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Assessment of Grain Color Parameters With Functional and SSR Markers in Bread Wheat (*Triticum aestivum* L.)

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ABSTRACT

This study aimed to examine the relationships between grain color parameters and functional markers of color-controlling genes in wheat. Grain color parameters (L^*, a^*, b^*, C^* and $h°$) of the segregated genotypes in the F_2 generations of Byrd (red) × Tosunbey (white) and Kayra (red) × Cumhuriyet 75 (white) hybrids were measured. The F_2 genotypes with parents were screened with wheat grain color SSR marker (Xgwm155) and functional color markers (Tamyb10-A1, Tamyb10-B1 and Tamyb10-D1). The color alleles possessed by the parents were determined by functional markers. While the parents with white color (Tosunbey and Cumhuriyet 75) were determined to be homozygous recessive in terms of all three-color alleles, Byrd variety with red grain color was found to have the R-B1b allele, and Kayra variety was found to have the red color allele in terms of all three loci. In terms of color parameters, the average color parameter values in the F_1 generation and F_2 population of both hybrid combinations gave results closer to the parent with red grain color. In the Byrd × Tosunbey F_2 population, the interaction of two markers (Xgwm155 × Tamyb10-B1) in the L^* color parameter and the Xgwm155 marker in the Hue angle ($h°$) parameter were found to be important and explained the observed variation by approximately 11%. In the Kayra × Cumhuriyet 75 F_2 population, the Tamyb10-B1 marker in the L^* parameter and Tamyb10-D1 in the b^* and C^* parameters were determined to be important and explained approximately 5% of the observed variation.

INTRODUCTION

Wheat varieties are divided into six classes according to simple hereditary genetic characteristics such as grain color, vernalization requirement and seed hardness. These are winter red hard wheat, summer red hard wheat, white soft wheat, red soft wheat, hard white wheat and hard white wheat. The inheritance of grain color in wheat is controlled by three independent genes located on chromosomes 3A,

3B and 3D. In the formation of grain color, red color is created by R-A1b, R-B1b and R-D1b alleles, and white color is created by R-A1a, R-B1a and R-D1a alleles (McIntosh et al., 1998; Sherman et al., 2008). Red color is dominant over white color, and carrying a dominant allele at a single locus is sufficient for the red color to occur (Metzger & Silbaugh, 1970). The degree of red color has an additive effect, and genotypes carrying homozygous dominant alleles at three loci (R-A1b, R-B1b and R-D1b) produce a darker red color. White color occurs only in genotypes carrying homozygous recessive alleles (R-A1a, R-B1a and R-D1a) at three gene loci (Sherman et al., 2008).

The red pigment found in the grain coat of wheat consists of catechins and proanthocyanidins synthesized through flavonoid biosynthetics pathway (Himi et al., 2011) and causes bitter flavor and lower hydrolytic enzyme activity (Lachman et al., 2017). In wheat, flour color plays an important role in the end-use quality of wheat as it affects consumer preference, market value and human nutrition. The color of the grain and the final products obtained depend on genetic, environmental and processing factors (Guzman et al., 2022). It is known that hard and dark colored grains generally have higher value in terms of protein content and protein quality (Ünal, 2003). Likewise, it is accepted that hard red wheat has higher protein content and quality, as well as higher flour quality and final product quality (Slaughter et al., 1992; Kaldy et al., 1993; Şanal et al., 2012). Pre-harvest germination in wheat causes a decrease in flour quality due to starch breakdown. Generally, wheat with red grain color is more tolerant to pre-harvest germination than wheat with white grain. The relationship between these two traits is due to the pleotropic effect of genes controlling grain color (Flintham, 2000; Warner et al., 2000; Himi et al., 2002, 2011).

The R-1 gene, which controls grain color as well as pre-harvest germination in bread wheat, is located in the end region of the long arms of homoeologous chromosomes 3A, 3B and 3D (Himi et al., 2011). It has been determined that the pigments in red wheat grain include flavonols and stilbenes, as well as catechin and proanthocyanidin (Miyamoto & Everson, 1958; McCallum & Walker, 1990; Matus-Cadiz et al., 2008; Himi et al., 2011). Identification of the polymorphism that causes functional differences between alleles controlling grain color in wheat, detection of relevant markers and their use have revealed the possibility of cloning the grain color locus (Sherman et al., 2008). Himi et al. (2011) isolated Tamyb10-A1, Tamyb10-B1 and Tamyb10-D1 genes, which encode the protein that controls proanthocyanidin synthesis and activate flavonoid biosynthetic genes in wheat, and are localized on chromosomes 3A, 3B and 3D of wheat, respectively, and they developed functional DNA markers for the genes in question.

In this study, the grain color characteristics of each genotype in the F_1 and F_2 generations of two different hybrid combinations made between the wheat varieties differing in grain color were measured. Genotyping was performed using functional markers of the genes controlling grain color in the same F_2 genotypes and their parents, and the relationships between color measurements and genotyping results were examined.

MATERIAL AND METHODS

The research was conducted in the 2020-2021 wheat growing season in the Field Crops Department of the Faculty of Agriculture of Ege University, İzmir, Türkiye. In the experiment, the F_1 seeds of the Byrd × Tosunbey and Kayra × Cumhuriyet 75 hybrid combinations, which were obtained from the crosses conducted previous year, were planted with their parents to obtain F_2 generations. While the Byrd and Kayra varieties have red grain structure, Cumhuriyet-75 and Tosunbey varieties are white grain varieties used as parents in the hybrid combinations.

In the experiment, manual planting was done in rows with 20 cm row spacing and 1 m row length. In sowing, NPK 15:15:15 fertilizer was applied at 8 kg pure nitrogen per decare and urea fertilizer was applied at 8 kg pure nitrogen per decare during the stem extension period. Plants were grown under rainfed conditions, no extra irrigation was applied. Leaf samples were taken for DNA extraction from the parents and 100 randomly tagged plants in the F_2 populations when the plants reached approximately 15-20 cm in length and DNA isolations were made

according to the mini CTAB extraction method of Doyle & Doyle (1987). The amount of isolated genomic DNA was measured using a biophotometer (BioPhotometer, Eppendorf) at 260 nm wavelength and diluted to 25 ng/µl.

In the study, kernel color was determined molecularly using functional markers of Tamyb10-A1, Tamyb10-B1 and Tamyb10-D1 genes defined by Himi et al. (2011) and also SSR marker Xgwm155 (Sherman et al., 2008) (Table 1). Polymerase chain reaction was performed in Eppendorf mastercycler gradient PCR device according to the protocol specified by Aykut Tonk et al. (2016). SSR touchdown program (Aykut Tonk et al., 2016) was used for Xgwm155 marker, while specific PCR programs suggested by Himi et al. (2011) were used for functional markers. PCR products of functional color markers were run in 2% agarose gel using 1×TBE buffer in Thermo Scientific electrophoresis device at 85 V for approximately 2 hours. PCR products of the SSR marker were run on an 8% non-denaturing acrylamide gel using the Bio-Rad Protean II xi Cell device for approximately 16 hours at 90 V. After the gels were stained in pure water containing 2 µg/ml ethidium bromide, they were viewed under UV light and images were taken with a digital camera.

Grain color measurements were made in terms of CIE L^* a^* b^* using a colorimeter (Chrometer CR-400, Minolta Co., Tokyo, Japan) on the harvested seeds of individually labelled 100 plants from the F_2 populations as well as the F_1 generations and their parents.

In molecular evaluation, with the help of the parental bands, the bands obtained in F_2 individuals were scored as maternal (A), paternal (B) or heterozygous (H). Analysis of color traits was performed using the R v4.2.0 statistical package program (R Core Team, 2021). Before performing linear regression and ANOVA analyses, the model assumptions were tested. The normality of residuals was tested using the Shapiro-Wilk test. Homoscedasticity (equal variance of residuals) was checked via Breusch-Pagan test. Independence was checked by the Durbin-Watson test. All tested models satisfied these assumptions. Relationships between the molecular markers and the color parameters were examined using the *aov* function. Each character was fitted to the linear regression model using the *lm* function of the lmer4 package (Bates et al., 2015).

RESULTS AND DISCUSSION

The color parameter values of the parents, F_1 and F_2 generations are given in Table 2. When the table is examined, it is seen that the L^* value, which expresses lightness-darkness, has higher values in Tosunbey and Cumhuriyet 75 parents with white grain color than Byrd and Kayra parents with red grain color. It is understood that grains obtained from F_1 plants had lower L^* values than both parents and closer values to parents with red grain color.

Table 1. Base sequences of functional color markers and Xgwm155

Tamyb10-A1	Tamyb10-LP1	CTGAGCAAGAGGATGCTGC
	Tamyb10-RP1	GATGCCCTCCAGATCAAGGT
Tamyb10-B1	Tamyb10-LP2	AGCAAGAGGAACCTGCAGTC
	Tamyb10-RP1	GATGCCCTCCAGATCAAGGT
Tamyb10-D1	Tamyb10-LP3	CGCTGAGCAAGAGGAACCA
	Tamyb10-RP3	AGGCACACCAGCTTATTTGG
Xgwm155	F	CAATCATTTCCCCCTCCC
	R	AATCATTGGAAATCCATATGCC

In terms of the a^* color parameter, where + indicates red and - indicates green on the horizontal axis of the color diagram, it was observed that all parents and F_1 plants had a +a^* value, and there were no major differences between the parents and F_1 generations except for the Cumhuriyet 75 parent. In terms of the b^* parameter, where + indicates yellow and - indicates blue on the vertical axis of the diagram, it was observed that the Byrd and Kayra parents with red grain color showed significantly lower values than the Tosunbey and Cumhuriyet 75 parents with white grain color, while the F_1 plants gave values closer to the parent with red grain color. In terms of the chroma (C^*) value, which indicates the vividness and dullness value of the color, the parents with red grain color had lower values than the parents with white grain color. It was observed that the C^* values of the F_1 generations of the hybrids made between these parents were closer to the parents with red grain color (Table 2). The hue angle ($h°$) value is the value that determines the color we perceive of the object, that is, the name of the color. When the $h°$ values observed in the parents and F_1 grains are examined, it is understood that there is a similar distribution for $h°$ like the L^*, b^* and C^* parameters. The hue angle value was lower in the parents with red grain color and higher in those with white grain color. The average values of color parameters in the F_2 populations also showed values closer to the red parent except for two parameters (b^* and C^*) in the Kayra × Cumhuriyet 75 population. Transgressive segregations were observed for all color parameters in both F_2 populations (Table 2). In studies of quantitative traits, the generation of extreme phenotypes in segregating hybrid populations is referred to as transgressive segregation (Rieseberg et al., 1999). Transgressive segregation is a complex phenomenon in inheritance of kernel color which are controlled by three major genes. The additivity of allelic effects between the loci and within a locus and also novel allelic combinations caused transgressive segregation in all color parameters.

Table 2. Results of color parameters in the parents, F1 and F2 generations

Parent, F_1 and F_2	L^*	a^*	b^*	C^*	h°	Color
Byrd	50.71	7.36	19.94	21.25	69.74	Red
Tosunbey	53.91	7.23	23.56	24.64	72.94	White
Byrd × Tosunbey F_1	48.51	7.81	19.10	20.64	67.76	-
Byrd × Tosunbey F_2	50.01	7.86	19.26	20.81	67.77	-
Kayra	48.61	7.24	18.76	20.11	68.90	Red
Cumhuriyet 75	59.18	6.00	22.94	23.71	75.34	White
Kayra × Cumhuriyet F_1	47.78	7.63	19.61	21.04	68.74	-
Kayra × Cumhuriyet F_2	53.66	7.17	21.47	22.66	71.42	-

Table 3. Allele distribution of the R1 color gene in the parents

Parent	R-A1	R-B1	R-D1	Former Notation	Color
Byrd	a	b	A	r1r2R3	Red
Tosunbey	a	a	A	r1r2r3	White
Kayra	b	b	B	R1R2R3	Red
Cumhuriyet 75	a	a	A	r1r2r3	White

Table 4. Distribution of color markers in the F2 populations and chi-square test results

Generation and Marker	Observed value	χ^2	P value
Byrd × Tosunbey F_2			
Xgwm155	21:47:27	0.77	*0.69>0.05*
Tamyb10-B1	20:74	0.70	*0.43>0.05*
Kayra × Cumhuriyet 75 F_2			
Tamyb10-B1	25:67	0.23	*0.56>0.05*
Tamyb10-D1	30:64	2.40	*0.13>0.05*

Table 5. p, R^2 values and regression coefficients of the markers for the color parameters in the Byrd × Tosunbey F_2 population

Marker	L*	a*	b*	C*	h°
Xgwm155	0.153374	0.2646	0.2453	0.4450	0.009781**
Tamyb10-B1	0.223949	0.7326	0.5666	0.6398	0.377644
Xgwm155 × *Tamyb10-B1*	0.007338**	0.1211	0.8736	0.8338	0.093165
R^2	0.1079	0.02358	-0.01761	-0.03224	0.1051
Regression Coefficients					
Xgwm155	7.35	0.51	3.57	3.52	9.75
Tamyb10-B1	6.07	0.26	1.37	1.36	8.33
Xgwm155 × *Tamyb10-B1*	-8.26	0.18	-2.01	-1.81	-9.98

The allele distributions of functional markers of Tamyb10-A1, Tamyb10-B1 and Tamyb10-D1 genes, which control grain color, in the parents are given in Table 3. According to the Tamyb10-A1 gene marker results, it was determined that both varieties Byrd and Tosunbey had the R-A1a allele. The same marker showed polymorphism between Kayra and Cumhuriyet 75 varieties, and it was determined that the Kayra parent had the R-A1b allele and the Cumhuriyet 75 parent had the R-A1a allele. Tamyb10-B1 gene color marker showed polymorphism between both Byrd / Tosunbey and Kayra / Cumhuriyet 75 varieties. It was determined that Tosunbey and Cumhuriyet 75 varieties with white grain color had the R-B1a allele. While the Tamyb10-D1 gene marker showed monomorphism in Byrd and Tosunbey varieties, it gave polymorphic results between Kayra and Cumhuriyet 75 varieties. When the results are examined; it was determined that Byrd, Tosunbey and Cumhuriyet 75 varieties had the R-D1a allele, while Kayra variety had the R-D1b allele. In terms of color alleles, among the varieties used as parents, it was determined that Byrd variety had the R-A1a, R-B1b and R-D1a alleles, Tosunbey variety had the R-A1a, R-B1a and R-D1a alleles, Kayra variety had the R-A1b, R-B1b and R-D1b alleles and Cumhuriyet 75 variety had R-A1a, R-B1a and R-D1a alleles (Table 3).

White grain wheat varieties are homozygous for R-A1a, R-B1a and R-D1a recessive alleles (r2, r3 and r1 in the old notation, respectively). The presence of one or more dominant alleles R-A1b, R-B1b and/or R-D1b (old notation R2, R3 and R1, respectively) causes red

pigmentation on the grains (McIntosh et al., 1998; Himi et al., 2011). While the Byrd variety with red grain color is dominant in terms of a single gene (R-B1b), the Kayra variety with the other red grain color is dominant in terms of all three genes. Tosunbey and Cumhuriyet 75 varieties, which have white grain color, are homozygous recessive in terms of all genes. The functional color markers have been successful in distinguishing color alleles of the parental genotypes. The obtained allele distributions of the varieties were found to be in agreement with the research results of Himi et al. (2011).

The Xgwm155 marker used in the study showed polymorphic results between Byrd / Tosunbey and Kayra / Cumhuriyet 75 varieties. This marker was developed by Sherman et al. (2008) and located on chromosome 3A in the wheat genome, the band sizes obtained differed in varieties with the same grain color. The researchers also stated that all of the bands obtained with Xgwm155 were different in genotypes with two white (MTHW0202 and MTHW0471) and two red grain colors (Chhoteau & Vida), but that this marker was useful in distinguishing individuals.

The distribution of color markers showing polymorphic results between the parents in F_2 population is given in Table 4. According to chi-square analysis, the results of the Xgwm155 marker in the Byrd × Tosunbey F_2 population, the genotypic segregation in the population was determined to be in accordance with the 1: 2: 1 ($\chi^2 = 0.77$, P = 0.69). In the same population, the genotypic segregation of the dominant Tamyb10-B1 marker was determined to be compatible with a 3:1 distribution ($\chi 2 = 0.70$, P = 0.43). Tamyb10-B1 ($\chi^2 = 0.23$, P = 0.56) and Tamyb10-D1 ($\chi^2 = 2.40$, P = 0.13) markers, both dominant, similarly differentiated F_2 individuals of Kayra × Cumhuriyet 75 in accordance with the 3:1 genotypic segregation (Table 4). The distributions of the markers in both populations were found to be compatible with the expected theoretical distributions. Himi et al. (2011), emphasized that grain color in wheat is determined by the phenotype of F_3 grains obtained from F_2 plants, and these are controlled by the F_2 genotype.

The effect of color markers for each color parameter used in the study was examined by using a linear regression model. The p and R^2 values obtained from the summary of the results performed for the Byrd × Tosunbey F_2 population are given in Table 5. It was determined that the interaction of two markers (Xgwm155 × Tamyb10-B1) in the L^* color parameter was significant at the 0.01 statistical level. In the hue angle (h°) parameter, the Xgwm155 marker was found to be significant at the 0.01 level in distinguishing the genotypes. The R^2 values for both parameters were found to be approximately 11%. In other words, the applied model explained approximately 11% of the phenotypic variation observed in these color parameters (Table 5).

Table 6. P, R^2 values and regression coefficients of the markers for the color parameters in the Kayra × Cumhuriyet 75 F_2 population

Marker	**L***	**a***	**b***	**C***	**h°**
Tamyb10-D1	0.29642	0.7071	0.01259*	0.01077 *	0.2026
Tamyb10-B1	0.03068*	0.6254	0.50129	0.52964	0.5025
Tamyb10-D1 × *Tamyb10-B1*	0.85638	0.4981	0.52395	0.59661	0.3483
R^2	0.033	-0.0254	0.0478	0.04901	-9.093e-05
	Regression Coefficients				
Tamyb10-D1	1.86	-0.18	1.94	1.79	1.99
Tamyb10-B1	-2.14	-0.06	0.19	0.14	0.39
Tamyb10-D1 × *Tamyb10-B1*	0.29	0.3	-0.83	-0.68	-1.41

Regression coefficients revealed that both markers, Tamyb10-B1 and Xgwm155, and their interaction exhibited variable effects on all color parameters in the Byrd × Tosunbey F_2 population. The most pronounced effects were observed for the L^* and $h°$ parameters, where both markers individually increased the respective color values, while their interaction had a strong negative effect. Moderate positive effects of Xgwm155 were also observed for b^* and C^* parameters.

The p and R^2 values obtained for Kayra × Cumhuriyet 75 F_2 population are shown in Table 6. In the L^* parameter, the Tamyb10-B1 marker was found to be important in distinguishing the genotypes. Tamyb10-D1 was determined to be important in b^* and C^* color parameters. The model applied for these two parameters explained approximately 5% of the variation observed in these properties (Table 6).

The regression analysis revealed that the effects of the markers on color parameters varied by trait. For the Kayra × Cumhuriyet 75 F_2 population, the presence of the Tamyb10-D1 allele showed a positive effect on all color parameters except a^*, with the strongest increase observed for b^*, C^* and $h°$. Tamyb10-B1 had relatively small and inconsistent effects, while the interaction between the two markers generally showed minor or negligible contributions (Table 6).

Grain color in wheat is controlled by three independent genes. Phenotypic color values, especially in the individuals in the F_2 population which contains extensive genetic diversity, were obtained by separately evaluating the color parameters that constitute the color. Therefore, the relationships between allele-specific markers and the color parameters are not very high in the applied model. Wheat grains can be in various colors including white, light yellow, yellow red, amber and brown. For wheat grain color and similar products, a special scale created from the parameters in the color diagram will help to reveal the marker-color relationship more clearly in the segregation population. Similarly, a study reported that the most accurate and effective method for measuring the color of products with a visible-NIR device was to apply calculations using the standard application based on the CIE system (Black & Panozzo, 2004).

In wheat, grain color and hardness provide advantages for products to be obtained from grain in milling and in end-use quality. In addition, red grain wheat genotypes are generally more resistant to pre-harvest sprouting than white grain wheat genotypes (Himi et al., 2011). In wheat breeding programs, plants can be selected from segregation populations developed from parents with different color in order to select genotypes suitable for grain color. For effective selection at this stage, functional color markers and a special scale developed from color parameters can be beneficial in segregation populations.

CONCLUSION

Digital color images, colorimetry and spectrometry have been used for a long time to measure grain color, flour color or final product color in wheat (Neuman et al., 1989; Peterson et al., 2001; Şahin et al., 2006; Adams et al., 2013). To measure color objectively, colorimeters that measure $L^* a^* b^*$ values defined by the Commission Internationale de L'Eclairage (CIE) are mostly used (Black & Panozzo, 2004). In this study, wheat grain color was measured colorimetrically and grain color differences between the genotypes were clearly revealed. Functional markers developed from the genes controlling grain color and the SSR marker were screened in the parents with different grain colors and their F_2 populations. Different markers showed importance in terms of different color parameters measured in the F_2 populations. Grain color in wheat shows polygenic inheritance and the formation of grain color occurs as a result of the effects of many genes. Therefore, it is expected that the markers used will be associated with different color parameters. As a result of the study, it was determined that functional markers, especially those related to the genes controlling grain color in wheat, were useful in determining color alleles and could be easily used in determining color alleles in future wheat breeding programs.

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Compliance with Ethical Standards

Authors' Contributions

SÖ: Investigation, Methodology

Dİ: Data curation, Formal analysis, Interpretation

FAT: Conceptualization, Investigation, Supervision, Writing – review & editing

All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

AI Disclosure

The authors confirm that no generative AI was used in writing this manuscript or creating images, tables, or graphics.

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