

Identification and candidate gene screening of qHC1.2 , a Major QTL associated with hull color in foxtail millet (*Setaria italica* L.)

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Abstract

Hull color is a crucial characteristic that helps assess the nutritional value and economic potential of foxtail millet. However, the reports on quantitative trait locus (QTL) mapping and map-based cloning for hull color are limited. Here, we mapped QTLs responsible for hull color by using 215 recombinant inbred lines (RILs) derived from Yugu 18 (Light yellow hull) × Hongjiugu (Red hull) and a high-density bin map. A total of 36 QTLs for hull color were detected in all four environments by four phenotypic evaluation methods. Among these QTLs, a major QTL for hull color (HC) named qHC1.2 was repeatedly mapped on chromosome 1 and explained 8.89 – 69.63% of the phenotypic variation. In addition, RNA sequencing (RNA-seq) was performed 7, 14, and 21 days after flowering for the YRRIL-145 and YRRIL-229, and three differentially expressed genes (DEGs) were detected in the candidate region. The qRT-PCR results showed the same expression patterns as the RNA-seq data. Among them, DEGs, only one gene, *Seita.1G057300*, encoding a cinnamyl alcohol dehydrogenase (CAD), was located in the candidate region of qHC1.2. Furthermore, sequence analysis revealed One SNP (A to G), located at the third exon, resulted in an amino acid change from isoleucine to valine in YRRIL-145 compared with YRRIL-229. Our results provide a foundation for further cloning of qHC1.2 and will be very useful in clarifying the regulatory mechanism for hull color synthesis in foxtail millet.

Key message

A major QTL, *qHC1.2*, that controls hull color was identified on chromosome 1 in foxtail millet, and based on RNA-seq data, *Seita.1G057300* was the most promising candidate gene.

Introduction

Foxtail millet (*Setaria italica* (L.) Beauv.) originated in China, and domestication and cultivation can be traced back to 11,500 years (Jia 2017). As an important food crop and feed crop, foxtail millet has high nutritional value, rich in a variety of vitamins, proteins, fats, carbohydrates, calcium, several minerals, phosphorus, iron, and other essential nutrients for the human body (Gao et al. 2022; Sachdev et al. 2021; Yadav et al. 2022; Zou et al. 2019). Moreover, with strong adaptability and drought resistance, foxtail millet is widely planted in arid and semi-arid regions of the world. It is an essential crop for coping with water resources shortage, food security, and developing water-saving agriculture in the future (Gao et al. 2022; Liu et al. 2011). Therefore, the promotion of foxtail millet cultivation plays a significant role in enhancing the dietary patterns of residents, reducing the strain on agricultural water resources in dry regions, and bolstering the economic gains of farmers in production zones.

In foxtail millet, the hull color is the most crucial aspect of its appearance and quality. Selection of high-nutrition varieties can be indirectly made through the observation of hull color (He et al. 2002; YANG et al. 2017). Foxtail millet varieties with different hull colors not only have large differences in nutrient content but also have different flavonoids (He et al. 2002; Winkel-Shirley 2001; Zhang et al. 2021). Flavonoids can enhance the ability of plants to resist adversity and improve the adaptability of foxtail millet to the external environment (Emiliani et al. 2013; Nakabayashi et al. 2014a; Nakabayashi et al. 2014b). Previous studies have shown that the alteration of rice hull color is impacted by flavonoids and lignin metabolism. At present, there are 8 rice hull color genes were cloned, including *GH2* (Zhang et al. 2006), *RBH1* (Wang et al. 2020), *GH1* (Hong et al. 2012), *OsDfr* (Furukawa et al. 2007; Sun et al. 2018), *Os1BGlu4* (Xu et al. 2022), *BH4* (Gu et al. 2005; Zhu et al. 2011), *BH6* (Shao et al. 2012), *BBH* (Yang et al. 2018). *RBH1* encodes a cinnamyl alcohol dehydrogenase (*CAD*) in rice, and *rbh1* mutant causes a reddish-brown color in the hulls due to the total flavonoids and anthocyanin content increased and lignin reduced (Wang et al. 2020). *GH2*, an allele of the *RBH1*, is involved in the lignin biosynthesis pathway, and lignin deficiency causes golden yellow hull (Zhang et al. 2006). *GH1* encodes chalcone isomerase *OsCHI* in rice, which is the only *CHI*, involved in the flavonoid biosynthetic pathway. The *gh1* mutant with loss of *OsCHI* expression causes a reddish-brown hull and internodes due to the total flavonoid content increased in the hull and internodes (Hong et al. 2012). In addition, the *bbh* mutant causes a black-brown hull due to the total flavonoids and anthocyanin content increased (Yang et al. 2018). *OsDfr* gene is involved in the flavonoid biosynthetic pathway and affects hull color in rice (Furukawa et al. 2007; Sun et al. 2018).

In recent years, there have been many related studies on QTL mapping of hull color in foxtail millet. Through linkage analysis and genome-wide association study, QTLs were identified on chromosomes 1, 2, 3, 6, 7, and 9. Additionally, 11 potential candidate genes were identified on chromosomes 1, 6 and 9 (*Seita.1G057300*, *Si1g06530*, *Seita.1G057100*, *Seita.1G059100*, *Seita.1G059300*, *Seita.6G226500*, *Seita.6G226800*, *Seita.6G228300*, *Seita.6G228600*, *Seita.9G515900* and *Seita.9G515900*) (Guo et al. 2023; Jia et al. 2013; Tian et al. 2022; Wang et al. 2017; Xie et al. 2021). *Sihc1* on chromosome 6 has been consistently identified in linkage analysis and genome-wide association studies (Jia et al. 2013; Wang et al. 2017). However, it is a very difficult and time-consuming task to screen the target gene due to the large physical interval of the QTL preliminary mapping (Patil et al. 2017). RNA-seq is a deep-sequencing technology for detecting differentially expressed genes in transcriptome profiling, which has the characteristics of high sensitivity and provides massive amounts of

sequence data(Habib et al. 2018; Li et al. 2022; Xu et al. 2015). Therefore, by combining QTL mapping with RNA-seq data, candidate genes can be screened efficiently and accurately using pathway analysis of differentially expressed genes. (Jiang et al. 2022a; Song et al. 2021). Presently, it is extensively utilized for screening potential genes associated with traits in plants such as brassica napus(Song et al. 2021; Xu et al. 2015), rice(Huang et al. 2021; Lei et al. 2020), tomato(Wen et al. 2019), capsicum(Park et al. 2019), and alfalfa(Jiang et al. 2022b). For example, Huang et al. identified the candidate gene *Os09g0551600* for rice, which controls the callus induction rate in anther culture QTL *qCLR9.1* through QTL mapping and RNA-seq(Huang et al. 2021). However, there has been no report regarding the combined use of QTL mapping and RNA-seq to identify candidate genes that regulate the color of foxtail millet hull.

In this study, we tried to detect QTLs related to hull color traits using a RIL population (YRRIL) derived from a cross between Yugu 18 and Hongjiugu based on four phenotypic evaluation methods. A total of 36 QTLs associated with hull color was detected. Combining the results of QTL mapping and RNA-seq showed that a total of three genes were screened. A major QTL, *qHC1.2*, controlling hull color was detected on chromosome 1. Gene sequencing and sequence alignment results indicate that *Seita.1G057300* was the most possible functional gene underlying *qHC1.2*. Our findings establish the foundation for additional cloning of hull color genes and aid in clarifying the regulatory mechanism of hull color synthesis in foxtail millet.

Materials and methods

Plant materials

The population, YRRIL, consisting of 215 recombinant inbred lines (RILs) used in this study was derived from a cross between Yugu 18 and Hongjiugu, and a high-density bin map was constructed as previously reported in Guo et al(Guo et al. 2023). Two sites, Yulin (YL, 109°21'E, 37°56'N, Shaanxi Province, China) and Baoji (BJ, 107°37'E, 34°26'N, Shaanxi Province, China), were used for planting and phenotypic evaluation in 2020 and 2021. Two lines, YRRIL-145 and YRRIL-229, were selected from the RIL population for RNA-seq analysis. The YRRIL-145 line exhibits the A genotype for Hongjiugu parent allele in the *qHC1.2* locus. The YRRIL-229 line exhibits the B genotype for Yugu18 parent allele in the *qHC1.2* locus. In the *qHC9.5* locus, YRRIL-145 and YRRIL-229 exhibit the A genotype for Hongjiugu parent allele. In addition, two parent plants, Yugu 18 and Hongjiugu, and eight foxtail millet varieties (four varieties with red hull and four varieties with yellow hull) have been used for *Seita.1G057300* gene sequencing. The plants were cultivated in the greenhouse specifically DNA extraction, and the leaves were collected four weeks after germination. All plant materials were obtained from the College of Agronomy Northwest A&F University.

Phenotypic evaluation

Four color evaluation methods were developed to identify the hull color of the RIL population in four different environments. These approaches are described briefly below.

Method I (MI) involves categorizing observed hull colors based on direct visual inspection and categorizing them as light yellow, yellow, dark yellow, and red, assigning values in turn from one to four (Fig. 1).

Method II (MII), the hull color of nine lines in the RIL population was chosen as the color evaluation criterion (Fig. 2). The color matching of the RIL population to nine colors was done and assigned values based on color ranging from light to dark, in which the darkest color is assigned a value of nine and the lightest color is assigned a value of one.

Method III used was as previously reported(Guo et al. 2023). The hull color of the RIL population was identified by a colorimeter (CS-820N, Hangzhou CHNSpec Technology Co., Ltd., Hangzhou, China).

Method IV, the color in RGB space is composed of three basic colors: red (R), green (G), and blue (B), which can form 256^3 colors. Three color parameters R, G, and B were selected in the colorimeter (CS-820N). The measurement method was the same as in the Method III.

Statistical analysis and QTL mapping

The basic statistics of the hull color of the RILs were calculated by using SPSS 21.0. Origin 8.0 and GraphPad Prism software were used to draw the frequency distribution map and histogram. The ANOVA module of QTL IciMapping v4.2 software was used to analyze the variance of genotype (σ_g^2), environment(σ_e^2), genotype $\times$ environment interaction (σ_{ge}^2), and error (σ_e^2). The broad-sense heritability (H^2) is calculated as: $H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{ge}^2 / n + \sigma_e^2 / nr)$, where n represents the number of environments and r represents the number of repeats in a single environment(Li et al. 2019; Zhang et al. 2021).

QTL analysis for hull color was performed by inclusive composite interval mapping (ICIM) in IciMapping v4.2 based on our previously constructed high-density bin map (Guo et al. 2023). The QTL scanning step length was set as 1 cM, and a logarithm of odds (LOD) threshold of ≥ 2.5 was utilized to identify QTL. The linkage map and QTL position were drawn using MapChart software (Olukolu et al. 2016).

RNA extraction and RNA-seq

At 7 (S1), 14 (S2), 21 days (S3) after flowering, the grains of the two lines (YRRIL-145 and YRRIL-229) were placed in three centrifuge tubes, immediately frozen in liquid nitrogen and stored at -80°C . A total of 17 samples were obtained after removing the samples with a low correlation coefficient between sample repetitions. Transcriptome sequencing analysis was performed by Wuhan GENOSEQ (Wuhan Genoseq Technology Co., Ltd., China). The total RNA in the sample was isolated with a TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Dalian, China). The quality of the RNA samples was validated utilizing the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and additionally confirmed by performing RNase-free agarose gel electrophoresis (Huang et al. 2021). The equivalent quantity of RNA from each sample was combined to construct a cDNA library. The cDNA libraries were sequenced using the Illumina HiSeq 4000 platform, which produced paired-end reads with a length of 150bp. After data quality control, the clean data were aligned to the reference genome sequences of the foxtail millet genome *Setaria italica* v2.2. The differentially expressed genes (DEGs) were identified using DESeq2 in the R software, with an FDR (false discovery rate) 0.05 and a $|\log_2(\text{fold change})| \geq 1$ between two samples (Love et al. 2014; Wang et al. 2021). The DEGs were assessed for their functional enrichment by mapping them to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using phyper in the R software (Schmittgen and Livak 2008).

Quantitative real-time PCR analysis

The three most promising candidate genes, *Seita.1G057300*, *Seita.1G157600*, *Seita.9G034700*, and two other DEGs involved in phenylpropanoid and flavonoid biosynthesis, were selected to validate the reliability and reproducibility of the RNA-seq results by quantitative real-time PCR (qRT-PCR). The primers were designed using Primer5 software, and *ACTIN* was selected as the reference gene. The primers for qRT-PCR analysis are listed in Supplementary Table S1. The PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) was utilized for cDNA synthesis, according to the manufacturer's instructions. TB Green® Premix Ex Taq™ II kit (Takara, Dalian, China) and Q7 real-time PCR system (Applied Biosystems™, America) were used to complete RT-PCR analysis. The relative expression of the gene was calculated by the $2^{-\Delta\Delta\text{CT}}$ method.

DNA extraction, candidate gene sequencing, and sequence alignment

The candidate genes were amplified in the cDNA using predesigned PCR primers in DY and LY. Primers are shown in the Supplementary Table S2. Genomic DNA was extracted from the leaves of parent plants, Yugu 18 and Hongjiugu, and eight foxtail millet varieties (four varieties with red hull and four varieties with yellow hull) using a TaKaRa MiniBEST Plant Genomic DNA Extraction Kit. The candidate gene *Seita.1G057300* in the ten foxtail millet varieties was cloned using polymerase chain reaction (PCR), followed by sequencing and sequence alignment analysis conducted with DNAMAN software. Primers are shown in the Supplementary Table S3. This primer set amplified the complete coding sequence (CDS) region of the *Seita.1G057300* gene.

Results

Phenotypic variations among the parents and the RIL population

The phenotypic characteristics of the parental lines and the YRRIL population were investigated across four environments using four phenotypic evaluation methods. Yugu 18 and Hongjiugu were classified as yellow and red, respectively, using Method I and Method II (Fig. 2). The L^* , b^* , R, G, and B of Yugu18 were significantly greater (t test with $P < 0.05$) than those of Hongjiugu, but a^* of Yugu18 was significantly lower ($P < 0.05$) than those of Hongjiugu using Method III and Method IV (Table 1). Four colors were identified in the RIL population by Method I, while nine colors were identified by Method II (Fig. 1). Method II is a more detailed identification of hull color relative to Method I. Lab color space was utilized in Method III, while RGB color space was employed in Method IV. Each of these separates colors into three different constituents. The MI, MII, L^* , a^* , b^* , R, G, and B values of the YRRIL population displayed a wide range of variation, continuous variation, and significant transgressive segregation, suggesting that the traits were approximately normally distributed (Table 1, Fig. 3).

MI and MII readings for hull color exhibited highly significant negative correlations with L^* , b^* , R, G, and B values, but highly significantly positively correlated with a^* value. The significant correlations among MI and MII for Lab and RGB suggest that there is a common genetic

mechanism controlling these color traits (Table 2, Table S4). The broad-sense heritability for all the traits ranged from 0.76 to 0.97 in all four environments, indicating that genetic factors played an important role in determining hull color (Table 1).

Table 1
Descriptive statistics of the foxtail millet hull color in parents and RIL population in four environments using four methods

Method	Trait	Environment	Parents		RIL population					
			Hongjiugu	Yugu 18	Means $\pm$ SD	Range	Skewness	Kurtness	Variable coefficient (%)	broad-sense heritability
Method I	MI ^a	E1	4.00	1.00	2.48 $\pm$ 1.19	1.00–4.00	-0.08	-1.52	47.81	0.97
		E2	4.00	1.00	2.45 $\pm$ 1.15	1.00–4.00	-0.08	-1.46	46.92	
		E3	4.00	1.00	2.54 $\pm$ 1.19	1.00–4.00	-0.21	-1.49	46.70	
		E4	4.00	1.00	2.40 $\pm$ 1.18	1.00–4.00	0.01	-1.53	49.19	
Method II	MII ^a	E1	8.00	2.00	4.04 $\pm$ 2.55	1.00–9.00	0.53	-0.98	63.16	0.96
		E2	8.00	2.00	4.00 $\pm$ 2.32	1.00–9.00	0.53	-0.80	58.12	
		E3	8.00	2.00	4.69 $\pm$ 2.55	1.00–9.00	0.34	-1.21	54.36	
		E4	8.00	2.00	3.94 $\pm$ 2.23	1.00–9.00	0.50	-0.89	56.72	
Method III	L [*]	E1	53.47 $\pm$ 0.59	62.6 $\pm$ 1.13	58.61 $\pm$ 4.97	49.46–67.21	-0.04	-1.16	8.48	0.92
		E2	54.46 $\pm$ 0.81	64.21 $\pm$ 1.51	59.17 $\pm$ 3.43	52.93–67.57	0.36	-0.60	5.79	
		E3	51.09 $\pm$ 0.19	63.93 $\pm$ 0.53	55.42 $\pm$ 3.41	49.08–62.78	0.19	-0.99	6.15	
		E4	52.9 $\pm$ 1.25	62.32 $\pm$ 0.7	57.73 $\pm$ 3.28	50.58–68.15	0.34	0.03	5.68	
	a [*]	E1	11.06 $\pm$ 1.05	6.06 $\pm$ 0.45	10.00 $\pm$ 3.83	4.77–18.75	0.71	-0.62	38.30	0.92
		E2	7.46 $\pm$ 0.53	5.49 $\pm$ 0.45	7.70 $\pm$ 2.18	2.73–14.44	0.29	-0.30	28.37	
		E3	13.83 $\pm$ 1	6.63 $\pm$ 0.48	7.65 $\pm$ 2.43	3.04–14.04	0.70	-0.15	31.76	
		E4	8.79 $\pm$ 0.29	5.77 $\pm$ 0.41	7.82 $\pm$ 2.39	2.58–14.42	0.34	-0.50	30.54	
	b [*]	E1	17.56 $\pm$ 0.54	23.44 $\pm$ 1.23	20.42 $\pm$ 2.92	12.50–27.35	-0.45	-0.32	14.31	0.83
		E2	12.79 $\pm$ 1.09	22.69 $\pm$ 0.69	18.91 $\pm$ 2.70	12.95–25.24	0.07	-0.75	14.30	
		E3	16.25 $\pm$ 0.61	25.25 $\pm$ 0.36	16.83 $\pm$ 2.45	10.68–22.66	0.06	-0.34	14.57	
		E4	14.05 $\pm$ 1.79	22.11 $\pm$ 0.86	18.43 $\pm$ 2.15	13.65–23.73	0.08	-0.50	11.68	
	R	E1	155.83 $\pm$ 1.34	176 $\pm$ 3.74	169.67 $\pm$ 9.87	150.00–189.33	-0.03	-0.96	5.82	0.87

E1: 2020 in Yulin; E2: 2020 in Baoji; E3: 2021 in Yulin; E4: 2021 in Baoji; ^a Phenotype was measured only once; SD Standard deviation

Method	Trait	Environment	Parents		RIL population					broad-sense heritability
			Hongjiugu	Yugu 18	Means $\pm$ SD	Range	Skewness	Kurtness	Variable coefficient (%)	
		E2	150.78 $\pm$ 2.97	179.22 $\pm$ 3.64	166.96 $\pm$ 8.65	147.67-186.67	0.07	-0.77	5.18	
		E3	153 $\pm$ 1.63	181.33 $\pm$ 1.25	155.70 $\pm$ 7.49	138.00-173.67	0.24	-0.75	4.81	
		E4	149.33 $\pm$ 4.5	174 $\pm$ 2.4	162.98 $\pm$ 7.29	146.00-188.33	0.47	0.35	4.47	
G	E1		119.67 $\pm$ 1.97	145.89 $\pm$ 3	133.38 $\pm$ 14.90	106.67-158.67	-0.09	-1.17	11.17	0.93
	E2		124.67 $\pm$ 1.94	150.44 $\pm$ 4.03	136.30 $\pm$ 9.71	118.33-161.33	0.39	-0.58	7.13	
	E3		111.67 $\pm$ 0.94	149 $\pm$ 1.63	126.81 $\pm$ 9.83	107.00-147.00	0.11	-0.98	7.75	
	E4		120 $\pm$ 2.83	145.56 $\pm$ 1.95	132.54 $\pm$ 9.56	111.67-161.67	0.27	-0.15	7.21	
B	E1		97.5 $\pm$ 1.12	109.56 $\pm$ 2.11	105.32 $\pm$ 9.30	91.00-126.33	0.52	-0.98	8.83	0.92
	E2		108.11 $\pm$ 2.28	115.11 $\pm$ 3.75	109.24 $\pm$ 6.76	96.00-128.00	0.54	-0.50	6.19	
	E3		94 $\pm$ 0.82	110 $\pm$ 1.41	103.55 $\pm$ 5.86	94.00-118.00	0.58	-0.61	5.66	
	E4		102.33 $\pm$ 0.47	111.22 $\pm$ 1.81	106.51 $\pm$ 6.50	96.00-125.33	0.68	-0.36	6.10	
E1: 2020 in Yulin; E2: 2020 in Baoji; E3: 2021 in Yulin; E4: 2021 in Baoji; ^a Phenotype was measured only once; SD Standard deviation										

Table 2
Correlation coefficients between eight values of hull color in YRRIL populations in E1

Traits	MI	MII	L*	a*	b*	R	G
MII	0.929**						
L*	-0.923**	-0.910**					
a*	0.898**	0.913**	-0.886**				
b*	-0.602**	-0.692**	0.712**	-0.587**			
R	-0.825**	-0.810**	0.954**	-0.713**	0.772**		
G	-0.934**	-0.923**	0.997**	-0.918**	0.695**	0.928**	
B	-0.879**	-0.810**	0.921**	-0.835**	0.382**	0.829**	0.925**
*, **P < 0.05, and 0.01, respectively							

QTL mapping for hull color

A total of 36 QTLs were detected by the four phenotypic evaluation methods, distributed on 9 chromosomes (Table 3). Among these QTLs, 13 QTLs related to hull color distributed on chromosomes 1, 3, 4, 5, 8, and 9 were repeatedly mapped in multiple environments that explained 0.95%-69.63% of the phenotypic variation, including 4 major QTLs ($R^2 \geq 10\%$) and 9 minor QTLs (Table 3, Table S5, Fig. 4).

The major QTLs *qHC1.1*, *qHC1.2*, *qHC9.1*, and *qHC9.5* were repeatedly detected in multiple environments by two, four, two, and four methods, respectively. *qHC1.1* was mapped to chromosome 1, where it was flanked by the markers c01b028 and c01b030 (31.5-33.5cM). Its LOD score ranged from 3.90 (C^* value) to 17.80 (b^* value), explaining 4.59% (C^* value) to 21.33% (b^* value) of the phenotypic variations. *qHC1.2* was mapped to chromosome 1, where it was flanked by the markers c01b037 and c01b040 (35.5-39.5cM). Its LOD score ranged from 10.92 (b^* value) to 81.65 (G value), explaining 8.89% (b^* value) to 69.63% (MI value) of the phenotypic variations. *qHC9.1* was mapped to chromosome 9, where it was flanked by the markers c09b008 and c09b017 (6.5-16.5cM). Its LOD score ranged from 3.58 (MII value) to 18.78 (b^* value), explaining 1.37% (MII value) to 19.67% (R value) of the phenotypic variations. *qHC9.5* was mapped to chromosome 9, where it was flanked by the markers c09b174 and c09b176 (168.5-172.5cM). Its LOD score ranged from 3.75 (R value) to 38.78 (G value), explaining 4.43% (C^* value) to 32.08% (MII value) of the phenotypic variations. In addition, the minor QTLs *qHC1.3*, *qHC3.3*, *qHC3.4*, *qHC3.6*, *qHC4.4*, *qHC5.2*, *qHC8.2*, *qHC8.3*, *qHC9.4* was repeatedly detected in multiple environments. Their LOD score ranged from 2.54 (*qHC8.2*) to 7.57 (*qHC1.3*), explaining 1.09% (*qHC1.3*) to 9.36% (*qHC3.3*) of the phenotypic variations.

Table 3
Summary of stable QTL for hull color identified in YRRIL population

QTL	Chromosome	Marker interval	Genetic position	physical position	LOD	R ²
			(cM)	(bp)		(%)
<i>qHC1.1</i>	1	c01b028-c01b030	31.5–33.5	4087036–4549167	3.90–17.80	4.59%-21.33%
<i>qHC1.2</i>	1	c01b037-c01b040	35.5–39.5	5254895–5690175	10.92–81.65	8.89%-69.63%
<i>qHC1.3</i>	1	c01b069-c01b074	66.5–72.5	21359082–25381735	3.25–7.57	1.09%-4.48%
<i>qHC3.3</i>	3	c03b034-c03b040	33.5–36.5	6303987–6747742	2.88–6.02	2.89%-9.36%
<i>qHC3.4</i>	3	c03b050-c03b052	42.5–44.5	8452554–9199812	3.68–5.94	1.20%-3.41%
<i>qHC3.6</i>	3	c03b117-c03b120	99.5-101.5	44893212–45500825	3.33–5.15	3.45%-7.97%
<i>qHC4.4</i>	4	c04b154-c04b160	124.5–128	39426935–39802274	2.83–5.25	5.47%-7.66%
<i>qHC5.2</i>	5	c05b004-c05b005	2.5–5.5	1918938–2114343	4.23–4.72	3.57%-6.90%
<i>qHC8.2</i>	8	c08b038-c08b047	26.5–31.5	5603776–7336883	2.54–6.82	1.89%-5.52%
<i>qHC8.3</i>	8	c08b091-c08b093	49.5–52.5	25069208–26168380	3.45–3.61	3.18%-3.21%
<i>qHC9.1</i>	9	c09b008-c09b017	6.5–16.5	988753–2049720	3.58–18.78	1.37%-19.67%
<i>qHC9.4</i>	9	c09b164-c09b167	157.5-160.5	49027777–49697847	3.24–7.15	3.08%-4.87%
<i>qHC9.5</i>	9	c09b174-c09b176	168.5-172.5	53717341–54694792	3.75–38.78	4.43%-32.08%

DEGs between YRRIL-145 and YRRIL-229

To reduce interference from unrelated genes and the complexity of multiple major QTLs interval genes, *qHC1.2* was selected as an important QTL interval for RNA-Seq analysis. Two lines, YRRIL-145 and YRRIL-229, were selected from the RIL population for RNA-seq analysis. YRRIL-145 mature stage hull color was dark yellow (DY), and YRRIL-229 mature stage hull color was light yellow (LY). The hull color of both DY and LY underwent a gradual darkening over the course of three developmental stages spanning from 7 to 21 days. Furthermore, the hull color of DY and LY showed different colors at each stage (Fig. 5). A total of 199.68 Gb of clean data was obtained, with an average of 11.09 Gb per sample (Table S6). Using HISAT2 to compare clean data with the *Setaria italica* v2.2 reference genome, an average of 85.68% of reads can be compared to the reference genome (Table S6). More than 94.52% of clean reads were concentrated in the exon region of the gene, indicating that the sequencing results were consistent with RNA-Seq analysis (Table S7). The Pearson correlation coefficient was utilized to assess the biological repeatability and correlation between samples. A total of 17 samples were obtained after removing the samples with a low correlation coefficient between sample repetitions (Figure S1). PCA analysis was conducted to reduce the dimensions of 17 DY and LY samples. The results revealed six distinct clusters with noticeable variation between

DY and LY samples, but minimal differences were observed between biological replicates (Figure S2). Thus, the quality of the sample is dependable and suitable for future analysis.

The differentially expressed genes (DEGs) of hull color between DY and LY at different developmental stages (S1-S3) were screened by DESeq2. Using DY as the control group and with an FDR (false discovery rate) 0.05 and a $|\log_2(\text{fold change})| \geq 1$ between two samples as the standard of DEGs. A total of 3183 DEGs were obtained in the three periods, of which 122 DEGs were detected at all three stages (Figure S3). The up-regulated genes exceeded the down-regulated genes at each stage, which were 432:295, 612:428, and 1291:793 respectively (Fig. 6a).

Comprehensive analysis of the genes involved in phenylpropanoid biosynthesis and flavonoid biosynthesis

To functionally categorize the DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed on 3183 DEGs by hypergeometric distribution phyper in the R software. The results of the KEGG analysis showed that a total of 114 KEGG pathways were enriched. As expected, DEGs with a relatively large number were found to be involved in phenylpropanoid biosynthesis, and flavonoid biosynthesis, known components of the pathways related to hull color (Table S8, Fig. 6b). Furthermore, the KEGG analysis of the DEGs between the three stages of DY and LY revealed consistent findings (Table S9, Table S10, Fig. 6c, Fig. 6d).

Compared with DY, 43 and 11 DEGs were involved in the biosynthesis of phenylpropanoid and flavonoid in the hull of LY, respectively. The genes regulating the phenylpropanoid biosynthesis pathway include *PAL*, *PTAL*, *C4H*, *4CL*, *COMT*, *CCR*, *CAD*, *E1.11.1.7* (Table S11, Fig. 7). We studied the expression levels of these genes, most of which showed significant differences at S2-S3. It is noteworthy that the *CAD* gene (*Seita.1G057300*) was significantly up-regulated at three developmental stages (S1-S3). The *PAL* gene (*Seita.1G240500*, *Seita.1G240600*) was significantly down-regulated at S3. The *PTAL* gene (*Seita.2G435800*) was significantly up-regulated at S1. *C4H* genes (*Seita.1G157600*, *Seita.5G361200*) were significantly regulated at different developmental stages. *Seita.1G157600* was significantly down-regulated at S1 and *Seita.5G361200* was significantly up-regulated at S2. The *4CL* genes (*Seita.1G283300*, *Seita.9G306300*) were significantly regulated at different developmental stages. *Seita.1G283300* was significantly up-regulated at S3 and *Seita.9G306300* was significantly down-regulated at S1. The gene regulating *COMT* (*Seita.8G126500*) was significantly up-regulated at S3. The genes regulating *CCR* (*Seita.1G360800*, *Seita.1G361000*) were significantly up-regulated at S2. Most of the genes regulating *E1.11.1.7* were significantly up-regulated or down-regulated at S3. The genes involved in the regulation of the flavonoid biosynthesis pathway include *CHS*, *CHI*, *CYP75B1*, and *ANR*. Most of the genes showed significant differences at S3. The *CHS* gene (*Seita.8G140200*), *CHI* gene (*Seita.9G034700*), *CYP75B1* gene (*Seita.9G244600*), *ANR* gene (*Seita.7G248900*, *Seita.7G249200*, *Seita.7G249500*, *Seita.7G249600*) were significantly up-regulated at S3. In addition, the *ANR* gene *Seita.7G249500* was significantly up-regulated at S1.

Candidate gene analysis

Among the DEGs between DY and LY, we focused on genes located within the QTLs to identify candidate genes related to hull color. We compared 1411 genes in 13 stable QTLs detected by four phenotypic evaluation methods with 3183 DEGs and obtained 87 differentially expressed genes in these candidate intervals (Table S12). All differentially expressed genes (DEGs) were functionally annotated using several databases, including the NCBI nonredundant protein database (Nr), KOG, and Swiss-Prot (Table S13). In the 87 DEGs, three genes of which may be hull color candidate genes. Among these DEGs involved in the phenylpropanoid metabolic pathway, only one gene, *Seita.1G057300*, encoding cinnamyl alcohol dehydrogenase (*CAD*), was located in the candidate region of *qHC1.2* (Table 4). Among these DEGs involved in the flavonoid biosynthesis pathway, only one gene, *Seita.9G034700*, encoding chalcone isomerase (*CHI*), was located in the candidate region of *qHC9.1* (Table 4). The cytochrome P450 gene *Seita.1G157600* is located in the candidate region of *qHC1.3* and is involved in the phenylpropanoid metabolic pathway and flavonoid biosynthesis pathway (Table 4). BLAST search against the protein databases of foxtail millet and rice using *Seita.1G057300*, *Seita.1G157600*, and *Seita.9G034700* was conducted in Phytozome. Furthermore, we used the NCBI Conserved Domain Database (CDD) (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to predict the conserved domains. The results showed that *Seita.1G057300* and *GH2 (LOC_Os02g09490)* were highly homologous and contained the same conserved domain PLN02514, belonging to the PLN02514 superfamily. *Seita.9G034700* and *GH1 (LOC_Os03g60509)* were highly homologous and belonged to Chalcone_3 superfamily.

To confirm the reliability of RNA-seq results, we subjected five genes to qRT-PCR analysis, including three candidate genes (*Seita.1G057300*, *Seita.1G157600*, *Seita.9G034700*) and two genes (*Seita.5G361200* and *Seita.5G462500*) involved in phenylpropanoid biosynthesis and flavonoid biosynthesis pathway (Fig. 8). All genes showed the same expression patterns as in the RNA-seq data, and we detected high correlations ($R^2 \geq 0.8817$) between the qRT-PCR data and the RNA-Seq data, further confirming the validity of the RNA-seq analysis.

Table 4
Basic information of candidate genes

Gene ID	Chr	Start	End	Description
<i>Seita.1G057300</i>	1	5494976	5498823	probable cinnamyl alcohol dehydrogenase
<i>Seita.1G157600</i>	1	21991750	21993572	Cytochrome P450
<i>Seita.9G034700</i>	9	1904180	1905485	chalcone–flavonone isomerase

Identification of candidate genes hull color in foxtail millet

To further obtain strong evidence to determine the most possible candidate gene, the three candidate genes were sequenced in DY and LY. No band was amplified from DY and LY for the *Seita.1G157600* gene due to low gene expression. Sequence analysis exhibited no difference in the cDNA sequence of *Seita.9G034700* between DY and LY. *Seita.1G057300* showed that in the CDS region, four single nucleotide polymorphisms (SNPs) were detected in DY compared with LY; however, we found that three of these SNPs caused no amino acid changes using Tofms (http://www.tofms.org/calmw/NR2AA_wkh0.asp). One SNP (A to G), located at the third exon, resulted in an amino acid change from isoleucine to valine in DY compared with LY (Fig. 9). Next, the candidate gene *Seita.1G057300* from two parents and eight varieties of foxtail millet, including four varieties with red hull and four varieties with yellow hull, underwent sequencing. This result indicates that *Seita.1G057300* showed that in the CDS region, four SNPs were detected in foxtail millet varieties with different hull colors. The three of them were synonymous mutations, while the remaining one was a non-synonymous mutation. This result comes in line with the sequence analysis between DY and LY. Non-synonymous mutations in the CDS region may result in differential expression of *Seita.1G057300* in foxtail millet varieties with distinct hull colors. Therefore, *Seita.1G057300* was considered the most possible functional gene underlying *qHC1.2*.

Discussion

Application of different color evaluation methods in grain

Grain color is an important quality trait, and Method I is a common phenotypic evaluation method. Method I is a widely used and simple phenotypic evaluation. It has been frequently applied to evaluate the color of seeds or fruits, including foxtail millet(Xie et al. 2021), cucumber(Kishor et al. 2021), wheat(Lin et al. 2016), and other plants. Xie et al. classified the hull color in foxtail millet into two categories, red and golden yellow, using Method I for QTL detection(Xie et al. 2021). Method II is a more detailed identification of hull color relative to Method I. Method I and Method II have the characteristics of possessing simple operation, rapid, and low cost that make them more appropriate for quick evaluation of phenotypes in large populations. However, the accuracy of Method I and Method II is affected by environmental and anthropogenic factors. Method III: LAB color space was used for phenotypic evaluation that it is generating colors as similar to as human visual system(Alam et al. 2014; Philipp and Rath 2002). This method has been widely used in black bean(Cichy et al. 2014), cherry(Sooriyapathirana et al. 2010), grape(Underhill et al. 2020), etc. Method IV: RGB color space was used for phenotypic evaluation. It has been widely used in sesame(Zhang et al. 2013), peanut(Zhang et al. 2021), grape(Underhill et al. 2020), etc. However, compared to LAB color space, RGB color space cannot simulate human perception color perception well(Vanrell et al. 2004). Existing research generally selects only one method for phenotypic evaluation and the application of many methods for phenotypic evaluation simultaneously has not been reported in foxtail millet. The lack of efficient methods for evaluating hull color in foxtail millet has impeded further genetic experiments.

Comparison the QTLs identified in this study with previous ones

To identify QTLs associated with hull color in foxtail millet, four phenotypic evaluation methods were utilized in all four environments. A total of 36 QTLs associated with hull color was detected. Among these QTLs, 13 QTLs were repeatedly mapped in multiple environments, with four major QTLs (*qHC1.1*, *qHC1.2*, *qHC9.1* and *qHC9.5*) and nine minor QTLs (*qHC1.3*, *qHC3.3*, *qHC3.4*, *qHC3.6*, *qHC4.4*, *qHC5.2*, *qHC8.2*, *qHC8.3* and *qHC9.4*) explaining 1.09% to 69.63% of the phenotypic variation. Two major QTLs *qHC1.2* and *qHC9.5* could be detected repeatedly in all methods. Among them, *qH1.2* could explain 8.89% to 69.63% of the phenotypic variations. *qHC9.5* could explain 4.43% to 32.08% of the phenotypic variations. These two QTLs explained a relatively high percentage of the phenotypic variation, which suggests that the genetic factors might be the major source of phenotypic variation. In addition, the broad-sense heritability for all the traits ranged from 0.76 to 0.97 in all four environments, indicating that genetic factors played an important role in determining hull color.

Previous studies also identified several QTLs associated with hull color in foxtail millet. In 2013, with multiple associated variants from a genome-wide association study, Jia et al. identified seven QTLs for foxtail millet hull color. Two of them, *qHC1.2* and *qHC9.5*, are consistent

with our findings (Jia et al. 2013). *Sihc1* was discovered at the end of chromosome 6 based on findings from two preceding studies. However, these specific QTLs did not align with the QTLs identified in this study. In our study, two QTLs namely, *qHC1.1* and *qHC1.2* were found to be in agreement with *qHC1* and *qHC2* previously reported by Xie et al. (Xie et al. 2021). Interestingly, both this study and the previous publication used RILs derived from Yugu 18 × Hongjiugu crosses and constructed a high-density bin map for QTL mapping. However, Guo's research performed QTL mapping for hull color by Method III, while our study performed QTL mapping for hull color by four phenotypic evaluation methods. Notably, six QTLs, namely, *qHC1.2*, *qHC1.3*, *qHC3.3*, *qHC9.1*, *qHC9.4*, and *qHC9.5*, were consistent from previously reported in Guo's research, however, there are six QTLs identified in this study are novel ones, namely *qHC3.4*, *qHC3.6*, *qHC4.4*, *qHC5.2*, *qHC8.2* and *qHC8.3*. This not only confirms that our results are reliable but also implies that multiple phenotypic evaluation methods are effective in QTL mapping for hull color.

Candidate genes identified for hull color

To identify candidate genes within the stable QTLs region, we performed RNA-seq on the hull of DY and LY revealing DEGs that may be related to hull color. Previous studies suggested that it was found that lignin metabolism and flavonoids are important factors that affect hull color. Of the two biosynthetic pathway genes, *CAD* (*LOC_Os02g09490*) and *CHI* (*LOC_Os03g60509*) have been identified as crucial for the biosynthesis of rice hull color. Correspondingly, we found that the expression level of *CAD* (*Seita.1G057300*) in the LY hull exceeded that of DY for three periods, while the expression level of *CHI* (*Seita.9G034700*) in the LY hull was lower than that of DY at S3. In addition, the cytochrome P450 gene *Seita.1G157600* is located in the *qHC1.3* candidate interval and is involved in the phenylpropanoid metabolic pathway and flavonoid biosynthesis pathway.

Research on rice hulls has demonstrated that changes in hull color are primarily caused by the accumulation of flavonoids and anthocyanins, as well as defects in lignin synthesis within the organism (Wang et al. 2020). Lignin is one of the important substances in the phenylpropanoid metabolic pathway in plants (Dong and Lin 2021). Thus, we examined the phenylpropanoid and flavonoid metabolic pathways that were significantly enriched by genes expressed differentially. Phenylalanine ammonia-lyase (*PAL*) catalyzes the formation of cinnamic acid from Phenylalanine, cinnamic acid-4-hydroxylase (*C4H*) catalyzes the formation of p-Coumaric acid from cinnamic acid (Humphreys and Chapple 2002). The *C4H* gene (*Seita.1G157600*) exhibited down-regulation at S1, potentially acting as a regulatory factor at this stage and impacting the synthesis of lignin and flavonoids. *GH2* (*LOC_Os02g09490*) is the only subA subfamily involved in constitutive lignin biosynthesis. It is the main *CAD* enzyme for the synthesis of coniferyl alcohol and sinapyl alcohol precursors in rice lignification tissues. The hull and stem segments of the *gh2* mutant exhibited a golden-yellow coloration attributable to a deficiency in lignin production (Zhang et al. 2006). As a result of the substitution of T with C in exon four of *RBH1*, the expression of genes regulating lignin and flavonoid metabolism was significantly up-regulated in the hull of the *rbh1* mutant. This further confirms that *GH2/RBH1* affects hull color by regulating lignin and flavonoid metabolism (Wang et al. 2020). Through phylogenetic analysis and sequence alignment, *Seita.1G057300* in foxtail millet showed high homology with *GH2* (*LOC_Os02g09490*) and included a conserved PLN02514 domain. This gene demonstrated significant up-regulation across all three developmental stages (S1-S3) and appears to be an essential player in lignin synthesis and metabolism throughout these stages of development. Another gene, *Seita.9G034700*, is involved in the flavonoid biosynthesis pathway and is homologous to the *GH1* gene (*LOC_Os03g60509*) in rice. This gene belongs to the Chalcone_3 superfamily. *GH1* is a key gene involved in the flavonoid pathway in rice, which encodes a relatively conserved protein of chalcone isomerase. The loss of *OsCHI* expression in the *gh1* mutant resulted in an increase in flavonoid content and a golden yellow hull color (Hong et al. 2012). *Seita.9G034700* exhibited significant upregulation during S1, potentially playing a crucial role in flavonoid biosynthesis and consequently affecting anthocyanin synthesis.

Seita.1G057300 sequence analysis in the CDS region revealed four SNPs were detected in DY compared with LY, and one SNP (A to G), located at the third exon, resulted in an amino acid change from isoleucine to valine. *Seita.1G057300* in the sequence analysis results of ten foxtail millet varieties are consistent with the sequence analysis between DY and LY. Non-synonymous mutations in the CDS region may result in differential expression of *Seita.1G057300* in foxtail millet varieties with distinct hull colors. Therefore, *Seita.1G057300* was considered the most possible functional gene underlying *qHC1.2*.

Declarations

Author Contribution statement PY designed the experiment, developed the RIL population, and revised the manuscript. SC did data analysis and drafted the manuscript. HS helped in the data analysis and discussion. BF and LZ supervised the field experiments. SC, XS, YG, SG, and CW collected the phenotype. All authors have read and approved the final version of the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest The authors declare that they have no competing interests.

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