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Use of microsatellite markers to detect heterozygosity in an F_2 generation of a black rice and white rice cross

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ABSTRACT This research aimed to identify the heterozygosity of an F_2 population of a black rice and white rice cross using microsatellite markers. Rice was grown and harvested in a field approximately 500 m^2 in size in Pakem (Sleman, Yogyakarta, Indonesia), and analyzed in the Genetics and Plant Breeding Laboratory of the Faculty of Agriculture, Universitas Gadjah Mada. The research materials consisted of an F_2 S $\times$ G population composed of a cross of black rice (S) and white rice Situbagendit (G), female parent of black rice (S), male parent of white rice (G), chemical and organic fertilizer, chemicals and tools for molecular activity and three microsatellite markers related to color properties (RM 220, RM 224, and RM 252). All of the plant populations (generation F_2, parent female, parent male) were planted in the field until their harvest. Young leaves (30 days in age after planting) were molecularly analyzed using three microsatellite markers (RM 220, RM 224, and RM 252). This encompassed DNA isolation, PCR reaction, and the visualization of the PCR results using Metaphore Agarose Gel Electrophoresis. The results showed that the percentage of the number of individual plants showing a heterozygous pattern in the F_2 S × G population was 50% (RM 220), 40% (RM 224), and 60% (RM 252). As such, the RM 252 microsatellite marker can be used for marker-assisted selection in the crossbreeding of black rice.

KEYWORDS black rice; F_2 generation; heterozygosity; microsatellite

1. Introduction

The black rice that is currently consumed as a functional food is a local black rice. Black rice has a high anthocyanin content in the pericarp layer, which provides a dark purple color (Ryu et al. 1998; Ichikawa et al. 2001). Black rice also contains higher levels of protein, vitamins, and minerals than white rice (Suzuki et al. 2004). On the other hand, local black rice has weaknesses such as a long harvest period and low yield potential. Efforts to obtain a superior black rice cultivar were through cross-breeding with white rice that has a high yield and short harvest period. Conventional breeding of black rice with white rice has been done to obtain black rice with a short harvest period and high potential yield.

Conventional plant breeding involves selection on segregated generations from an F_2 to F_5 or F_6 generation, which overall takes about 5–10 years (Allard and Allard 1999). The long duration of this process has necessitated the development of new breakthroughs to reduce the amount of time required. Azrai (2006) suggests that selection of breeding activities can be conventionally accelerated when synergized with DNA marker technology known as Marker Assisted Selection (MAS). With MAS, selection activities become more effective and efficient because selection is based only on plant genetic characteristics, and not influenced by environmental factors. Susanto et al. (2009) stated that MAS can be applied to plants, even when the plants are young, in greenhouses, and in the field without being affected by seasonal changes. One of the MAS markers that is commonly used is the simple sequence repeat (SSR) or microsatellite marker.

SSR markers can aid in the estimation of genetic diversity between cultivars, e.g. between parents of the gene pool or between plants derived from a population or between populations. Mi(Lapitan et al. 2007). Zhou et al. (2003) investigated the genetic diversity and genetic structure of natural populations of *Oryza rufipogon* in China using SSR markers, and found the results to be significant.

One of the advantages of selection based on DNA markers is that it can potentially provide more accurate results compared with selection based on the phenotypes of plants that are influenced by seasons, microclimates, specific plant parts, and plant growth stages. Therefore, the use of MAS has potential as a breakthrough breeding method that can reduce the time and cost of plant breeding.

The selection-assisted marker can be identified after a matching marker information is obtained and is linked to the desired traits. According toUtami et al. (2009), there are several microsatellite markers that are known to be linked to the Quantitative Trait Loci (QTL) of the red color in rice, particularly in the pericarp. These microsatellite markers are RM 180 found on chromosome 7 of the rice genome, adrift with QTL accID: AQGD029; RM 224 found on chromosome 11 of the rice genome for the color/pigment trait on the pericarp of rice; and RM 220 found on chromosome 1 of the rice genome, adrift with QTL accID: AQGF019. The results of research by Wiriyasuk (2005) confirmed that the location of genes controlling the color properties of rice was on chromosome 4 and mapped in the position of RM 317–RM 241 and RM 252–RM 241.

In a study by Utami et al. (2009) on five rice cultivars from the Special Region of Yogyakarta, Indonesia, namely Mandel Handayani, Segreng, Cempo Merah, Andel Merah, and Saodah, PCR analysis with four microsatellite markers (RM 252, RM 220, RM 180, and RM 224) revealed that the microsatellites used in the analysis were associated with the color properties of the pericarp. In addition, as Kristamtini and Purwaningsih (2009) observed when using the RM 220 microsatellite marker, red rice cultivars, namely Mandel, Segreng, and Aeksibundong can also be distinguished.

2. Materials and methods

The research materials consisted of the female parent of black rice (S), male parent of white rice (Situbagendit/G), F_2 S $\times$ G and reciprocal G $\times$ S plant populations. All of the populations were planted and grown in a field approximately 500 m^2 in size in Pakem (Sleman, Yogyakarta, Indonesia) until they were harvested. All samples were maintained under field conditions in accordance with integrated crop management. Young leaves from each plant population (S, G, F^2 S $\times$ G, and G $\times$ S) were molecularly analyzed using three microsatellite markers (RM 220, RM 224, and RM 252), the stages of which encompassed DNA isolation, PCR reaction, and visualization of the PCR results using Metaphore Agarose Gel Electrophoresis (MAGE).

2.1. DNA isolation

Young leaves (three weeks after transplanting) were taken from each plant population (S, G, F^2 S $\times$ G, and G $\times$ S) for analysis, specifically 10 samples per population. The genomic DNA isolation was done from the leaves of rice plants using the CTAB method (Doyle (1990), with modification). Approximately 0.05 g leaf sample was homogenized with 800 µL of the heated CTAB buffer (at 65°C) and placed into a microtube. The tube was immersed for 60 minutes at 65°C with inversion every 10 min. Subsequently, 400 µL CIAA solution (24/L; v/v) was added and mixed by vortex, then centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to the new microtube. Sodium acetate 3 M was added to the tube as much as 1/10 of the supernatant volume. After the addition of cold isopropanol as much as 2/3 of the total volume (supernatant + sodium acetate), the tube was turned back and stored in the freezer for 24 h. Furthermore, the tube was centrifuged at 12,000 rpm for 10 min and the supernatant was discarded. 500 µL ethanol 70% was added to the sample and the sample was centrifuged at 12,000 rpm for 5 min. The pellet was air dried and diluted with 50 µL sterile aquabides. The obtained DNA was quantified using a spectrophotometer.

2.2. DNA amplification (PCR)

Three microsatellite primers (RM 220, RM 224, and RM 252) related to color properties (Table 1) were used in this study. DNA amplification was performed in 10 µL total volumes with 5 ng/µL DNA concentration in 2.5 µL, 5 µL 2x GoTaq Green PCR mixture; 0.25 µL 10 mM forward primer; and 0.25 µL 10 mM reverse primer. The PCR program was 5-min denaturation at 94°C, followed by 35 cycles consist of denaturation for 1 min at 94°C, annealing process for 1 min at 55°C, extension process for 2 min at temperature 72°C, repetition of 2–4 steps 13 times, with touchdown program (temperature drop regularly) with difference of 0.5°C for each cycle, followed by last extension at 72°C for 7 min. The incubation step was at 4°C for 1 h and the last step was incubated at 10°C.

2.3. Visualization of amplified DNA from PCR

The Metaphore Agarose Electrophoresis Gel Electrophoresis (MAGE) method was used to examine the

TABLE 1 Microsatellite marker primers linked to the rice color.

No.	Primer	Chromosome	Product size (bp)	Sequence
1	RM 224	11	157	F : ATCGATCGATCTTCACGAGG R : TGCTATAAAAGGCATTCGGG
2	RM 220	1	127	F : GGAAGGTAACTGTTTCCAAC R : GAAATGCTTCCCACATGTCT
3	RM 252	4	216	F : TTCGCTGACGTGATAGGTTG R : ATGACTTGATCCCGAGAACG

Source: McCouch et al. (2002).

results of DNA amplification. To prepare the 2% gel, 2 g metaphore agarose was added in 100 mL 1x TBE buffer and heated slowly until clear and DNA dye (DNA stain 0.01%) was added to the gel solution. The gel solution was compressed by pouring into the mold, stored at 4°C for 30–60 min before use to obtain a better resolution. After the gel was ready to be used, the PCR results were loaded into the gel well and soaked into a 1X TBE buffer solution into a horizontal electrophoresis device and electrophoresed for about 60 min at 80 V and 400 A. The DNA amplification results were visualized under UV transilluminator and photographed.

2.4. Observation

The observation was performed to analyze DNA bands (homozygotes or heterozygous) of each individual including the female parent, male parent, and F_2 of the crossbreed on each microsatellite marker and then the resulting DNA bands were grouped into 3 groups: A = DNA band corresponding to the female parent (homozygous), B = DNA band corresponding to the male parent (homozygous), and H = DNA band corresponding to female parent and male parent (heterozygous). In addition to the grouping, observations were also made on the size of the DNA band and then the consequent data were converted to binary data (no band = 0; band present = 1).

Observation of the seed pericarp color from the F^2 population was morphologically determined by scoring (Table 2). In addition, quantification of seed color was performed to obtain the L*, a*, and b* color parameter numbers. Due to the limited seed samples, color quantification was done with a digital camera and Adobe Photoshop CS3 (Adobe Systems, USA) software.

TABLE 2 Microsatellite marker primers linked to the rice color.

Characteristic	Score	Description
Black	1	≥ 50% black color in one grain of rice; called of Black = B
Medium black	2	< 50% black color in one grain of rice; called of Medium Black = MB
Red	3	100% red color in one grain of rice, called of Red = R
White	4	100% white color in one grain of rice, called of White = W

2.5. Data analysis

Clustering analysis of the F_2 generation population of the crossbreed between black rice and white rice was calculated based on a percentage of heterozygous (H) offspring of the total sample. Regression analysis for color quantification (parameter L*, a*, and b*) with binary data from microsatellite marker for color properties were performed with multiple linear regression models (Gomez and Gomez 2007). Individual regression analysis for each microsatellite marker to determine the relationship between a certain microsatellite marker with the total anthocyanin content (which was visualized as the color of rice that was in the L *, a *, and b * color parameters) was also performed.

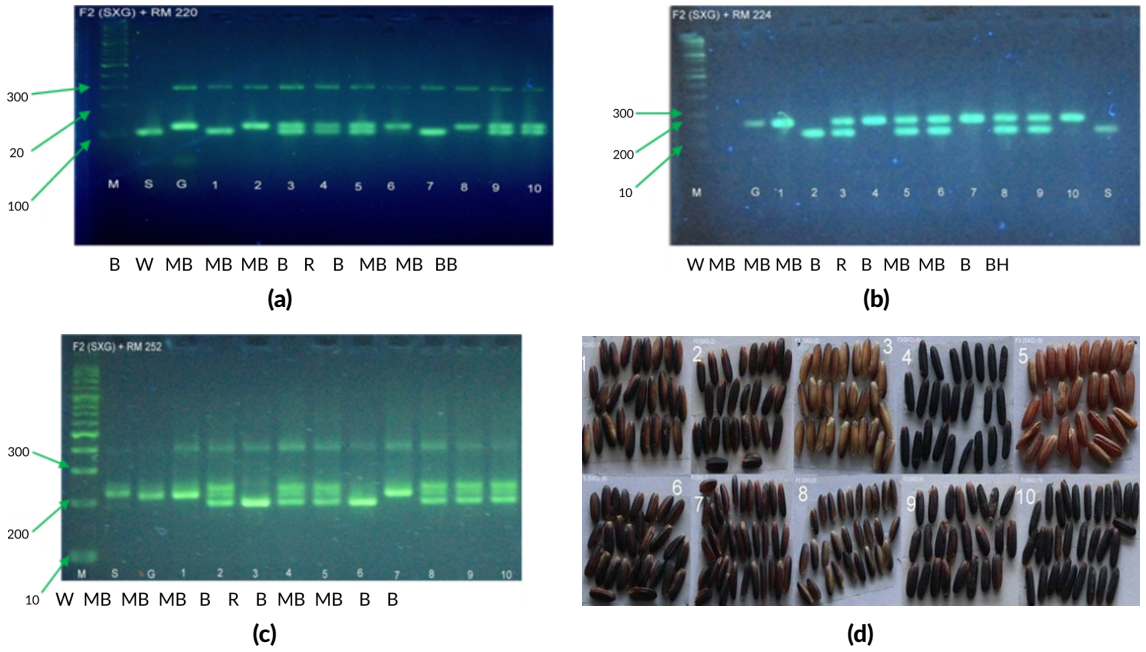

FIGURE 1 Profiles of DNA band amplified by three microsatellite markers using 2% metaphore agarose on the F_2 S × G plant. A1. RM 220; A2. RM 224; A3. RM 252; and A4. Pericarp color of seed grains. Scale 100 bp; S. Local black rice; G. White rice Situbagendit; 1-10 plant sample of F_2 S × G; B = Black; MB = Medium Black; R = Red; W = White.

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \quad (1)$$

where Y is the total anthocyanin content that was visualized as the color of the rice was is in L*, a*, and b* color parameters, $\beta_1 \cdots \beta_3$ is the regression coefficient of X_1 to X_3 variable, X_1 represents RM 220, X_2 represents RM 224, and X_3 represents RM 252.

The model used did not include intercepts and analyses were performed with SPSS version 16.1 (SPSS, USA) software.

3. Results and discussion

The parent population (black rice/S and white rice/G) and F_2 plant population were analyzed using microsatellite markers. A microsatellite marker can be used as an early selection tool to assess the heterozygosity of an F_2 generation resulted from crossbreeding between black rice and white rice. This molecular analysis was performed on a combination of cross S × G and reciprocal G × S (Figures 1 and 2). Methaphore agarose was used due to its high resolution in the DNA band separation. Based on the amplified microsatellite DNA bands, the heterozygosity analysis was performed.

The link between total anthocyanin content (expressed through the rice color parameters L*, a*, and b*) and a microsatellite marker was determined using regression analysis. Amplified DNA band data resulting from microsatellite markers (RM 220, RM 224, and RM 252) on F_2 S × G and F_2 G × S as independent variables (X) (Appendix A), and total anthocyanin content expressed through the color of F_2 S × G and F_2 G × S (L*, a*, and b* parameters) (Appendix B) as the dependent variable (Y) produced the regression equation that was good enough (Table 3).

Based on the regression equation, the color parameters (L*, a*, and b*) as the expression of the total antho-

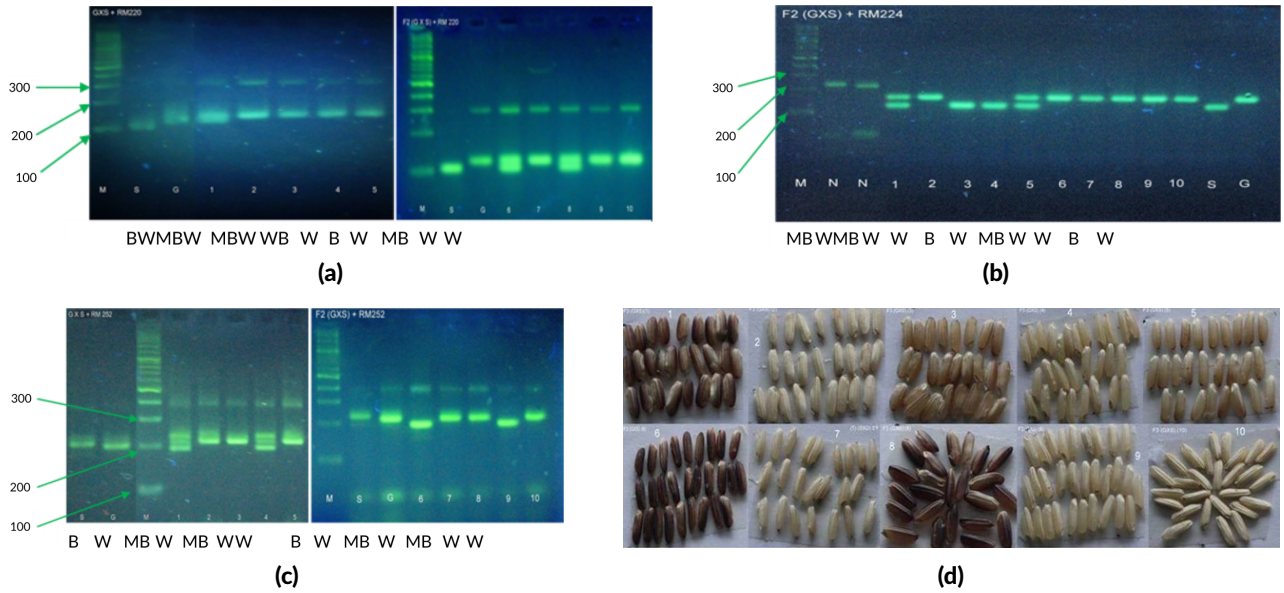

FIGURE 2 Profiles of DNA band amplified by three microsatellite markers using 2% metaphore agarose on the F_2 S × G plant. A1. RM 220; A2. RM 224; A3. RM 252; and A4. Pericarp color of seed grains. Scale 100 bp; S. Local black rice; G. White rice Situbagendit; 1-10 plant sample of F_2 S × G; B = Black; MB = Medium Black; R = Red; W = White.

TABLE 3 Microsatellite marker primers linked to the rice color.

F_2 generation	Dependent Variable (Y)	Independent Variable (X) Regression coefficient			R^2	r
		RM 220	RM 224	RM 252		
S × G	L*	10.0	10.61	−1.72	0.8967*	0.9469*
	a*	0.474	3.45	2.29	0.6507*	0.8066*
	b*	0.57	5.58	−4,29	0.5271	0.7726
G × S	L*	44.0*	0.054	0.1*	0.8354*	0.914*
	a*	2.669*	4.374	−1,08	0.6419*	0.8011*
	b*	11.96*	−3.813	0.1*	0.8999*	0.9486*

L*: lightness, a*: green color (negative value) to red (positive value), b*: blue color (negative value) to yellow (positive value).
*: significant at α = 5%.

TABLE 4 The value of determination coefficient (R^2) in the regression analysis for each microsatellite marker on the color parameter on F_2 S × G and F_2 G × S.

F_2 generation	Dependent Variable (Y)	Independent Variable (X) R^2 Value		
		RM 220	RM 224	RM 252
S × G	Parameter L*	0.8903*	0.9190*	0.9014*
	Parameter a*	0.5912	0.6502	0.6480*
	Parameter b*	0.3128	0.4089	0.4345
G × S	Parameter L*	0.8466*	0.7759	0.8543*
	Parameter a*	0.4984**	0.6178*	0.6625**
	Parameter b*	0.9051*	0.8972*	0.9644*

* = significant at α = 5%
** = significant at α = 10%

cyanin content had a close relationship (r value) with the three microsatellite markers, greater than 75% in F_2 S × G and F_2 G × S. The diversity of the color parameters (L*, a*, and b*) of more than 50% (R^2 value) was caused by the microsatellite markers in the crossbreeds of F_2 S × G and F_2 G × S. Table 2 shows that the apparent regression coefficients were significant in the RM 220 and RM 252 microsatellite markers with the parameters L* and b*, whereas the crossbreed of F_2 S × G was not significant. This implies that the RM 220 and RM 252 microsatellite markers were related to the total anthocyanin content disclosed in the L* and b* color parameters.

The regression equation of Table 3 together from the three microsatellite markers as the X variable and the color parameters (L*, a*, and b*) as partial Y variable was not significant in either the F_2 S × G or F_2 G × S cross. However, in separate analysis for each microsatellite marker showed the relationship between X and Y variable based on the coefficient of determination (Table 4). Tuinstra et al. (1996) used R^2 values to explain the percentage of phenotype diversity that can be explained by RAPD markers for dry properties in sorghum.

The use of microsatellite markers in the F_2 plant population resulting from crossbreeding black rice with white rice had two purposes; the first purpose was to verify the microsatellite marker using the color properties of the rice, and the second purpose was to verify the results of the crossbreeding to determine whether the F_2 offspring were heterozygous. The heterozygous character can be observed in the resulting DNA bands in that there are DNA bands like those of both parents (both female and male parent). This is an advantage of microsatellite markers that have the character of codominant means to determine a hererozygous or homozygous of individuals. An interpretation of the observations based on the results of DNA amplification using the RM 220, RM 224, and RM 252 microsatellite markers (Figures 1 and 2) is shown in Table 5 and Table 6.

The number of individual plants showing a pattern in the F_2 S × G plant generation was 50% (RM 220), 40% (RM 224), and 60% (RM 252). As for F_2 G × S, the percentage of individuals was 20% for RM 220, RM 224, and RM 252 (Table 6). These results suggest that both the RM 220 and RM 252 microsatellite markers have the potential to be used as microsatellite markers associated with the black color in rice, and therefore can be used in Marker Assisted Selection (MAS).

TABLE 5 Results of DNA amplification with microsatellite marker on F_2 S × G plant.

Microsatellite marker	Parent and individual of F_2 S × G												Percentage of heterozygous (H)
	S	G	1	2	3	4	5	6	7	8	9	10	
RM 220	A	B	A	B	H	H	H	B	A	B	H	H	50%
RM 224	A	B	B	A	H	B	H	B	B	H	H	B	40%
RM 252	A	B	B	H	B	H	H	B	A	H	H	H	60%

S. Female parent; A: DNA band as parent S; G. Male parent; H: Heterozygous, DNA band as S and G parent; B: DNA band as parent G.

TABLE 6 Results of DNA amplification with microsatellite marker on F_2 G × S plant.

Microsatellite marker	Parent and individual of F_2 G × S												Percentage of heterozygous (H)
	S	G	1	2	3	4	5	6	7	8	9	10	
RM 220	B	A	B	B	B	B	B	H	B	H	B	B	20%
RM 224	B	A	H	B	A	A	H	B	B	B	B	B	20%
RM 252	B	A	H	A	A	H	A	B	A	A	B	A	20%

S. Female parent; A: DNA band as parent S; G. Male parent; H: Heterozygous, DNA band as S and G parent; B: DNA band as parent G.

4. Conclusions

Microsatellite markers (RM 220 and RM 252) were linked to the color properties of rice (parameters L*, a*, and b*), as well as the total anthocyanin content. The number of individual plants showing a pattern in the F_2 S × G plant generation was 50% (RM 220), 40% (RM 224), and 60% (RM 252). The RM 252 microsatellite marker was effectively used for DNA-assisted selection (Marker Assisted Selection) on the crossbreed of black rice with white rice.

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Authors' contributions

K, T, PB, RHM designed the study. K carried out the laboratory work. K, T, PB, RHM analyzed the data. K wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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Appendix A. Binary data of DNA amplification results

TABLE A1 Binary data of DNA amplification results using three microsatellite markers on F_2 S × G plants.

Sample no.	RM 220			RM 224			RM 252			
	120	.40	300	200	210	200	210	230	240	400
1	1	0	1	0	1	0	0	0	1	1
2	0	1	1	1	0	1	0	0	1	0
3	1	1	1	1	1	1	0	0	0	1
4	1	1	1	0	1	1	0	0	1	1
5	1	1	1	1	1	1	0	0	1	1
6	0	1	1	1	1	1	0	0	0	1
7	1	0	1	0	1	0	0	0	1	1
8	0	1	1	1	1	0	1	0	1	1
9	1	1	1	1	1	0	1	0	1	1
10	1	1	1	0	1	0	1	0	1	1
Parent S	1	0	0	1	0	0	0	0	1	0
Parent G	0	1	1	0	1	0	0	1	0	0

TABLE A2 Binary data of DNA amplification results using three microsatellite markers on F_2 G × S plants.

Sample no.	RM 220			RM 224			RM 252		
	110	130	300	120	155	200	220	250	400
1	0	1	1	1	1	1	1	1	1
2	0	1	1	0	1	0	1	0	1
3	0	1	1	1	0	0	1	0	1
4	0	1	1	1	0	1	1	1	1
5	0	1	1	1	1	0	1	1	1
6	1	1	1	0	1	0	0	0	1
7	0	1	1	0	1	0	0	0	1
8	1	1	1	0	1	0	0	0	1
9	0	1	1	0	1	1	0	0	1
10	0	1	1	0	1	0	0	0	1
Parent S	1	0	0	1	0	0	1	0	0
Parent G	0	1	1	0	1	1	0	0	1

Appendix B. Observation data of color parameters

TABLE B1 Observation data of color parameters of molecular example in plant F_2 S × G.

No.	Color parameter		
	L*	a*	b*
1	13.17	3.83	2.42
2	10.00	3.92	0.83
3	31.17	7.92	13.5
4	10.75	−0.50	−2.00
5	30.75	14.42	12.17
6	14.33	2.83	0.08
7	23.50	6.08	1.50
8	21.92	3.83	2.50
9	17.33	2.08	−0.17
10	17.42	1.67	−1.33

TABLE B2 Observation data of color parameters of molecular example in plant F_2 G × S.

No.	Color parameter		
	L*	a*	b*
1	30.08	5.75	6.58
2	63.92	-1.08	8.08
3	34.00	6.25	14.42
4	54.00	0.67	9.50
5	42.92	2.75	11.75
6	2.07	5.67	3.12
7	55.58	0.42	9.12
8	23.23	5.50	3.67
9	56.00	-0.08	9.58
10	63.75	-1.00	8.83