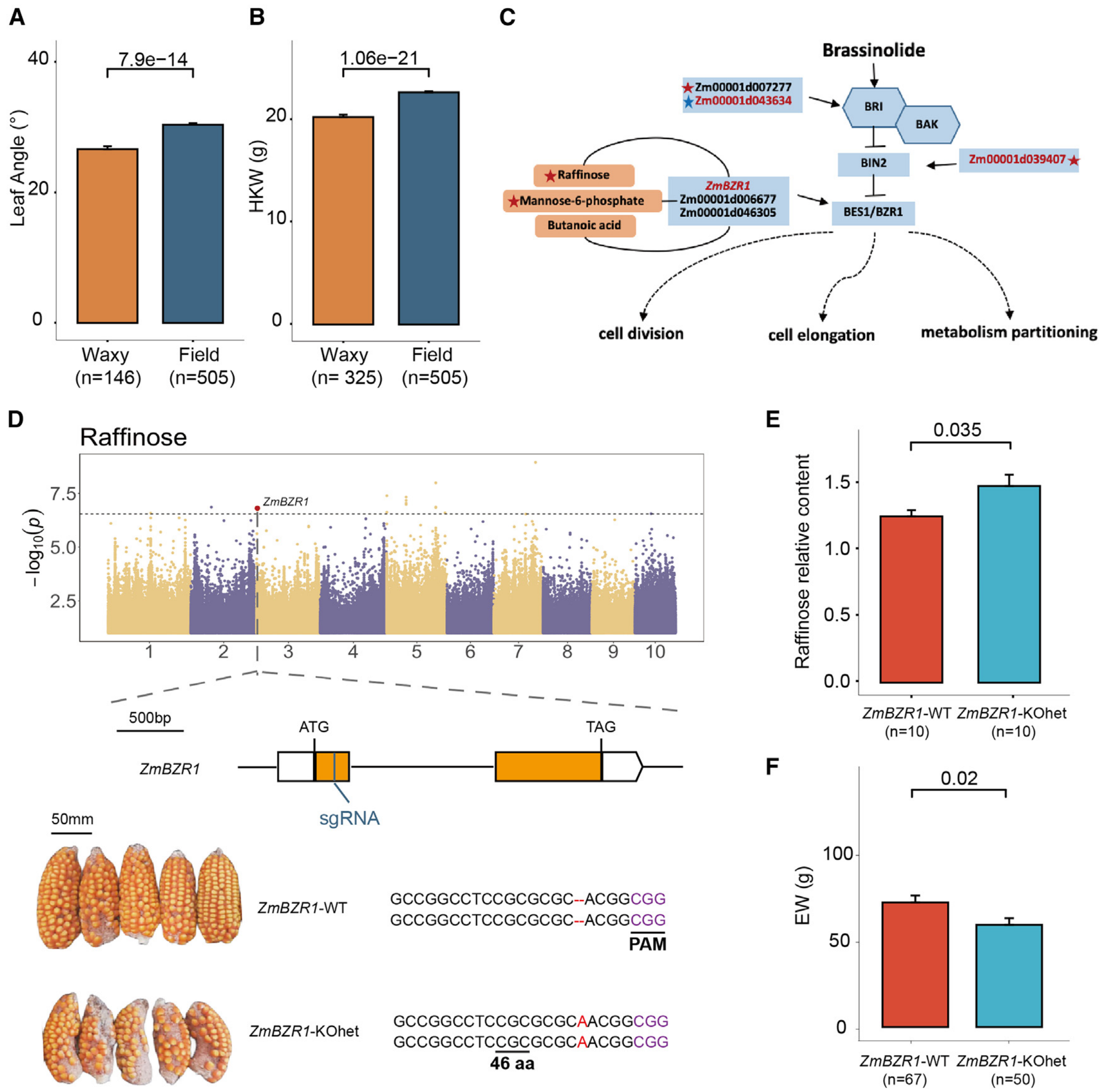

Figure 3. Possible pleiotropic effects of altered brassinosteroids in waxy and field corn populations.

(A and B) Phenotypic differences of **(A)** leaf angle and **(B)** hundred-kernel weights (HKW) between waxy corn and field corn.

(C) Speculative BR signal transduction pathway in maize. The red genes are selection candidates; genes and metabolites marked with stars were significantly correlated with waxy corn taste scores; red indicates a positive correlation; blue indicates a negative correlation. GWASs of the metabolites marked by the orange boxes are mapped to related genes connected by lines.

(D) Manhattan plot of the GWAS result for raffinose content. The candidate gene *ZmBZR1* was identified and knocked out by CRISPR-Cas9. The gene model of *ZmBZR1* is shown under the Manhattan plot. Also shown are photographs of the ears and gene sequences of *ZmBZR1* wild-type (*ZmBZR1*-WT) lines and *ZmBZR1* knockout heterozygous (*ZmBZR1*-KOhet) lines.

(E and F) Raffinose relative content **(E)** and ear weight (EW) **(F)** in *ZmBZR1*-WT and *ZmBZR1*-KOhet lines.

quality as well as important information for the next step of coordinated improvement (Figure 2D and 2E).

Trade-offs between flavor quality and grain yield are associated with the brassinosteroid biosynthetic and signaling pathway

There are significant differences in agronomic and yield phenotypes between the waxy corn and field corn populations, such as leaf angle, length/width of grains, and grain weights, among others (Figure 3A and 3B; Supplemental Figure 7). We found that genes in the brassinosteroid (BR) biosynthetic and signaling pathways were highly enriched in genomic selection analysis based on KEGG pathway annotation analysis (p = 0.0030, Supplemental Figure 8). We therefore speculated that the differences in phenotypic differentiation between waxy corn and field corn populations might be due to the differences in the BR signaling pathway. Our analysis revealed that many genes in the BR pathway may also affect the accumulation of flavor-related metabolites in waxy corn, including raffinose and mannose-6-phosphate (Figure 3C and 3D; Supplemental Figures 8 and 9). The GWAS analysis of the flavor-related metabolite raffinose identified *ZmBZR1* as the underlying gene affecting raffinose abundance (p = 1.82E−07, Figure 3D). *ZmBZR1* encodes a transcription factor downstream

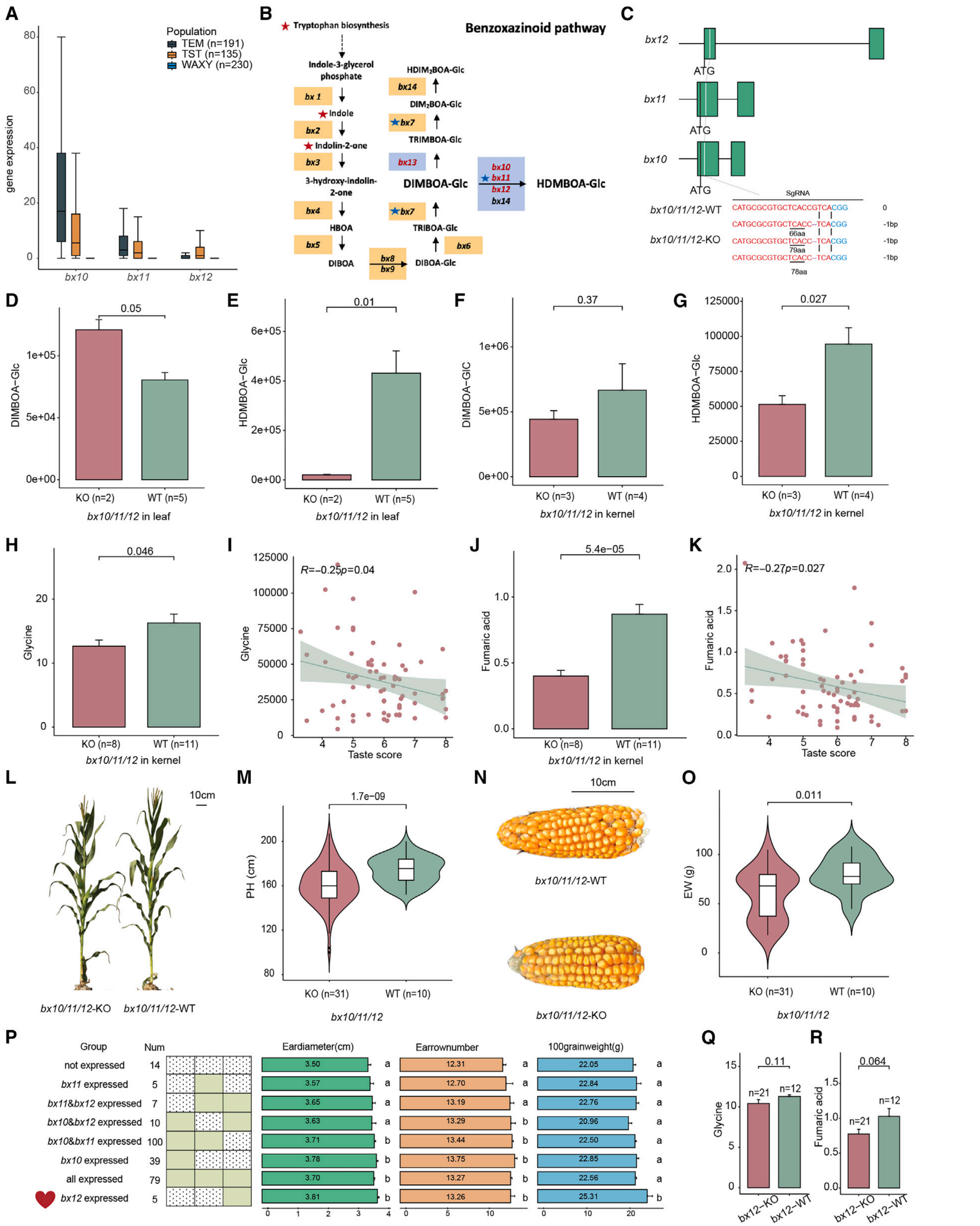

Figure 4. Divergence of the *bx* genes cluster between waxy and field corn populations.

(A) The expressions of *bx10*, *bx11*, and *bx12* were significantly different between the temperate (TEM) and tropical (TST) field corn and waxy corn (WAXY) populations.

(B) The benzoxazinoid pathway in maize. The genes in red are those selected for further genetic investigation; the metabolites marked by stars are significantly correlated to the transcript abundance of those genes or the waxy corn tasting score; red indicates a positive correlation; blue indicates a negative correlation.

(legend continued on next page)

of the brassinolide pathway. Surprisingly, a *ZmBZR1* knockout mutant was homozygous lethal and did not develop seeds, leading to a substantial decrease in yield (Figure 3D and 3F; Supplemental Table 16). Following this observation, metabolite profiles of heterozygous mutants and wild-type lines were acquired to assess the influence of *ZmBZR1* on metabolite levels. As expected, the mutants displayed a significantly higher level of raffinose than the wild type (Figure 3E and Supplemental Table 16). These results suggest that the differentiation of *ZmBZR1* and BR-related pathways may be caused by the artificial selection of waxy corn grains for flavor quality. This also indicates that the breeding history of waxy corn may have sacrificed yield-related traits to preserve quality traits and retain such "less desirable" germplasm. It will be important to try to circumvent such trade-offs between flavor quality and yield in future breeding of waxy corn.

Regulating *bx* genes can achieve flavor–yield balance in waxy corn

Three paralogous genes, *bx10*, *bx11*, and *bx12*, which encode enzymes catalyzing the conversion of DIMBOA-Glc to HDMBOA-Glc, were found within a 200-kb region on chromosome 1 (McMullen et al., 2009; Meihls et al., 2013). These three genes exhibit sequence variation between the waxy and field corn populations. Importantly, in most waxy corn, the expression of *bx10*, *bx11*, and *bx12* are almost absent compared to tropical and temperate field corn varieties (Figure 4A). It has been reported that benzoxazinoid compounds played a role in maize defense mechanisms (Tian et al., 2019). DIMBOA-Glc can be converted into DIMBOA, the aglucone form, by treatment with press juice extracted from seedlings or through heating. Regarding human taste sensitivity, the perceived sweetness of DIMBOA is approximately 400 times that of sucrose (Hamilton et al., 1962; Zhou et al., 2018). The accumulation of DIMBOA-Glc may, therefore, be related to the preference for sweetness in waxy corn. Further examination of the metabolic pathways of benzoxazinoid derivatives revealed a number of upstream metabolites, such as indole and indolin-2-one, that positively correlate with flavor (Figure 4B). Conversely, the expression level of the *bx11* gene is significantly negatively correlated with the flavor of waxy corn. Therefore, we speculate that the differences in the metabolism of these compounds between waxy and field corn may be due to the abundant accumulation of DIMBOA-Glc, the precursor to the very sweet aglucone DIMBOA in waxy corn kernels, making them more appetizing. This investigation also expands our understanding of the role of benzoxazinoid compounds. To further investigate the role of the genes of the DIMBOA-Glc metabolic pathway, single- and triple-knockout mutants of *bx10*, *bx11*, and *bx12* genes were generated (Figure 4C and Supplemental Figure 10). We observed significant changes in the metabolites DIMBOA-Glc and HDIMBOA-Glc in leaf tissues in *bx* gene mutants, although the change in abundance of these metabolites in grains themselves was not statistically significant (Figure 4D–4G). However, other metabolites such as glycine and fumarate exhibited a statistical decrease in accumulation in grain from the *bx* knockout mutants, which could potentially influence grain flavor (Figure 4H–4K). There were also changes in some grain-yield-related metabolites, including ethanimidic acid, malonic acid, glycine, mandelic acid, phenylalanine, glucose-1-phosphate, arabinopyranose, and plant-height-related metabolites, including ornithine, which explains the reduced grain production of mutants (Supplemental Table 17). Regarding the agronomic and yield traits of the mutants, we found that the loss of function of these *bx* genes significantly reduced plant height and yield, indicating antagonistic effects between the flavor and yield traits at this locus (Figure 4L–4O). Also, the yield gradually decreased in the single- and triple-mutant types, and the more severe the mutation, the greater the yield decrease (Supplemental Figure 12). To provide breeders with favorable haplotypes that can be used for practical breeding, yield traits of different expression haplotypes of *bx* genes in a field corn population were analyzed (Figure 4P). It was found that when the *bx12* gene is expressed alone, the yield is optimal (Figure 4P). The changes in the taste-related metabolites glycine and fumarate in the *bx12* gene mutants were also investigated (Figure 4Q and 4R). We found that the impact of the *bx12* single gene mutation on these metabolites was not significant, which suggests that the expression of this gene alone may not affect flavor. Therefore, the haplotype with the *bx12* gene expressed alone may represent the best option for balancing yield and flavor. Collectively, these results indicate that when one of the three *bx* genes is not expressed, or its functionality is lost, it can provoke a misbalance between yield and flavor quality.

ZmUGP1 is an ideal target for waxy corn flavor improvement

The *ZmUGP1* gene was identified in the GWAS of the flavor-associated metabolite raffinose (Figure 5A and 5C), and its

(C) Candidate genes *bx10*, *bx11*, and *bx12* were knocked out by CRISPR-Cas9. The gene model of *bx10*, *bx11*, and *bx12* is shown. Sequences of *bx10*, *bx11*, and *bx12* wild-type (*bx10/11/12*-WT) lines and *bx10*, *bx11*, and *bx12* triple-knockout (*bx10/11/12*-KO) lines are also shown.
(D) Relative DIMBOA-Glc content in *bx10/11/12*-WT and *bx10/11/12*-KO leaves.
(E) Relative HDIMBOA-Glc content in *bx10/11/12*-WT and *bx10/11/12*-KO leaves.
(F) Relative DIMBOA-Glc content in *bx10/11/12*-WT and *bx10/11/12*-KO kernels.
(G) Relative HDIMBOA-Glc content in *bx10/11/12*-WT and *bx10/11/12*-KO kernels.
(H) Relative glycine content in *bx10/11/12*-WT and *bx10/11/12*-KO kernels.
(I) Glycine content was significantly positively correlated with taste score in the waxy corn population by Pearson linear regression.
(J) Relative fumaric acid content in *bx10/11/12*-WT and *bx10/11/12*-KO kernels.
(K) Fumaric acid content was significantly positively correlated with taste score in waxy corn population by Pearson linear regression.
(L) Representative images of maize plants of *bx10/11/12*-WT and *bx10/11/12*-KO.
(M) Plant height of *bx10/11/12*-WT and *bx10/11/12*-KO lines.
(N) Representative images of ears of *bx10/11/12*-WT and *bx10/11/12*-KO lines.
(O) Ear weight of *bx10/11/12*-WT and *bx10/11/12*-KO lines.
(P) Yield traits of different haplotypes of *bx* genes in the field corn population.
(Q and R) Glycine content **(Q)** and fumaric acid content **(R)** in *bx12* gene wild-type (*bx12*-WT) lines and *bx12* gene knockout (*bx12*-KO) lines.

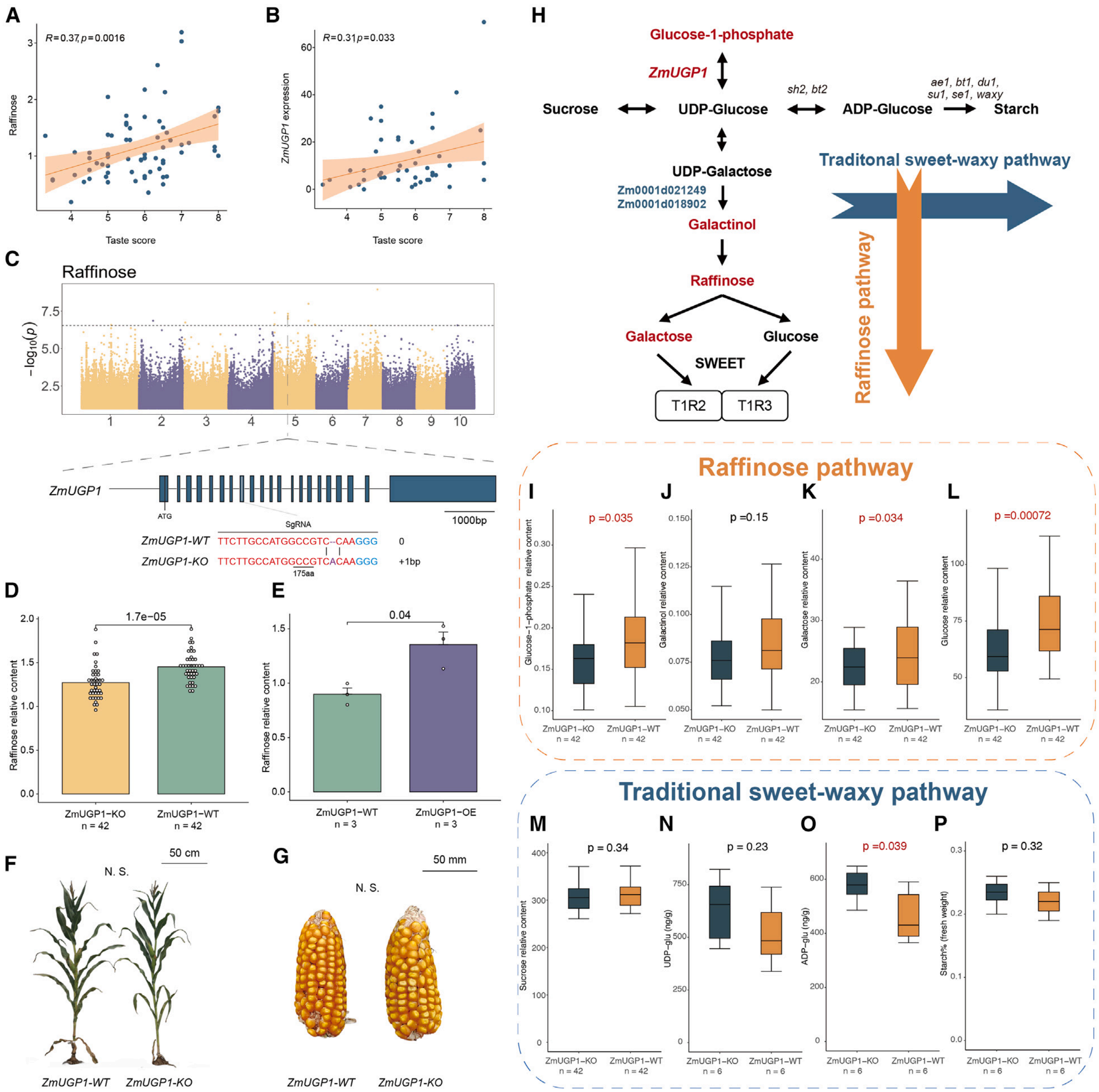

Figure 5. Functional analysis of the flavor-related gene *ZmUGP1*.

(A) Raffinose content was significantly positively correlated with taste score by Student's *t*-test ($r = 0.37$, $p = 0.0016$).

(B) *ZmUGP1* gene expression was significantly positively correlated with taste score ($r = 0.31$, $p = 0.033$).

(C) Manhattan plot of the GWAS results for raffinose content. The candidate gene *ZmUGP1* was identified and knocked out by CRISPR-Cas9. The gene model of *ZmUGP1* is shown. Filled blue boxes represent exons and UTRs. Sequences of *ZmUGP1* wild-type (*ZmUGP1*-WT) and *ZmUGP1* knockout (*ZmUGP1*-KO) lines are shown. sgRNA targets are indicated in red.

(D) Relative raffinose content of *ZmUGP1*-WT and *ZmUGP1*-KO lines from an experiment in Jilin province in 2021.

(E) Relative raffinose contents of *ZmUGP1*-WT and *ZmUGP1* overexpression (*ZmUGP1*-OE) lines in an experiment in Gansu province in 2022.

(F) Representative images of ears of *ZmUGP1*-WT and *ZmUGP1*-KO lines.

(G) Representative images of *ZmUGP1*-WT and *ZmUGP1*-KO lines.

(H) Possible metabolic pathways affecting the sweetness of waxy corn. Metabolic pathway analysis of raffinose and the *ZmUGP1* gene is shown vertically. Metabolites and genes marked in red were positively correlated with taste, while those marked in blue were negatively correlated with taste. The horizontal axis represents the traditional pathways for improving corn sweetness along with key metabolites and genes.

(I–P) Sucrose **(I)**, UDP-glucose **(J)**, ADP-glucose **(K)**, Starch **(L)**, glucose-1-phosphate **(M)**, galactinol **(N)**, galactose **(O)**, and glucose **(P)** content of *ZmUGP1*-WT and *ZmUGP1*-KO lines from an experiment in Jilin province in 2021.

expression was significantly correlated with the flavor score of waxy corn (Figure 5B). This gene was identified as a likely positive regulator influencing the flavor of waxy corn, and subsequent mutant analyses validated its gene function. Analysis of knockout and overexpression lines confirmed that *ZmUGP1* positively influences raffinose content in maize kernels (Figure 5D and 5E). Meanwhile, no obvious differences were observed in plant architecture, flowering time, and yield traits between the mutants and wild type (Figure 5F and 5G; Supplemental Table 18), suggesting that the *ZmUGP1* gene is a good target for improving the quality traits of waxy corn. Further investigation into the raffinose metabolic pathway revealed that *ZmUGP1* is an upstream regulator of raffinose metabolism (Figure 5D and 5E). Raffinose can be further metabolized into galactose and glucose, affecting sweetness perception by humans. Thus, the *ZmUGP1* gene may affect the flavor of waxy corn by influencing grain sweetness. Moreover, three other related metabolites (glucose-1-phosphate, galactinol, and galactose) and two genes (Zm00001d021249 and Zm00001d018902) in the raffinose metabolic pathway also showed significant correlations with the flavor score of waxy corn, suggesting that the raffinose-related pathway is a crucial pathway affecting flavor perception (Figure 5H and Supplemental Figure 12). The compounds most usually thought of as the main influences on corn grain sweetness are sucrose and starch, and these metabolites intersect with the raffinose pathway through some of the intermediate products (Figure 5H). We subsequently investigated the impact of the *ZmUGP1* gene on these pathways of starch and sugar metabolism. When *ZmUGP1* was mutated, in addition to the target metabolite raffinose, some flavor-related substances in the raffinose pathway, such as glucose-1-phosphate and galactose, were also altered (Figure 5I–5L and Supplemental Figure 13). However, sucrose and starch levels were not affected by knockout or overexpression of *ZmUGP1* (Figure 5M–5P and Supplemental Figure 13), indicating that manipulating the expression of this gene could increase sweetness without compromising glutinous properties and yield traits, respectively. *ZmUGP1* is therefore considered an ideal target for improving the flavor of waxy corn and has the potential to overcome the breeding dilemma caused by the high linkage of sucrose and starch in the breeding of ''sweet-waxy'' corn.

DISCUSSION

In the process of traditional waxy corn breeding, there has been a concerted effort to enhance its waxiness and starch quality, given their direct impact on its consumption and processing. However, waxy corn transcends its classification as a mere corn type, encompassing various factors contributing to its unique characteristics. Beyond its starch composition, waxy corn boasts a plethora of flavor components, setting it apart from conventional field corn varieties. In the pursuit of developing waxy corn as a consumer vegetable, breeding efforts have targeted and enhanced many essential substances, resulting in a superior edible flavor compared to field corn. As research progresses, many genes and pathways have emerged as crucial contributors to waxy corn's flavor profile. Notably, the divergence in pathways such as BRs and benzoxazinoids in waxy and field corn can be attributed to targeted artificial selection for flavor-enhancing traits in waxy corn. However, these pathways exhibit pleiotropy, influencing not only flavor but also plant growth, development, and defense mechanisms. The secondary metabolites benzoxazines are essential for defense against microbial pathogens and insects and have allelopathic effects (Meihls et al., 2013; Tian et al., 2019), which underscores the multifaceted nature of these pathways. Similarly, BRs, a class of steroidal hormones, are indispensable for plant growth and development, controlling cell elongation, division, and differentiation (Clouse, 1996, 2011; Vriet et al., 2012; Hu et al., 2017). Their regulatory reach extends to key agronomic traits such as seed germination, root development, flowering time, leaf angle, and even grain quality and yield (Hong et al., 2005; Lisso et al., 2006; Morinaka et al., 2006; Vicentini et al., 2009; Weichert et al., 2010; Jiang et al., 2013; Xu et al., 2015). Furthermore, our findings indicate that selection pressures during breeding often diverge from maximizing yield, revealing a nuanced interplay between flavor and productivity traits. The differential responses of waxy and field corn to breeding selection pressures underscore the importance of considering various traits beyond yield in crop improvement strategies. This underscores the intricate genetic pathways and regulatory mechanisms involved in flavor development, reflecting the complexity of its genetic makeup and breeding history.

Through rigorous metabolomic analyses and consumer taste evaluations, we uncovered the complex genetic and molecular underpinnings that underlie the desirable attributes of waxy corn. Our research has identified multiple metabolites, covering sugars, amino acids, and specific secondary compounds, as critical contributors to their unique flavor. Notably, 20 distinct sugars and carbohydrate derivatives, among them dulcitol, *meso*-erythritol, and *myo*-inositol—all renowned for their sweetening effects—displayed robust correlations with the waxy corn flavor (Supplemental Table 13). In addition, we detected various amino acids that significantly contribute to flavor, especially alanine and threonine, which were classified as sweet amino acids (Solms, 1969; Nishimura and Kato, 1988), and leucine, a branched-chain amino acid associated with the formation of volatile compounds in melon, thereby increasing perceived sweetness (Gonda et al., 2010). These amino acids may synergistically intensify the sweetness perception in waxy corn. Aspartate and glutamate, on the other hand, either confer umami directly or indirectly modulate flavor by serving as precursors for other flavor-enhancing amino acids (Solms, 1969; Nishimura and Kato, 1988). Phenylalanine is a crucial precursor to a range of secondary metabolites, including flavonoids, cinnamic acids, and anthocyanins, which are essential for aroma biosynthesis (Peled-Zehavi et al., 2015; Ilahy et al., 2019). It also plays a crucial role in forming key volatile compounds such as benzaldehyde and phenylethanol (Tieman et al., 2006; Klee and Tieman, 2013). Glutathione, a Kokumi-active compound, had the strongest association with flavor scores. It can enhance flavor continuity when combined with glutamate and inosine-5′-monophosphate (Ueda et al., 1997; Goto et al., 2016), although its direct sensory effects can be subtle. Interestingly, metabolites in the phenylpropanoid pathway were negatively correlated with the flavor, suggesting a potential regulation of lignin synthesis that ultimately affects the texture of waxy corn (Fornalé et al., 2010; Li et al., 2022). This highlights the multifaceted nature of waxy corn's attractive taste, formed by a harmonious interplay of sweetness, aroma, texture, and other factors. Identifying these flavor-linked loci highlights the great potential of targeted breeding strategies to optimize the flavor profiles of waxy corn varieties. Our findings further challenge conventional wisdom, revealing that viscosity and starch quality are not significantly associated with waxy corn's flavor, possibly due to their relative stability across different inbred lines.

The application of multi-omics methodologies in aiding breeding endeavors represents a pivotal development trajectory. Each omics technology, including GWASs, transcriptomics analyses (exemplified by eQTL and DEG analyses), and selection techniques (such as XP-CLR), illuminates distinct facets of genetic mechanisms. Their synergistic application enables a more holistic discernment of vital genetic loci and their intricate architectures. GWASs can pinpoint genetic markers associated with specific traits, facilitating the precise mapping of QTLs relevant to these attributes. Meanwhile, eQTL and DEG analyses delve into the interaction between gene expression and genetic variation, uncovering genetic variations that regulate gene expression, gene-regulatory networks, and potential causal relationships. Generally, the agreement between GWAS and eQTL results highlights natural variations in regulatory domains, as evidenced by our discovery of the *ZmUGP1* site that affects raffinose metabolism. The polymorphism of *ZmUGP1* provided an opportunity to improve waxy corn germplasm. In contrast to GWASs, selection strategies such as XP-CLR reveal gene regions under natural selection by surveying the genetic diversity of a population, highlighting genes that are critical for adaptive traits. Instead of targeting a specific phenotype, XP-CLR emphasizes genes of adaptive significance. The *ZmBZR1* gene, identified by both XP-CLR and GWASs, controls raffinose content, and its phenotypic effects may result from sequence changes rather than expression regulation. Meanwhile, the *bx* genes, also affected by selection, were detected by XP-CLR and DEG analysis, but their expression was limited in waxy maize populations. Integrating chromatin accessibility data suggested that regulatory elements controlling these genes might reside in adjacent sequences. The phenotypic evaluation confirmed that ablation of all three genes affected plant height and yield characteristics, demonstrating the complexity of this regulatory locus for breeding optimization. However, the single mutation of *bx12* presented a balanced haplotype, resulting in improved yield and quality. By combining these multi-omics data, we can gain a deeper understanding of the function of genetic variation and can specify precise breeding targets, thus promoting more efficient and precise breeding practices. In essence, the converged application of multi-omics approaches in breeding research highlights its paramount value, empowering us to reveal key genetic loci, decipher their genetic blueprints, and propel breeding forward with heightened precision and efficacy.

The importance of sweetness has recently been re-evaluated and is now considered one of the core factors affecting the texture and flavor of waxy corn. In recent years, corn varieties combining sweetness and glutinousness have gradually gained popularity in Southeast Asia and China and occupy one-third of the Chinese specialty corn market (Dong et al., 2019; Song et al., 2023). These so-called "sweet-waxy" corns are F1 hybrids derived from double or multifold recessive genes of *Waxy* and sugary (*du*, *su1*, *su2*, *se1*, or *sh2*) parental combinations, with each corn cob carrying grains in a ratio of 3:1 or 9:7 for glutinousness and sweetness (Yang et al., 2021). The sugary genes encode enzymes in the sucrose biosynthesis pathway that links directly to starch biosynthesis (Figure 5H). This may explain the accepted conclusion that sweet and waxy types cannot coexist in the same kernel. The two types of grains coexisting in the same ear differ greatly in moisture and dry matter content, resulting in inconsistent grain types, which seriously affects the appearance of the product and is not conducive to processing, storage, and transport. One of the remarkable discoveries in our study was the identification of certain sweet substances, such as DIMBOA and raffinose, as potential sweeteners for waxy corn. Surprisingly, these metabolites, which are not closely linked to sucrose or starch content and do not impact the "glutinous" nature of the grain, can be harnessed to develop novel "sweet-waxy" corn varieties. This innovative approach offers a pathway to explore and exploit a balance between sweetness and glutinous texture in waxy corn, presenting a promising solution for further enhancement of glutinous corn varieties. Furthermore, our research has uncovered important loci and regulatory genes, such as *ZmUGP1,* associated with these flavor-related metabolites. These metabolites and genes serve as valuable genetic markers, facilitating the efficient selection of desirable flavor traits in waxy corn breeding programs. Leveraging molecular marker-assisted selection technology, waxy corn breeding materials with desired characteristics can be rapidly and effectively developed. In summary, our study provides reliable molecular markers for enhancing glutinous corn and opens up new avenues for improving the breeding of high-nutrient-content corn varieties.

METHODS

Plant materials and sequencing

A set of 318 waxy corn inbred lines from a global collection developed at the Shandong Academy of Agricultural Sciences was employed for the present study, and the pedigree of the inbred lines is presented in Supplemental Table 1. All inbred lines were grown in Jinan (36°40′ N, 117°00′ E) in June 2017. For leaf samples, the leaves of three plants from the same line were collected and pooled before flash freezing in liquid nitrogen for whole-genome resequencing. For kernel samples, kernels 17 days after pollination from three ears of the same line were collected and pooled before flash freezing in liquid nitrogen for transcriptomic sequencing. Genomic DNA from each sample was extracted with the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980) and sequenced with the Illumina HiSeq 3000 platform with 150-bp paired-end reads, generating more than 45 Gb of sequenced base pairs per sample. The poly(A) transcriptome collected from kernels at 17 days after pollination of 230 waxy corn lines in the population was sequenced, and over 4 Gb of sequenced base pairs per sample were obtained. The DNA extraction, RNA extraction, and sequencing work described was carried out by BGI Group (Shenzhen, China).

Reads mapping, variant discovery, and genotype analysis of waxy corn population

Adaptor sequences and low-quality reads were trimmed by Trimmomatic (version 0.33; Bolger et al., 2014) as the first step of analysis, using the following parameters: LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:15, MINLEN: 36. The clean reads were mapped to the maize B73 reference genome (V4; downloaded from http://plants.ensembl.org; Jiao et al., 2017) using Bowtie2 (v.2.1.0; –very-fast; Langmead and Salzberg, 2012), which has a good balance between runtime, memory usage, and accuracy. The unique mapped reads extracted from SAM files were sorted and indexed using Picard (version 1.119, http://broadinstitute.github.io/picard/). The RealignerTargetCreator and IndelRealigner modules from GATK (version 3.5; Mckenna et al., 2010) were used to perform local realignment around InDels to correct mapping artifacts. SAMtools (version 0.1.19; Li et al., 2009) and UnifiedGenotyper from GATK were first used to generate a high-quality SNP set as known sites to build the covariance model and estimate empirical base qualities for each individual. Next, BaseRecalibrator and PrintReads tools from GATK were used to recalibrate base quality scores to correct sequencing errors and other potential experimental artifacts. The recalibrated BAM files were processed using SAMtools and UnifiedGenotyper from GATK. For GATK, the parameter "-glm" was set as "BOTH" to obtain SNPs and InDels simultaneously. Variant calls from the SAMtools mpileup package were identified using default parameters. Variants identified by both programs were retained only if they satisfied mapping quality (MQ $\geq$ 20.0) and sequencing coverage (DP $\geq$ 3 and DP $\leq$ 100). GATK CombineVariants further combined the filtered variants of each sample, and another re-calling process of each individual using GATK UnifiedGenotyper was performed to obtain initially integrated genotypes at the population level. Next, SNP sites with a "Lowqual" label were excluded, and all filtered variants were combined by GATK CombineVariants to a single variant calling file again. Missing genotypes were imputed using an IBD algorithm with Beagle (version 4.0; Browning and Browning, 2007). SNPs with a missing rate of more than 75% before imputation were removed after imputation. Eventually, a set of 21,744,459 high-confidence SNPs of waxy corn were retained for further analysis.

Genotype integration with the field corn population

The SNPs of 507 maize inbred lines were collected from a previous study (Chen et al., 2022). SNPs in two datasets were recalled by GATK, in which we filtered out all low-quality and high-missing-rate loci for subsequent comparative analysis of different populations. Finally, a high-density variation hapmap of waxy and field corn population, including 31,175,750 SNPs, was obtained.

Phylogenetic tree construction and population structure analysis

Non-missing SNPs in waxy and regular maize were annotated with VEP (McLaren et al., 2016), and the non-synonymous sites were used in phylogenetic tree construction using the SNPhylo software package (v.20140701; Lee et al., 2014). To evaluate the structure of the panel, the genome-wide complex trait analysis tool (GCTA, version 1.25.0; Yang et al., 2011) was used to perform principal-component analysis, and the software Admixture (v1.3.0; Alexander et al., 2009) was used for population structure analysis.

LD, FST, and nucleotide diversity calculation

LD (r^2) of waxy and field corn was estimated within 500 kb by Haploview (v.4.2; Barrett et al., 2005) with parameters -minMAF 0.05, -hwcutoff 0, -missingCutoff 0.5. The VCFTools program (Danecek et al., 2011) was used to calculate F_{ST} statistics (–windows 20 000, –steps 2000). The top 5% of the regions were considered divergence regions, and contiguous windows were merged to obtain a larger region. The nucleotide diversity of waxy and field corn was also calculated by VCFTools (–windows 20 000, –steps 2000). To eliminate the underestimation of nucleotide diversity in low-coverage regions, windows with missing rates of more than 0.8 were filtered.

Selective sweeps

The genetic distance of each SNP in waxy corn and field corn was calculated based on the B73 × By804 genetic map (Pan et al., 2016). Genetic distances of SNPs located between the genetic markers were averaged based on their physical distance. Whole-genome scanning for the regions of waxy corn that had undergone selection from field corn was implemented by XP-CLR (-w1 0.005 100 1000 -p0 0.7; Chen et al., 2010). The corresponding top 5% XP-CLR score regions were regarded as the candidate selection regions. Genes within these regions or strongly in LD with these regions were the presumed selection candidates.

Quantification of known genes and transcripts and differentially expressed gene analysis

Raw reads were first filtered to remove the poor-quality base calls and adaptors by Trimmomatic (LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4: 15, MINLEN: 36). Reads were then aligned to the B73 reference genome (V4) using the Ultrafast STAR Aligner7 (Dobin et al., 2013) using a standardized pipeline. Read counts aligned to known exonic regions based on the B73 V4 annotation were quantified using the HTSeq package (Anders et al., 2015). The HTSeq-count script was executed using intersection_nonempty mode, which excluded ambiguous reads that map to regions of multiple genes. All other reads mapping to single genes were included in the corresponding gene counts. Finally, quantile normalization was applied to the remaining genes to obtain normalized gene counts using DESeq2 (Love et al., 2014). Different expression of genes between waxy corn and field corn were identified by the edgeR package (Robinson et al., 2010) with $\log_2$ fold change (maximum-likelihood estimate [MLE]) >2, odds ratio < -2, p value <0.01.

eQTL mapping and enrichment analysis

All gene-expression levels of the population were combined, and the gene-expression matrix of the population was normalized using DESeq2 software to obtain the normalized gene counts. After obtaining the gene-expression matrix, genes expressed in more than 70% of the population were selected for eQTL mapping in the population. The eQTL analysis was carried out using EMMAX software (Legarra et al., 2018), and the mixed linear model was adopted to consider the population structure and genetic relationship among materials. GEC (Genetic Type 1 Error Calculator software (Li et al., 2012) was used to determine the threshold of eQTLs.

Starch viscosity evaluation

Samples were milled and subsequently sifted through a 0.15-mm sieve. Milling was conducted in the same conditions to avoid the effects of moisture content for rapid viscosity analyzer (RVA; Rva-Tedmaster, Perten Instruments, Sweden) measurement. The experiment was carried out strictly following the recommended method specific to corn starch. In brief, distilled water (25 ± 0.01 mL) was added to the milled maize flour sample (3 ± 0.01 g) in an aluminum RVA canister. A paddle was placed in the canister and rotated at 160 rpm for 10 seconds to disperse the maize sample. A constant paddle rotation of 160 rpm was then used for the viscosity evaluation. The sequential temperature curve for a 13-min test was as follows: (1) incubate at 50 for 1.0 min; (2) increase to 95 °C using a 3.7 °C/min increment; (3) keep at 95 °C for 2.5 min; (4) cool down to 50 °C with 3.8 °C/min decrement; and (5) hold at 50 °C for 2 min. Starch viscosity characteristics comprised five primary components (pasting temperature, peak viscosity, time to peak, minimum viscosity, and final viscosity) and two secondary components (breakdown and setback). Breakdown values were calculated by subtracting minimum paste viscosity from peak viscosity, while setback values were calculated by subtracting minimum paste viscosity from final viscosity.

GC–MS and LC–MS metabolite profiling

Samples for metabolite profiling were planted with a randomized complete experimental design in Zhangye (38°55′ N, 100°27′ E) in April 2018. Approximately ten kernels from the middle of the ear of 243 waxy corn lines were collected 22 days after pollination and freeze-dried under nitrogen before metabolite extraction. For GC–MS metabolite profiling, 50 mg of each freeze-dried sample powder was extracted following established procedures with minor modifications (Salem et al., 2016; Wang et al., 2019). Dried extract was derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide as described previously (Yan et al., 2018), and further analyzed using GC–MS (7890A-5975C; Agilent, USA). One microliter of liquid mixture was taken from each sample and injected into GC–MS at 270 °C in a split mode (50:1) with helium carrier gas (>99.999% purity) flow set to 1 mL/min and separated by a DB-35MS UI (30 m × 0.25 mm, 0.25 μm) capillary column. The metabolite data were further analyzed according to the protocol described by Yan et al. (2018). Agilent Mass Hunter Quantitative Analysis software (version B.07.01) was used for GC–MS data analyses. The NIST library, built with authentic standards, was used together with an internal database for metabolite identification. For LC–MS profiling, 100 mg of freeze-dried samples was extracted as described previously before analysis with an LC–electrospray ionization–MS/MS system (Chen et al., 2013). The extracts were adsorbed onto a solid-phase extraction cartridge (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; Shanghai ANPEL Scientific Instrument Co., Ltd.). Quantification of metabolites was carried out using a scheduled multiple reaction monitoring (MRM) method (Dresen et al., 2010), with an MRM detection window of 80 s and a target scan time of 1.5 s. Metabolite data was further analyzed according to the protocol previously described (Chen et al., 2014).

Tasting experiment

All inbred lines involved were planted in two stages in Zhangye (38°55′ N, 100°27′ E) in April 2018 for the experiment. The first stage (for the expert group) was planted 5 days earlier than the second one (for the consumer group). Three ears of each inbred line from the first stage were harvested 25 days after pollination. After cleaning and 15 min of steaming, the medial part of each was cut into five pieces and tasted by the five experts. The scoring criteria involved four aspects: peel color (10%), peel thickness (25%), fruit flavor (30%), and glutinousness (35%). A total of 79 representative lines, according to the score of experts, were selected from 318 inbred lines for the next experiment. The second step of the typical material-tasting experiment was conducted among a consumer group of about 100 people. The consumer group comprised teachers and students from Zhangye Normal University with a balanced gender ratio, whose ages ranged from 18 to 67 years old (mostly around 20 years old). The tasting experiment was divided into two parts due to the long pollination period of the population. The ears of each tasting were harvested and stored at −20 °C (4 °C) until the day before tasting, and the storage time was not more than a week. The cooking and experimental processes were the same as already described. The consumer group gave a score for the overall flavor intensity of each material based on their preference. The group was given a standard explanation and training before the start of the experiment. Moreover, throughout trials, we prepared water for gargling to ensure fairness of all independent scoring for materials.

Correlation and regression analysis of metabolites or gene expression with flavor scores

A total of 69 genotypes with available metabolic data and flavor scores and 47 genotypes with available gene-expression data and flavor scores were subjected to correlation and regression analyses. Simple Pearson correlation analysis was conducted using the cor.test function in the R statistical scripting language. Additionally, multiple regression analyses of Metabolites and gene expression were used to model the relationship between overall flavor intensity using the lm function in R. The *p*-value threshold was set at 0.05 for both analyses.

Genome-wide association analysis

The population structure of the waxy corn population was calculated with Admixture (v.1.3.0; –cv = 10; Alexander et al., 2009), *K,* with the lowest coefficient of variation (K = 5) being used in the downstream analysis. Kinship of the waxy corn population was calculated by GCTA (version 1.25.0; Yang et al., 2011). The association between the genome-wide SNPs (with minor allele frequency [MAF] $\geq$5%) and each trait was tested. A mixed linear model was used to assess the population structure (Q) and familial relationship (K) implemented in TASSEL3.0 software (Bradbury et al., 2007). The *p*-value threshold was 2.85E−07 for the entire population.

Transgenic maize generation and functional validation of *bx* genes and *ZmBZR1*

To obtain single- and triple-knockout mutants of *bx* genes, we designed three single-guide RNAs targeting the exon of *bx10*, *bx11*, and *bx12*, respectively (sgRNA-*bx10*, 5′-GTC GTC GAG CAA CCA CCA GCA GG-3′; sgRNA-*bx11*, 5′-GCG ATC CAC CAC CAC GGC GCC GG-3′; sgRNA-*bx12*, 5′-GAG CGA TTT GGC GAA GCA CAA GG-3′), and one single-guide RNA targeting the same site of three genes (sgRNA-*bx10/11/12*, 5′-CAT GCG CGT GCT CAC CGT CAC GG-3′). For gene *ZmBZR1*, one single-guide RNA (sgRNA-*ZmBZR1*, 5′-CAT GCG CGT GCT CAC CGT CAC GG-3′) was designed for targeting the first exon of *ZmBZR1*, to produce knockout lines. These targets were cloned into the binary vector, pCPBZmUbi-hspCas9. The final constructs were transformed into *Agrobacterium tumefaciens*, EHA105 (Weimi Biotechnology Co. Ltd, Changzhou, China) for infection of KN5585 immature embryos. Sampling and phenotypic investigation of T_3 generations of *bx* genes and *ZmBZR1* mutants were conducted in 2022 in Gansu province (100° E, 38° N). The genotype of gene-edited lines (Supplemental Tables 16 and 17) was identified by PCR amplification and Sanger sequencing using target-specific primers. The RNA-seq work with these transgenic lines was carried out by Wuhan GrandOmics Biosciences Co., Ltd.

Transgenic maize generation and functional validation of *ZmUGP1*

Knockout mutants were generated from a high-throughput genome-editing design (Liu et al., 2020; http://www.wimibio.com/). In brief, line-specific sgRNAs were filtered based on the assembled pseudogenome of the KN5585 genotype, and a double sgRNAs pool approach was used to construct vectors. The vectors were transformed into the wild-type line KN5585, and the targets of each T_0 individual were assigned by barcode-based sequencing. Sampling and phenotypic investigation of T_3 and T_4 generations were conducted respectively in 2021 in Jilin province (125° E, 44° N) and in 2022 in Gansu province (100° E, 38° N) and Jilin province (125° E, 44° N). The genotype of gene-edited lines (Supplemental Table 18) was identified by PCR amplification and Sanger sequencing using target-specific primers. Overexpression of *ZmUGP1* was achieved as follows. The full-length coding sequence of *ZmUGP1* and the ZmUbiquitin promoter was cloned into the pZZ01523 vector and confirmed by sequencing. All transformations of KN5585 were carried out by Weimi Biotechnology. The overexpression lines were confirmed by RT–qPCR assay. The mRNA was isolated from transgenic individuals at the five-leaf stage using TRIzol reagent (Invitrogen). RT–qPCR was performed using SYBR premix (ABClonal) and the CFX96 Real-Time System (Bio-Rad). All experiments were done at least three times. For each assay, the experiment was repeated at least three times with similar results. Sampling and phenotypic investigation of T_3 and T_4 generations were conducted respectively in 2021 in Jilin province (125° E, 44° N) and in 2022 in Gansu province (100° E, 38° N).

DATA AND CODE AVAILABILITY

All datasets that support the findings of this study have been deposited into the CNGB Sequence Archive (CNSA) of the China National GeneBank DataBase (CNGBdb) with the following accession numbers: all raw genomic sequencing data for the 318 waxy corn accessions, CNP0003601; all raw RNA-seq data for the 230 waxy corn accessions, CNP0004315.

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AUTHOR CONTRIBUTIONS

J.Y. and L.W. designed this study. C.H., F.Z., and L.W. prepared the materials. C.H., J.L., and W.L. performed field experiments. J.L. performed most of the bioinformatics data analysis. S.G. and N.Y. helped with genomic data analysis. Y.X. and W.Y. helped with statistical analysis. K.L. performed starch viscosity evaluations. S.Y. and W.H. performed GC-MS metabolite profiling. Y.Z., and L.Z. helped with LC-MS metabolite profiling. S.W. and A.R.F. helped with the metabolite data analysis. J.L., C.J., and A.C. conducted gene validation experiments. J.L., C.H. and J.Y. wrote the manuscript. All authors critically read and approved the manuscript.

SUPPLEMENTAL INFORMATION

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