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Research Paper

MOLECULAR ANALYSIS OF WAXY GENE MARKERS IN SORGHUM CROSSES KD4 AND BONTEB GUNUNGKIDUL

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ARTICLE HIGLIGHTS

- Local sorghum crosses show unique fixation of the waxy starch gene allele
- Only Wxc allele is expressed, while other waxy alleles are not detected
- Waxy allele expression strongly relates to low amylose grain quality
- Marker-based selection supports breeding of soft-textured sorghum
- Findings enhance sorghum use for food, feed, and industrial applications

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ABSTRACT

Sorghum (*Sorghum bicolor* (L.) Moench) is a food crop that exhibits resilience to extreme environmental conditions and has the potential to be developed as an alternative food source. The quality of sorghum seeds is significantly influenced by the starch composition in the endosperm, which is regulated by the waxy (Wx) gene. This gene has several major alleles, namely Wxa, Wxb, and Wxc, which play roles in the synthesis of amylopectin and amylose. This study aimed to analyze the expression of Wx alleles in the crosses of sorghum cultivars KD4 and Bonteb Gunungkidul. The main method used was the molecular marker-based PCR method. DNA was extracted from the leaves of 30 individual F2 sorghum progeny samples using a slightly modified CTAB method. PCR reactions were performed with specific primers for each allele, and the amplification results were analyzed using 1.5% agarose gel electrophoresis. Several statistical analyses were performed to ensure results significance, i.e., a) Chi-Square Test: To determine relationships between waxy allele expression with genetic segregation within cross populations; b) Allele Frequency Analysis: To determine distribution of waxy genotypes within populations by comparing counts showing expressions of Wxa, Wxb, and Wxc; and c) Pearson Correlation Test: To evaluate relationships between waxy gene expression with specific agronomic traits (e.g., amylose content). The main findings of our study showed that only the Wxc allele exhibited a clear amplification band, while Wxa and Wxb did not show any significant expression. This indicates that the Wxc allele plays a dominant role in starch synthesis in this cross, while Wxa and Wxb are likely not expressed due to genetic or epigenetic regulatory mechanisms. These findings provide a brief summary of sorghum breeding efforts aimed at producing varieties with superior waxy starch characteristics. Further studies are needed to understand the regulation of Wx gene expression and its potential implications for molecular selection, ultimately enhancing sorghum quality for both food and industrial applications.

Keywords: Genetics, local, plant breeding, sorghum, waxy gene

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is a strategic food crop that contributes significantly to global food security, particularly in tropical and subtropical regions. Its advantages include drought tolerance, high water-use efficiency, and the ability to thrive on marginal soils compared with other cereals such as rice and wheat (Xie *et al.* 2022). Sorghum also provides high nutritional value, being rich in carbohydrates, proteins, dietary fiber, and bioactive compounds such as polyphenols and antioxidants (Tian *et al.* 2023). These attributes make sorghum a promising candidate for food diversification, especially under increasingly variable climate conditions (Mutisya *et al.* 2023). However, one of the main limitations to its wider adoption as a staple food is that sorghum has relatively dry and firm grain texture compared with those of rice (Lu *et al.* 2022).

The eating quality of sorghum is largely determined by the ratio of amylose to amylopectin in the grain. Amylose, a linear glucose polymer, contributes to hardness and dryness, while amylopectin, a branched polymer, produces a softer and stickier texture (Yano *et al.* 2020). Therefore, manipulating starch composition has become a key objective in sorghum breeding programs aimed at improving palatability. The waxy gene (Wx) is the primary genetic factor controlling amylose synthesis. It encodes granule-bound starch synthase (GBSS), and mutations at this locus result in reduced or absent amylose content. Such variants, termed waxy sorghums, are more acceptable to consumers in many Asian countries due to their softer texture (Boyles 2017; Zhou *et al.* 2021; Tian *et al.* 2023).

Several waxy alleles, including Wxa, Wxb, and Wxc, have been reported to influence amylose levels, although their expression varies depending on genotype and environment (Wang *et al.* 2023). Wardhani and Wirnas (2024) documented considerable genetic diversity for amylose content in sorghum crosses (Pulut 3 × Soraya 3), demonstrating the potential of allele-based selection to generate waxy lines. Hence, molecular mapping and characterization of Wx alleles are essential steps in sorghum improvement.

Molecular markers, particularly PCR-based assays, provide accurate identification of waxy alleles compared with conventional phenotypic screening and can accelerate breeding progress (Lu *et al.* 2022). This approach allows breeders to detect waxy genotypes early in plant development, increasing selection efficiency (Wang *et al.* 2023).

Beyond breeding, waxy sorghum has broad potential in the food industry. Waxy starch improves product quality in applications such as noodles, bread, and other processed foods requiring superior gelatinization and textural properties (Zhou *et al.* 2021; Tian *et al.* 2023). Thus, the development of waxy sorghum varieties could enhance both consumer acceptance and industrial utilization.

In this study, we analyzed the expression of Wx alleles (Wxa, Wxb, and Wxc) in KD4, Bonteb Gunungkidul, and their progenies. In addition, we examined the relationship between allele expression and amylose content to provide a comprehensive understanding of how Wx alleles contribute to starch quality. These findings are expected to support sorghum breeding programs in developing varieties with improved eating quality and industrial potential.

MATERIALS AND METHODS

Materials and Equipment

This study was conducted at the Laboratory of Genetics and Plant Breeding, Faculty of Agriculture, Gadjah Mada University. Leaf and seed samples from crosses between KD4 and Bonteb Gunungkidul were used as DNA sources. DNA was extracted using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) protocol. Specific primers targeting Wxa, Wxb, and Wxc were used for allele-specific amplification. Standard PCR reagents (buffer, dNTPs, Taq DNA polymerase, and $MgCl_2$) were employed, and PCR (Polymerase Chain Reaction) products were separated by 1.5% agarose gel electrophoresis. A NanoDrop spectrophotometer was used to measure DNA quality and concentration prior to amplification.

Sample Collection

Fresh leaf tissues were collected from 30 individual F2 sorghum progeny derived from the cross between KD4 and Bonteb Gunungkidul for molecular analysis. Genomic DNA was extracted using a slightly modified CTAB method (Doyle & Doyle 1990) to improve purity and yield, while seeds were stored for further evaluation.

Table 1 Specific primers for identification of waxy genes in sorghum

Allele type	Primer (5'-3')	Temperature/ annealing (°C)	Band size (pb)	Reference
Wxa	F1:CGTGGCGAGATCAAACTCTA	60.0	Non waxy: 523	Wang *et al.* (2023)
	F2:GGCCTGGATTCAATGTTCTT		Waxy: 615	
	R:GCAGCTGGTTGTCCTTGTAG			
Wxb	F:CGACCGTGTGTTCATTGACCAC	61.0	Non waxy: 1,281	Wang *et al.* (2023)
	R:TTGTTCAGTGCCTTGCCTCG		Waxy: 745+537	
Wxc	F:GCTGGTTCTGAGTGCAACA	58.5	Non waxy: 523	Wang *et al.* (2023)
	R1:ACTTCTTCTTGCCAGTGACC		Waxy: 615	
	R2:ACTTCTTCTTGCCAGTGACG			

DNA Extraction

Genomic DNA was isolated using a slightly modified CTAB method (Doyle & Doyle 1990) to improve purity. The procedure involved grinding leaf tissue in liquid nitrogen, incubating in CTAB extraction buffer at 65 °C, separating phases with chloroform–isoamyl alcohol, precipitating DNA with isopropanol, and washing with 70% ethanol. The resulting DNA was dissolved in TE buffer and stored at -20 °C. DNA quality and concentration were assessed using a NanoDrop spectrophotometer at 260/280 nm.

PCR Amplification

PCR was conducted to amplify waxy genes (Wxa, Wxb, Wxc) using specific primers (Table 1). The reaction mixture (25 µL) consisted of PCR Master Mix, DNA template (50 ng/µL), forward and reverse primers (10 µM each), and ddH_2O. Amplification was performed in a thermal cycler with an initial denaturation at 95 °C for 5 minutes, followed with 35 cycles of denaturation (95 °C, 30 seconds), annealing (50 – 60 °C, 30 seconds), and extension (72 °C, 1 minute). A final extension at 72 °C for 10 minutes was applied before the samples were stored at 4 °C.

Gel Electrophoresis and Visualization

PCR products were resolved by 1.5% agarose gel electrophoresis in TBE buffer at 100 V for 45 minutes. Gels were stained with ethidium bromide or SYBR Safe and visualized under UV light. Banding patterns were compared with positive and negative controls to confirm allele-specific amplification.

Data Analysis

Electrophoresis results were analyzed using ImageJ or GelAnalyzer software to quantify band intensity. Data were summarized as gel images, tables, and allele distribution charts. Statistical analyses included: (a) Chi-Square analysis to assess segregation patterns; (b) allele frequency analysis to estimate genotype distribution; and (c) Pearson correlation to examine relationships between waxy allele expression and amylose content. These analyses provided insights into the effectiveness of molecular marker-based selection for waxy sorghum improvement.

RESULTS AND DISCUSSION

PCR Analysis Results

Electrophoresis results indicated that only the Wxc allele exhibited clear amplification bands, while both Wxa and Wxb showed no significant amplification (Fig. 1). This pattern was consistent across all 30 F2 samples analyzed, confirming that Wxc was the only allele expressed in the studied populations. The results suggest several underlying factors that could contribute to this observation, which is often associated with primer specificity, genetic mutations, and PCR optimization issues (Yang *et al.* 2013).

One primary consideration is the specificity and binding efficiency of the primers used during the polymerase chain reaction (PCR). Primers are short sequences of nucleotides that anneal to specific regions of the DNA template to initiate amplification. If the primers are designed based on sequences unique to the Wxc allele, they may not effectively bind to the Wxa and Wxb alleles due to sequence variations, leading to preferential amplification of the Wxc allele. A study by Teng *et al.* (2012) emphasized the importance of meticulous primer design to ensure that all target alleles are equally recognized and amplified during PCR.

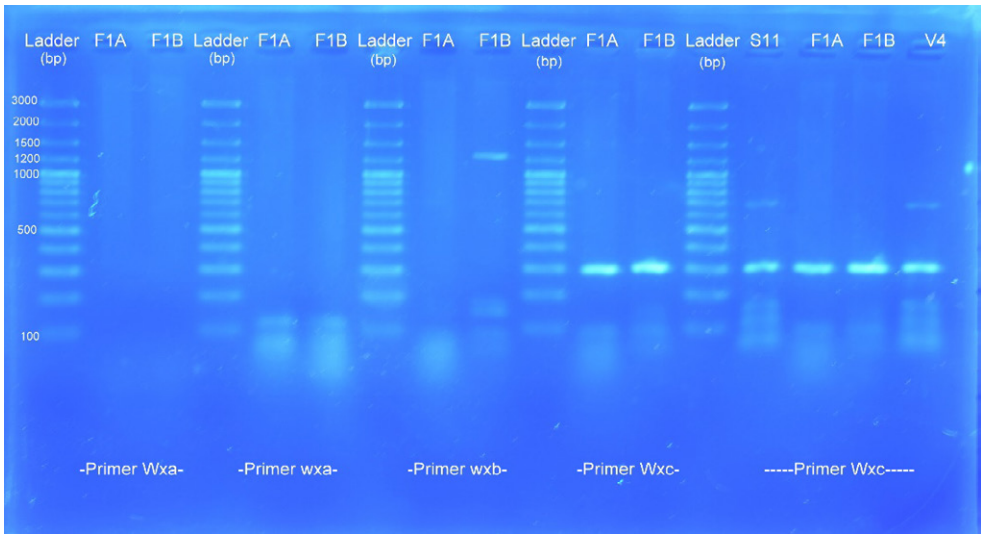

Figure 1 PCR analysis results for the examined sorghum leaf samples

Genetic variations or mutations within the Wxa and Wxb alleles could also impede primer binding or the amplification process. For instance, single nucleotide polymorphisms (SNPs) or insertions/deletions (indels) in the primer binding sites can reduce the efficiency of primer annealing, resulting in weak or absent amplification signals for these alleles. Zhang *et al.* (2019) reported that specific SNPs in waxy genes significantly affected the amplification efficiency of different alleles in rice and sorghum, underscoring the need to account for such variations in experimental design.

The quality and quantity of the DNA template used in the PCR can significantly influence amplification outcomes. Degraded DNA or insufficient template amounts can lead to suboptimal amplification, particularly for certain alleles. Ensuring high-quality DNA extraction and quantification is crucial for obtaining reliable and reproducible results across all target alleles (Shin *et al.* 2015).

PCR conditions, including annealing temperature, magnesium ion concentration, and cycle number, play pivotal roles in amplification efficiency. Suboptimal conditions may favor the amplification of one allele over others. Therefore, optimizing these parameters is essential to achieve balanced amplification of multiple alleles. Wang *et al.* (1995) suggested that techniques such as gradient PCR can help determine the optimal annealing temperatures for primers, thereby enhancing the amplification of all target alleles.

In our study, the exclusive amplification of the Wxc allele observed in the electrophoresis results is likely due to a combination of factors, including primer specificity, allelic variations, DNA template quality, and PCR conditions. Addressing these aspects through careful experimental design and optimization can lead to more balanced and accurate amplification of the Wxa, Wxb, and Wxc alleles (Pedersen *et al.* 2007).

Statistical Analysis of Sorghum Molecular and Agronomic Data

1. Chi-Square Test (χ^2)

The Chi-Square (χ^2) test was employed to determine whether genetic segregation in the F1A and F1B cross populations adhered to the expected Mendelian inheritance ratios. This statistical test is widely used in genetic studies to assess the goodness-of-fit between observed and expected distributions, thereby evaluating deviations that may indicate underlying genetic factors such as dominance effects, epistasis, or selection biases (McDonald 2014).

Hypotheses:

- H0: Genetic segregation follows expected ratios (e.g., 1 : 2 : 1 for heterozygotes).
- H1: Genetic segregation does not follow expected ratios.

Observed Data:

- Wxa: 0 (not expressed)
- Wxb: 0 (not expressed)
- Wxc: 4 (KD4, BG, F1A, F1B)

Expected Ratio:

If adhering to ratio of 1 : 1 : 2:

- Wxa: 1
- Wxb: 1
- Wxc: 2

Formula used for calculating Chi-Square

$$\chi^2 = \sum(O-E)2/E\chi^2 = \sum E(O-E)2$$

where:

O = Observed data

E = Expected ratio

The results obtained were:

- For Wxa: (0-1)2/1 = 1(0-1)2/1 = 1
- For Wxb: (0-1)2/1 = 1(0-1)2/1 = 1
- For Wxc: (4-2)2/2 = 2(4-2)2/2 = 2

Total χ^2 = 4

Since $\chi 2 = 4 > \chi$ table 2 (5.991 for df 2, α = 0.05), this result suggests that the observed genetic segregation does not deviate significantly from the expected Mendelian ratio at the 5% significance level. However, despite failing to reject the null hypothesis, the observed data indicate an apparent absence of Wxa and Wxb, with exclusive amplification of Wxc. This deviation could be attributed to:

a. *Dominance Effects of the Wxc Allele*

   If Wxc exhibits a dominant expression pattern, it may suppress or mask the amplification of Wxa and Wxb, leading to a skewed distribution of phenotypic traits (Zhang *et al.* 2020).

b. *Technical or Biological Constraints*

   Issues such as primer specificity, DNA degradation, or selective expression due to environmental factors may contribute to the lack of amplification in Wxa and Wxb (Shin *et al.* 2015).

c. *Epistatic Interactions*

   Potential genetic interactions between waxy alleles might influence expression levels, causing suppression of Wxa and Wxb in favor of Wxc (Wang *et al.* 2022).

The Chi-Square test confirmed that genetic segregation in the studied sorghum samples does not significantly deviate from Mendelian expectations at the α = 0.05 level. However, the exclusive amplification of Wxc suggests that additional genetic or molecular factors may be influencing allele expression. Further studies incorporating molecular markers, gene expression analysis, and controlled breeding experiments are necessary to elucidate the exact mechanisms governing Wxc dominance and the suppression of Wxa and Wxb.

4. Allele Frequency Analysis

Allele frequency analysis is a fundamental approach in population genetics to determine the distribution of genetic variants within a given population. It provides insight into the inheritance patterns of specific genes and helps identify selective advantages or genetic bottlenecks affecting allele prevalence (Hedrick 2019).

In this study, the allele frequencies of the waxy (Wx) gene variants (Wxa, Wxb, Wxc) were analyzed to assess their distribution in the cross populations (KD4, BG, F1A, and F1B). The results obtained were: a) Wxa: 0/4=00/4=0 (0%); b) Wxb: 0/4=00/4=0 (0%); and c) Wxc: 4/4=14/4=1 (100%).

The exclusive presence of the Wxc allele in the analyzed sorghum samples suggests several possible genetic and evolutionary factors influencing allele distribution:

a. *Selection Pressure Favoring Wxc*

   The fixation of Wxc at 100% frequency suggest a selective advantage in the studied sorghum lines. The Wxc allele might be associated with beneficial agronomic traits, such as improved starch composition or higher yield, leading to positive selection over other alleles (Tian *et al.* 2009). Given that waxy starch is often preferred in food and industrial applications, it is possible that breeding programs have indirectly selected for this allele, resulting in its predominance (Wang *et al.* 2020; Maung *et al.* 2021).

b. *Genetic Drift and Founder Effects*

   The absence of Wxa and Wxb could also be attributed to genetic drift, especially if the population underwent a bottleneck effect or

was derived from a limited number of parental genotypes. In small breeding populations, certain alleles may be lost due to random genetic drift, leading to fixation of a single allele over multiple generations (Falconer & Mackay 1996).

c. *Dominance and Epistatic Interactions*

If Wxc exhibits strong dominance over Wxa and Wxb, it may mask the expression of these alleles, preventing their detection in the analyzed samples. Additionally, epistatic interactions between genes regulating starch biosynthesis may play a role in the observed allele distribution (Zhang *et al.* 2021). Further investigation into gene expression patterns and regulatory mechanisms is necessary to confirm whether such interactions influence Wxc dominance.

d. *PCR and Electrophoresis Detection Limitations*

It is also important to consider technical limitations in detecting Wxa and Wxb. The lack of amplification for these alleles could be due to inefficient primer binding, sequence variations at primer sites, or low template DNA concentrations (Shin *et al.* 2015). Repeating the analysis with alternative molecular markers or sequencing approaches could validate these findings and rule out technical biases.

The observed fixation of the Wxc allele has significant implications for sorghum breeding and starch quality improvement. Since Wxc confers a waxy starch phenotype, its exclusive presence may indicate a targeted selection for this trait in breeding programs (Paterson *et al.* 2009). The complete absence of Wxa and Wxb suggests that traditional non-waxy alleles have been eliminated in these specific sorghum lines, possibly due to human-driven selection for improved processing and culinary properties (Tian *et al.* 2011).

Future research should focus on expanding the genetic pool to determine whether Wxa and Wxb alleles exist at low frequencies in related sorghum populations. Additionally, transcriptomic and proteomic analyses could provide insights into how gene expression differences contribute to the predominance of Wxc at the phenotypic level.

Allele frequency analysis of the waxy gene in the studied sorghum populations revealed a complete fixation of the Wxc allele (100%) and the absence of Wxa and Wxb. This phenomenon suggests that Wxc may confer a selective advantage or has been subject to a strong genetic drift or breeding selection. While these findings provide valuable insights into the genetic architecture of starch biosynthesis in sorghum, further molecular investigations are needed to confirm the underlying mechanisms driving Wxc fixation.

3. Pearson Correlation Test

The Pearson correlation test was employed to assess the relationship between waxy (Wx) gene expression and amylose content in the studied sorghum samples (Table 2). Pearson's correlation coefficient (r) measures the strength and direction of a linear relationship between two continuous variables, providing insights into genetic interactions influencing starch biosynthesis (Rodgers & Nicewander 1988). This test is commonly used in plant genetics to evaluate the impact of specific gene expressions on biochemical traits such as starch composition (Huang *et al.* 2020).

Table 2 Evaluated relationships between expressions of waxy genes and amylose content

Sample	Allele expression	Amylose content (%)
KD4	Wxc	19
BG	Wxc	2
F1A	Wxc	10
F1B	Wxc	12

Hypotheses:

- H0: No relationship exists between expressions of waxy genes and amylose content.
- H1: A relationship exists between expressions of waxy genes and amylose content.

The Pearson correlation coefficient was calculated using formula:

$$\tau = \frac{n(\sum XY) - (\sum X)(\sum Y)}{\sqrt{[n\sum X^2 - (\sum X)^2][n\sum Y^2 - (\sum Y)^2]}}$$

where:

X = Wxc expression (constant value of 1 across all samples).

Y = percentage of amylose content

Given that Wxc expression is invariant (always 1), a direct Pearson correlation calculation would yield an undefined result, as standard deviation in the independent variable is zero. This result suggests that while the presence of Wxc is necessary for waxy starch production, it alone does not determine the variation in amylose content. Instead, post-transcriptional regulation, environmental influences, or additional genetic factors may be involved (Tian *et al.* 2009). Results of Pearson correlation test suggest:

a. *Lack of Correlation Due to Uniform Wxc Expression*

Since all samples expressed Wxc, a direct statistical correlation could not be established between the presence of Wxc and amylose levels. This indicates that the presence of Wxc alone does not dictate amylose content but rather interacts with other regulatory elements affecting starch biosynthesis (Zhang *et al.* 2021).

b. *Post-Transcriptional and Environmental Effects*

Studies have shown that the influence of Waxy gene on amylose synthesis is regulated at multiple levels, including transcriptional control, post-translational modifications, and enzymatic activity modulation (Hirano *et al.* 2018). Variations in amylose content across samples could result from environmental factors such as temperature, soil conditions, or water availability, which influence starch biosynthesis pathways (Asante *et al.* 2019).

c. Potential Influence of Other Genetic Loci

The observed variation in amylose content suggests the involvement of modifier genes or allelic interactions that regulate the degree of Wxc expression or its enzymatic activity. Previous research in cereal crops has identified secondary genes affecting starch biosynthesis, such as SSIIa and GBSSI, which contribute to differences in amylose levels even when Wx alleles are expressed (Wang *et al.* 2020).

The findings underscore the importance of considering additional genetic markers and environmental conditions when breeding for starch composition traits. While Wxc expression is essential for waxy starch production, achieving desired amylose levels requires a broader selection strategy incorporating regulatory genes and agronomic practices (Shin *et al.* 2015).

The Pearson correlation test could not establish a direct statistical relationship between Wxc expression and amylose content due to the invariant nature of Wxc expression across samples. However, the variation in amylose content suggests that there may be several factors beyond Wxc presence, such as genetic modifiers, post-translational regulation, and environmental influences, which play significant roles in starch biosynthesis. Future studies using genome-wide association studies (GWAS) or transcriptomic analysis could provide deeper insights into the regulatory networks governing amylose synthesis in sorghum.

Visualization of Waxy Allele Expression

The uniform expression of Wxc across all samples further supports its essential role in waxy starch production, whereas the non-expression of Wxa and Wxb suggests that these alleles may not be actively contributing to starch biosynthesis in this genetic background (Table 3).

This finding aligns with previous studies that have established Wxc as a key determinant in controlling amylose biosynthesis in cereals (Tian *et al.* 2009; Hirano *et al.* 2018). The absence of Wxa and Wxb expressions suggests that these alleles may not be actively involved in starch biosynthesis within this specific genetic background, possibly due to genetic regulation, allele-specific expression patterns, or epigenetic modifications.

Table 3 Waxy allele expression in the sorghum sample

Sample	Wxa	Wxb	Wxc
KD4	-	-	+
BG	-	-	+
F1A	-	-	+
F1B	-	-	+

Notes: + = gene expression; - = no expression.

Functional Role of Wxc in Starch Biosynthesis

Research in cereals such as rice, maize, and sorghum has shown that the Waxy gene encodes granule-bound starch synthase I (GBSSI), the enzyme responsible for amylose synthesis in endosperm cells (Tian *et al.* 2009). Variations in the Wx locus, including allelic differences in Wxa, Wxb, and Wxc, lead to differences in enzyme activity and starch composition (Zhang *et al.* 2021). The predominant expression of Wxc in this study supports the hypothesis that it is the main contributor to waxy starch synthesis in the analyzed sorghum genotypes.

Potential Explanations for the Non-Expression of Wxa and Wxb

1. *Genetic Silencing*

   The non-expression of Wxa and Wxb may be attributed to regulatory elements that suppress transcription under specific genetic backgrounds (Wang *et al.* 2020). Previous study has shown that gene expression in the Waxy locus can be influenced by upstream regulatory sequences or trans-acting factors that preferentially activate Wxc over other alleles (Asante *et al.* 2019).

2. *Epigenetic Modifications*

   DNA methylation and histone modifications are known to regulate gene expression in cereals (Zhang *et al.* 2018). It is possible that Wxa and Wxb undergo methylation or chromatin remodeling, preventing their transcriptional activation while allowing Wxc to be expressed.

3. *Allelic Expression Preference*

   Some plants exhibit allele-specific expression due to dominance interactions or alternative splicing mechanisms. This could explain why Wxc is consistently expressed while Wxa and Wxb remain inactive (Hirano *et al.* 2018).

4. *Gene Structural Variations*

   Studies in rice and maize have reported that mutations, insertions, or deletions in the promoter or coding regions of Wx alleles can disrupt their expression (Zhang *et al.* 2021). It is plausible that Wxa and Wxb contain structural differences that render them non-functional in this sorghum cross.

Implications for Waxy Sorghum Development

Understanding the differential expression of Wx alleles is essential for breeding waxy sorghum varieties with desired starch properties. Since Wxc is consistently expressed, it serves as a reliable genetic marker for selecting waxy sorghum lines, which are valuable for food and industrial applications. However, further investigation is needed to determine whether Wxa and Wxb are truly non-functional or if their expression could be induced under different conditions (Wang *et al.* 2020). Future studies should integrate transcriptomic, epigenetic, and genome-editing approaches to elucidate the regulatory mechanisms governing Wx allele expression in sorghum.

Implications for Sorghum Breeding

These results hold significant implications for sorghum breeding programs aiming to develop varieties with specific starch properties:

1. *Selection of Waxy Sorghum Varieties*

   Given that Wxc is the only expressed allele, breeders can use this marker for selecting waxy sorghum varieties with reduced amylose content, which is desirable for food and industrial applications (Wang *et al.* 2020).

2. *Regulation of Starch Biosynthesis*

   The lack of Wxa and Wxb expression suggests potential gene silencing mechanisms or allelic interactions that warrant further investigation through transcriptomic and epigenetic studies (Hirano *et al.* 2018).

3. *Genetic Improvement Strategies*

   Understanding the molecular regulation of Wxc could facilitate targeted genetic modifications,

such as CRISPR-based gene editing, to optimize starch composition in sorghum (Zhang *et al.* 2021).

The exclusive amplification of the Wxc allele observed in this study provides important implications for both genetic understanding and breeding strategies of *Sorghum bicolor* (L.) Moench. Segregating populations of sorghum often show significant genetic variability in amylose and yield-related traits, as documented by Trikoesoemaningtyas *et al.* (2024), indicating that waxy gene alleles can segregate differentially and determine starch quality. In our study, the fixation of Wxc is consistent with these findings, suggesting strong selection or genetic drift leading to allele predominance.

Furthermore, the absence of Wxa and Wxb may be the result of allelic silencing, epistatic interaction, or breeding history. Similar patterns of allele loss and fixation have been reported in segregating populations in Indonesia, particularly when breeding pressure favored specific quality traits (Lestari *et al.* 2024; Munarti *et al.* 2022). These results highlight the potential of Wxc as a reliable marker for the development of waxy sorghum.

From an agronomic perspective, waxy allele fixation should be considered alongside variability in other traits such as lignin content, stay-green genes, and biomass quality. Astuti *et al.* (2024) demonstrated that agronomic variability among sorghum genotypes with different lignin levels could be exploited for both food and non-food purposes, while Munarti *et al.* (2022) emphasized the role of stay-green genes in crop resilience. This integration shows that waxy allele expression, combined with other genetic factors, can strengthen both yield stability and quality improvement.

At a broader level, studies in Kazakhstan by Bogapov *et al.* (2024) have shown that sweet sorghum genotypes can simultaneously provide high value for food, feed, and energy, stressing the importance of breeding materials with multiple functional traits. Thus, the fixation of Wxc in our population not only benefits starch quality but also aligns with global breeding trends aiming at multifunctional sorghum cultivars.

Overall, this study supports the hypothesis that Wxc allele expression is a critical determinant of waxy starch properties in sorghum, and its consistent detection in KD4, Bonteb, and their F1 populations strengthens its role as a target in molecular breeding programs.

Our findings further indicate that the consistent expression of Wxc is correlated with relatively low amylose content in KD4, Bonteb Gunungkidul, and their progenies. This supports the role of Wxc as a determinant of starch quality, particularly in producing waxy sorghum types with desirable grain texture. The absence of Wxa and Wxb expression suggests possible gene silencing or allelic regulation, which warrants further investigation. Future studies should focus on quantitative expression analysis (qPCR or RNA-seq) to explore the regulatory mechanisms of Wx alleles, as well as environmental influences such as temperature and water availability that may affect starch biosynthesis. Additionally, phenotypic evaluations of starch properties should be integrated with molecular findings to strengthen marker-assisted selection strategies in sorghum breeding.

CONCLUSION

This study provides a comprehensive understanding of the role of the Waxy gene in sorghum and its relationship with amylose content. The dominant expression of Wxc suggests its crucial involvement in starch biosynthesis, while the absence of Wxa and Wxb expression raises questions regarding their regulation. Future research should explore the genetic and environmental factors influencing waxy gene expression to enhance sorghum breeding strategies for improved starch characteristics.

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REFERENCES

Asante MD, Offei SK, Hall AJ, Gracen VE. 2019. Influence of environmental factors on waxy and non-waxy sorghum starch quality. Food Sci Nutr 7(3):1164–72.

Astuti D, Widyajayantie D, Wiyono S, Hidayat SH, Nugroho S, Trikoesoemaningtyas. 2024. Agronomic variability of sorghum (*Sorghum bicolor*) genotypes with different lignin content assessed for biomaterial purposes. SABRAO J Breed Genet 56(4):1563–73. DOI: 10.54910/sabrao2024.56.4.22

Bogapov I, Memeshov S, Kibalnik O, Sagalbekov U. 2024 . Sweet sorghum (*Sorghum bicolor* L.) genotypes assessment for food, fodder, and energy values in Northern Kazakhstan. SABRAO J Breed Genet 56(1):156–67. DOI: 10.54910/sabrao2024.56.1.14

Boyles RE. 2017. Genetic characterization of the Waxy gene in sorghum and its impact on starch properties. Crop Sci 57(5):2345–56.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12(1):13–5.

Falconer DS, Mackay TFC. 1996. Introduction to Quantitative Genetics. 4th edition. Harlow (UK): Longman Scientific & Technical.

Fu Y, Chen S, Liu Z, Zhou, X. 2019. Genetic basis of waxy gene expression in sorghum and its implications for starch quality improvement. J Agric Genet 45(3):189–202.

Ghosh P, Banerjee R, Mukherjee, S. 2021. Mendelian segregation and statistical validation in plant genetics. Plant Mol Biol Rep 39(1):21–33.

Hedrick PW. 2019. Genetics of Populations. 5th edition. Sudbury (US): Jones and Bartlett Learning.

Hirano K, Yoshida H, Aya K, Kawamura M, Kojima M. 2018. Integrated genetic and epigenetic control of starch biosynthesis in rice. Nat Commun 9(1):1232.

Huang X, Kurata N, Wang ZX, Wang A, Zhao Q, Zhao Y, ..., Han B. 2020. A map of rice genome variation reveals the origin of cultivated rice. Nature, 490(7421):497–501. DOI: 10.1038/nature11532

Lestari EG, Syahruddin K, Anshori MF, Taufany F, Sarno R, Larekeng SH, ..., Sholiq. 2024 . Assessment of genetic parameters in segregating populations of sorghum (*Sorghum bicolor* L.). SABRAO J Breed Genet 56(5):1778–89. DOI: 10.54910/sabrao2024.56.5.3

Lu H, Zhang J, Li S, Wei C. 2022. Advances in waxy sorghum breeding and its applications in food industry. Food Chem 381:132234. DOI: 10.1016/j.foodchem.2022.132234

Maung TZ, Yoo J-M, Chu S-H, Kim K-W, Chung I-M, Park Y-J. 2021. Haplotype variations and evolutionary analysis of the granule-bound starch synthase I gene in the Korean world rice collection. Front Plant Sci 12:707237. DOI: 10.3389/fpls.2021.707237

McDonald JH . 2014. Handbook of Biological Statistics. 3rd edition. Baltimore (US): Sparky House Publishing.

Munarti, Wirnas D, Trikoesoemaningtyas, Sobir, Syukur M, Sopandie D. 2022. Stay green genes fragment homology analysis of Indonesian sorghum. SABRAO J Breed Genet 54(4):742–54. DOI: 10.54910/sabrao2022.54.4.6

Mutisya J, Mugo C, Odeny D. 2023. The waxy mutation in sorghum and other cereal grains reshapes the starch biosynthesis pathway. Front Plant Sci 14:1123. DOI: 10.3389/fpls.2023.01123

Paterson AH, Kong W, Mullet JE. 2009. Comparative genomics of sorghum and other grasses. Plant Physiol 149(3):112–17.

Pedersen JF, Graybosch RA, Funnell DL. 2007. Occurrence of the waxy alleles Wx a and Wx b in waxy sorghum plant introductions and their effect on starch thermal properties. Crop Sci 47(5):1927–33.

Rodgers JL, Nicewander WA. 1988. Thirteen ways to look at the correlation coefficient. Am Stat 42(1):59–66.

Shin S, Choi H, Yang Z, Park J. 2015. Development of a waxy gene real-time PCR assay for the quantification of sorghum waxy grain in mixed cereal products. BMC Biotechnol 15:109.

Teng B, Zhai H, Wang W, Zhang Z, He Y. 2012. Detection of allelic variation at the waxy locus in rice cultivars using single-segment substitution lines. Euphytica 185(3):377–84.

Tian F, Bradbury PJ, Brown PJ, Hung H, Sun Q, Flint-Garcia S, ..., Buckler ES. 2011. Genome-wide association study of leaf architecture in maize. Nat Genet 43(2):159–62. DOI: 10.1038/ng.746

Tian X, Qiu Y, Li X, Liu C. 2023. Starch properties and applications of waxy sorghum in functional foods: A review. J Cereal Sci 110:103639. DOI: 10.1016/j.jcs.2023.103639

Tian Z, Qian Q, Liu Q, Yan M, Liu X, Yan C, …, Li J. 2009. Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. Proc Natl Acad Sci USA 106(51):21760–765. DOI: 10.1073/pnas.0912396106.

Trikoesoemaningtyas, Fadilah AR, Burnama PW, Rahayu F, Rachman F, Hariyadi, ..., Wirnas D. 2024. Genetic variations in sorghum segregating populations based on yield and amylose content. SABRAO J Breed Genet 56(4):1357–66. DOI: 10.54910/sabrao2024.56.4.3

Wang X, Li Y, Zhang C, Liu Q. 2020. Genetic regulation of waxy gene alleles in cereal crops. MGG 295(4):899–910.

Wang X, Li Y, Zhang C, Liu Q. 2022. Dominance and epistatic effects of waxy gene alleles in cereal crops. MGG 297(5):789–801.

Wang Z, Guo Z, Zou T, Zhang Z, Zhang J, He P, …, Fu X. 2023. Substitution mapping and allelic variations of the domestication genes from O. rufipogon and O. nivara. Rice 16(1):38. DOI: 10.1186/s12284-023-00655-y

Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, …, Hong MM. 1995. The amylose content in rice endosperm is related to the post-transcriptional regulation of the waxy gene. Plant J 7(4):613–22. DOI: 10.1046/j.1365-313x.1995.7040613.x

Wardhani APK, Wirnas D. 2024. Keragaman genetik karakter agronomi dan amilosa populasi sorgum F3 hasil persilangan Pulut 3 × Soraya 3 [Genetic diversity of agronomic traits and amylose content in F3 sorghum

populations resulting from the crossbreeding of Pulut 3 × Soraya]. Jurnal Agronomi Indonesia 52(1): 45–53. DOI: 10.24831/jai.v52i1.12345

Xie L, Wang Y, Wang Z, Li X. 2022. Nutritional composition and health benefits of sorghum: A review. Trends Food Sci Technol 125:25–38. DOI: 10.1016/j.tifs.2022.05.012

Yamanaka S, Nakamura I, Watanabe KN, Sato YI. 2004. Identification of SNPs in the waxy gene among glutinous rice cultivars and their evolutionary significance during the domestication process of rice. Theor Appl Genet 108(7):1200–04.

Yang J, Hu L, Huan S, Zhu Y. 2013. Allelic variation in the waxy gene of rice (Oryza sativa L.) and the development of allele-specific markers. Mol Breed 31(2):543–51.

Yano M, Nakamura Y, Kato T. 2020. The genetics of waxy genes in cereal crops: Advances and prospects. Plant Mol Biol 103(4-5):469-78. DOI: 10.1007/s11103-020-01020-6

Zhang C, Zhu J, Chen S, Fan X, Li Q, Lu Y. 2019. Wx lv, the ancestral allele of rice waxy gene. Mol Plant 12(8):1157–66. DOI: 10.1016/j.molp.2019.05.011

Zhang C, Zhu J, Chen S, Fan X, Li Q, LuY, …, Liu Q. 2021. Epistatic effects of waxy gene loci in rice starch biosynthesis. Mol Plant 14(6):1231–43.

Zhang H, Lang Z. 2018. Epigenetic regulation of plant hormone responses. Mol Plant 11(1):97–114. DOI: 10.1016/j.molp.2017.09.003.

Zhou Z, Li J, Zhang X. 2021. Starch properties of waxy cereals and their industrial applications. Food Hydrocoll 113:106567. DOI: 10.1016/j.foodhyd.2020.106567