



Relationship between physicochemical characteristics and gene expression in lettuce as a tool for genetic improvement

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Abstract

Lettuce (*Lactuca sativa* L.) is a leafy vegetable that contains phenolic compounds, flavonoids and anthocyanins, which are useful for human health when consumed. These physico-chemical characteristics gives to the plant a significant potential to be improved genetically. Thus, the objective of the present work was to study the relationship between physical-chemical characteristics (color parameters and anthocyanins contents) and gene expression in lettuce, to identified simple tools to be used in breeding programs. The study was carried out in a segregating population (F₂) from the cross between two lettuce cultivars contrasting in terms of leaf color (red and green). A high heritability for the studied characteristics, was identified, which would facilitate the selection of genotypes of interest. The anthocyanin content in lettuce leaves is controlled by more than one gene with partial dominance of the genes that confer higher levels. The intense red color can be used as an alternative in lettuce breeding programs to identify superior lettuce genotypes with a high anthocyanin content. Transgressive segregation in the F₂ population allows to select superior individuals with a high expression on physical and chemical characteristics of interest.

Keywords: *Lactuca sativa* L, bioactive compounds, phytochemicals

Introduction

Among the leafy vegetables, lettuce stands out as one of the most consumed in the world (Baslam et al., 2013; Pinto et al., 2014; López et al., 2014). In Brazil, lettuce crops occupies approximately 35,000 hectares (Valeriano et al., 2016). In addition to contributing with essential micronutrients, lettuce also has compounds that does not have a nutritional function but promote benefits to human health, such as preventing the emergence of chronic diseases. Such compounds, known as bioactive compounds, results from secondary metabolism and have roles in plant defense against ultraviolet radiation, pests and pathogens (Taiz & Zieger, 2009; Kruger et al., 2014; Zhang et al., 2015; Kim et al., 2016). On human diet, they also present antioxidant activity, disarming free radicals, that can be reactive oxygen species (ROS), which are oxidant agents with damage potential to some macromolecules, such as: lipids, proteins, and DNA

(Zhang et al., 2015).

In vegetable tissues, the orange-red to blue-violet colors are determined by the presence of anthocyanin, polyphenols from the flavonoid class that belongs to the phenolic compounds group and have antioxidant activity (Rocha, 2015; Figueiredo & Lima, 2015; Dai et al., 2016).

A few works propose the expression of genes that controls the lettuce leaf color, such as the classical work of Thompson (1938), cited by Ryder (1999). According with this work, a complementary gene pair controls the anthocyanin presence or absence (CcGg) and a multiple allelic system controls the content distribution on the plant. Zhang et al. (2016) identified 34 genes related to the anthocyanin metabolic route, implying the quantitative nature of the genic expression, which fit a Poisson normal distribution. Thus, it is important to know whether these same genes that control color, also control

the anthocyanin content on leaves.

Mou (2005) suggested indirect selection for high carotenoids content in lettuce, through selection for higher chlorophyll content or green color. It can facilitate the work of lettuce breeders, due to the greater practicality and the lower cost in comparison to the classical quantification methods. In this case, breeders could perform selection for color values, obtained by a colorimeter.

Thus, the objective of the present work was to study the relationship between physical-chemical characteristics (color parameters and anthocyanins contents) and gene expression in lettuce, to identified simple tools to be used in breeding programs.

Material and Methods

The research was carried out from 2015 to 2017, at the Universidade Federal de São João del - Rei, campus Sete Lagoas, located in Sete Lagoas City, Minas Gerais State, Brazil (19° 27' 57" South and 44° 14' 48" West).

In order to obtain the segregant population, two contrasting genotypes regarding leaf color were crossed by the wash depollination method (Nagai, 1980), since lettuce is an autogamous cleistogamic species.

The parents used was both curly leaf lettuces: a commercial cultivar (Cristal) that have light green leaves, and an experimental genotype of intense red color (Rubra). The male parent was Rubra, donor of the marker gene. Generations F1 and F2 were obtained from the combination mentioned above. Posteriorly, plants from the breeding generations (F1 and F2) and his parents (P1 and P2) were cultivated simultaneously, providing material for the assessments.

Seedlings production occurred on 128-cell polystyrene trays filled up with commercial substrate. Leaf color on the seedling stage provide identification of F1 plants. On open field, 200 plants of the segregant generation (F2) plus F1 generation and the parents were cultivated and assessed for the following characteristics:

Leaf color: measured with a colorimeter (Konica Minolta model CR- 410- China) according with the classification proposed by the CIE (Commission Internationale de l'Eclairage), represented by the following equations:

$$L^*C^*_{ab}h_{ab}, \text{ where}$$

L^* = Luminosity;
 C^*_{ab} = Chroma;
 h_{ab} = hue angle.

$L^*a^*b^*$, where:

L^* = Luminosity;
 a^* = red/green coordinate (+a indicates red and - a indicates green);
 b^* = coordinate yellow / blue (+b indicates yellow and - b indicates blue).

Color analysis were performed at the Laboratory of Food Conservation after harvest, where three leaves of every single plant were analyzed to obtain the color patterns of the 200 segregating genotypes. Analysis was standardized by using the central region of the 600 leaves.

Genetic parameters: dataset from the populations P_1 , P_2 , F_1 , and F_2 were submitted to the software GENES (Cruz, 2013), in order to obtain genetic (V_G), environmental (V_E), and phenotypic (V_P) variances, such as broad-sense heritability (h^2b), according to the following expressions:

$$V_E = (V_{F1} + V_{P1} + V_{P2})/3$$

$$V_G = V_P - V_E$$

$$h^2b = V_G/V_P$$

Anthocyanin content: determined by the method described by Francis (1982), where a homogenized sample (0.5 g) is add to a medium of 10 ml of ethanol acidified with HCl (85:15 v/v) and a standby of 24 hours on a refrigerator and light absence. Absorbance of the samples were determined in spectrophotometer (FENTO 700S) at 535nm wavelength. Using the absorbance (Abs) reads, we estimated the anthocyanin contents by the following equation:

$$At = (Abs * f) / \epsilon,$$

where:

At = anthocyanin content, in mg of cyanidin-3-glycoside.100g⁻¹ of fresh matter;

f = dilution factor;

ϵ = molar absorptivity coefficient of cyanidin - (98.2 L.mol⁻¹.cm⁻¹).

Flavonoid content: for quantification of flavonoids is used the same methodology of anthocyanin, but on the 374 nm wavelength.

Spearman's correlation: calculated by the formula

$$\rho = 1 - \frac{\sum_{i=1}^n d_i^2}{n^3 - n}$$

where:

ρ is the Spearman's correlation coefficient;

d_i is the difference between the ordinations;

n is the number of ordination pairs.

Results and Discussion

On color analysis (L^* , a , h_{ab} , a^* , b^*) the Cristal cultivar presented the higher values, with exception to a^* , which indicates plants with a light green color (Table 1).

Table 1. Mean values of L^* , h_{ab} , a^* , and b^* .

Genotype	L^*	h_{ab}	a^*	b^*
Cristal	53.23	106.29	-7.80	26.70
Rubra	36.09	54.26	4.52	6.27
F1 Generation	45.51	93.71	-1.35	16.39

L^* = Luminosity; h_{ab} = tonality angle of color diagram; a^* = horizontal axis of color diagram; b^* vertical axis of color diagram.

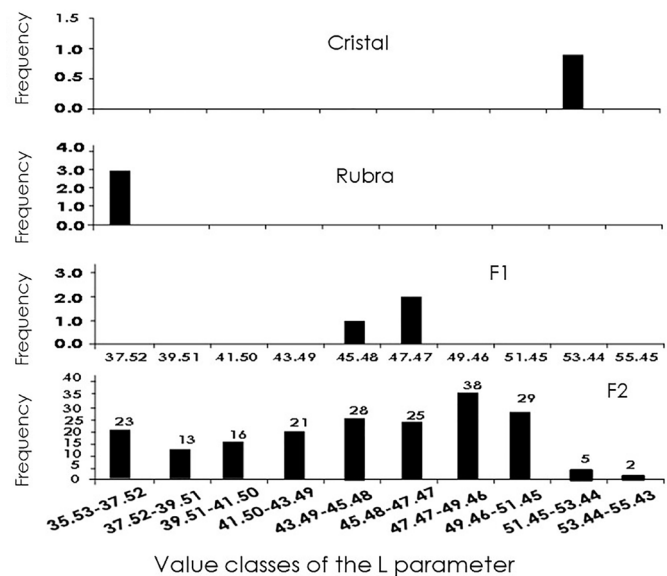


Figure 1. Distribuição de frequência para o parâmetro L^* em alface referente ao cruzamento Cristal x Rubra, parentais e gerações F1 e F2.

Martins et al. (2017) working with the same parent genotypes, have found similar data regarding luminosity, with a mean value of 52.88 for Cristal and 23.20 for Rubra. The F1 generation presented intermediate values in relation to the parents (Figures 1, 2, 3 and 4), which indicates that the color parameters assessed (L^* , h_{ab} , a^* , b^*) does not have complete dominance (Table 2).

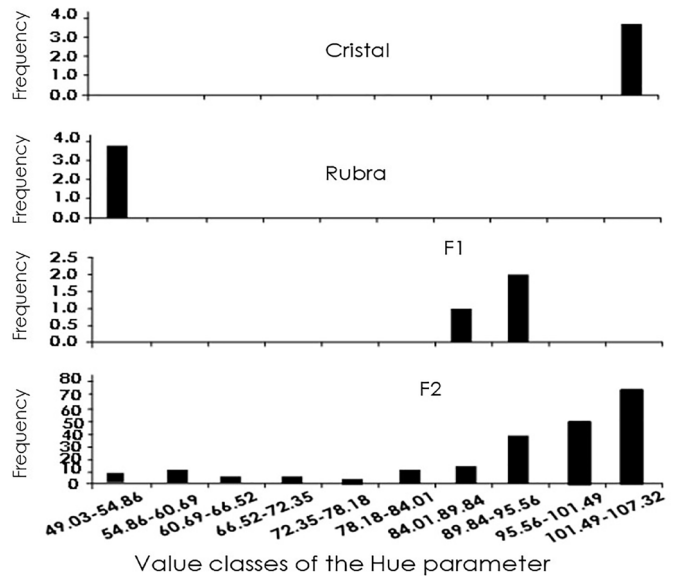


Figure 2. Distribuição de frequência para o parâmetro h_{ab} em alface do cruzamento "Cristal x Rubra", parentais, gerações F1 e F2.

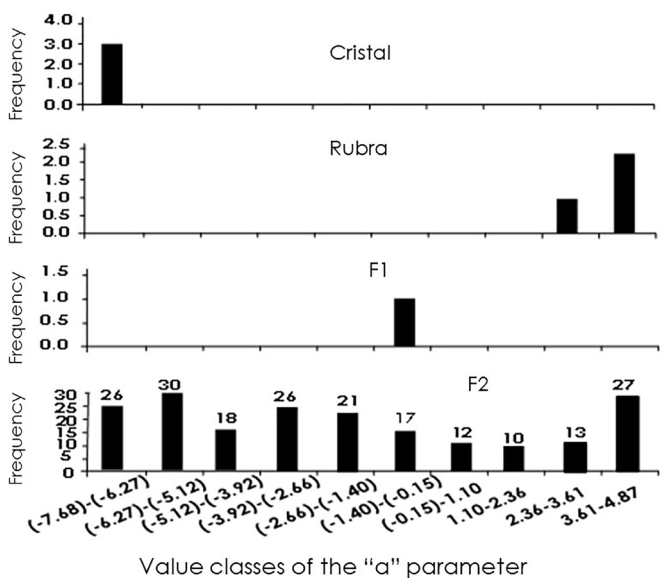


Figure 3. Distribuição de frequência para o parâmetro a^* em alface do cruzamento "Cristal x Rubra", parentais, gerações F1 e F2.

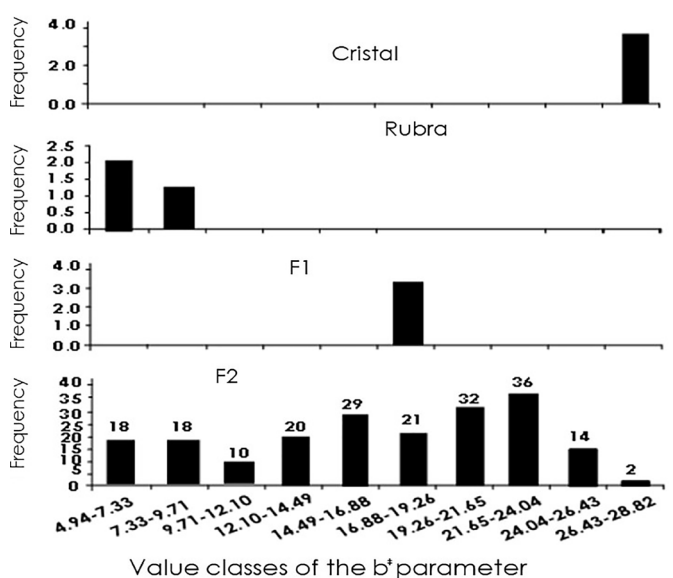


Figure 4. Distribuição de frequência para o parâmetro b^* em alface do cruzamento "Cristal x Rubra", parentais, gerações F1 e F2.

Table 2. Additive (a), and dominance (d) effects, plus average degree of dominance (GMD) for L*, h_{ab}, a*, and, b*.

	Allelic interaction parameters			
	L*	h _{ab}	a*	a*
a	8.57	26.02	6.16	10.22
d	0.85	13.44	0.29	-0.09
ADD**	0.10	0.52	0.05	-0.01

**Average degree of dominance (d/a).

According to Ramalho et al. (2012), to characterize complete dominance, the F1 generation must have a mean value equal to the parent with the dominant allele.

Additive allelic interaction not occurred in the studied parameters, since none "d" value was equal to "a" (Table 2). It seems to occur allelic interaction of partial dominance type, considering that the "d" values are higher than zero and lower than "a", except for b*. The ADD values higher than zero shows allelic interaction of partial dominance type: the closer to zero it is, lower is the degree of dominance and closer to one, greater is the degree of dominance.

Segregation of F2 generation ranged from the lower L value (35.53) from the higher value (55.43) observed in all generations studied. Considering that the F2 plants present variable distribution, it is possible to identify a relative higher number of plants in the higher classes (Figure 1), that corroborates with data of Table 2, which shows partial dominance of the characteristic.

On this sense, the segregating generation (F2) show a tendency to present plants more similar to Cristal due to partial dominance. Transgressive segregation on both directions occurred for this characteristic, once there are extreme values higher to Cristal and extreme values lower to Rubra. Among the F2 plants, four plants presented values of L* higher than Cristal, and other seven plants presented values of L* lower to Rubra.

In relation to the parameter h_{ab} (Hue angle), observed values in the F2 segregating generation varied from 49.03° to 107.3° (Figure 2). Gazula et al. (2007), evaluating 12 lettuce varieties with color tones from green to dark-red, obtained a variation that ranged from 6.59° to 133.18°. Cristal presented a mean value of 106.29°, which comprises a region between green and yellow. Rubra hue angle placed on an intermediate position to red (0°) and yellow (90°), with a value of 54.25°. This indicates a partial dominance of the characteristic, which corroborates with Ryder (1999).

We also observed a higher number of plants in the classes of high values, similar to Cristal cultivar with a green color (Figure 2). Eight plants presented values lower than Rubra, indicating transgressive segregation for this

characteristic.

According to obtained data, focusing on the a* values, the plants ranged the color patterns corresponding from red (positive values) to green (negative values), with a variation comprising of 7.8 to 4.82 (Figure 3). Based on the a* frequency, it is possible to observe that there is a slight tendency of a higher number of plants similar to Cristal (Figure 3). This suggests dominance over the green color, but with a low dominance degree, as shown in Table 2.

The b* values shows a distribution with a slight tendency to positive extreme values (Figure 4) as expected, since a partial dominance exists, as shown by the ADD, "a", and "d" values. As values of "a" e "d" classifies the type of genic interaction, for the a* parameter of colorimeter, it is possible to conclude that exists a partial dominance of 'Rubra' color in relation to green color.

In relation to anthocyanin content in the leaves, the dataset does not fit a Poisson's normal distribution (Figure 5). That probability happened due to action of more than one gene over the characteristic with different intensities (Ryder, 1999). Anthocyanin content of the hybrid (F1) was intermediate in relation to the parents (Figure 5), with a little superiority in relation to green parent (Cristal), indicating partial dominance of character (Table 3), like the observed in color parameters (Table 2).

Table 3. Additive (a), and dominance (d) effects plus average degree of dominance (ADD) for anthocyanin content.

Allelic interaction parameters	
a	46.93
d	-14.27
ADD**	-0.30

**Average degree of dominance (d/a).

On the F2 segregant population, the anthocyanin content ranged from 6.37 to 141.68 mg.100 g⁻¹ on fresh sample (Figure 5). Occurred a higher tendency in classes with low anthocyanin content, like the obtained in the parameter h_{ab}, which had higher occurrence of plants similar to parent 'Cristal'.

The non-normal distribution of this character may present an allelic interaction of partial dominance type, as shown by the values of "a", "d" and "ADD" (Table 3). The anthocyanin content of Cristal (12.64 mg.100g⁻¹), shown in Table 4, is similar to found by Gazula et al. (2007) (13 mg.100g⁻¹). Probably, this value was not sufficient to express a visible 'Rubra' color to the leaves, predominating the green color.

Table 4. Means of Anthocyanin content.

Genotype	Anthocyanin*
Cristal	12.64
Rubra	106.49
F1 Generation	45.30

*Values expressed in mg.100 g⁻¹ of sample.

In the F2 population, eight plants presented transgressive segregation, with values higher to the male parent (Rubra). According with Oliveira et al. (2019), an oligogenic or polygenic pattern is responsible for the anthocyanin content in lettuce leaves. The genic interactions related to expression of chlorophyll and anthocyanin contents, are predominantly a partial

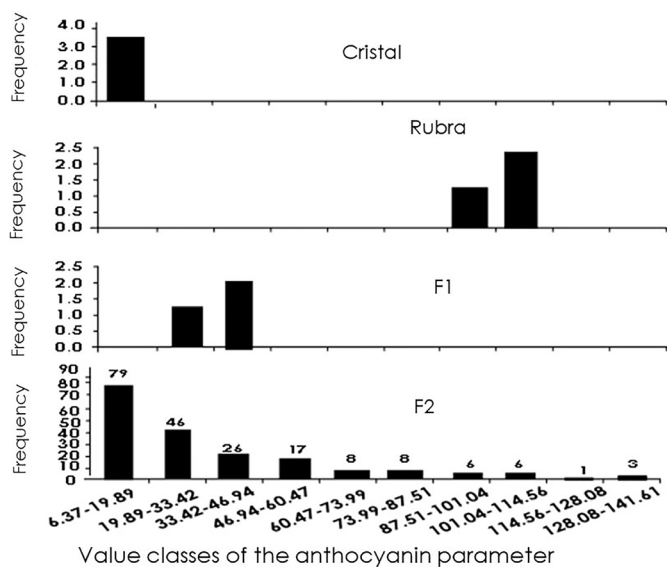


Figure 5. Distribuição de frequência para teores de antocianinas em alface do cruzamento "Cristal x Rubra". Parentais, gerações F1 e F2.

As can also be observed, most of F2 genotypes presented lower values than the mean. The distribution follow the expected according to "a", "d" and "ADD" values (Table 5), being that explained by the occurrence of partial dominance of 'Rubra' over 'Cristal' for this character.

Table 5. Additive (a), and dominance (d) effects plus average degree of dominance (ADD) for flavonoid content.

Allelic interaction parameters	
a	107.64
d	-10.46
ADD**	-0.10

**Average degree of dominance (d/a).

Discrepancy between segregation of two characteristics (flavonoid and anthocyanin), even that one is included on other, may be explained by the fact that this class present at least eight compounds (Bilyk & Sapers, 1985; Hertog et al., 1992; Ferreres et al., 1997; Crozier et al., 1997; Nicolle et al., 2004), which probably contributes to change the compounds contents in different directions.

dominance type.

Histograms of the h_{ob} parameter and anthocyanin content are similar, with a slight difference on classes with extreme values, in which was observed polarization on higher classes for h_{ob} , and on lower classes for anthocyanin content (Figures 2 and 5). This occur because the variation in leaf color affects anthocyanin content.

In the segregant population (F2), total flavonoid content ranged from 26.99 to 393.91 mg.100g⁻¹ of fresh sample (Figure 6). Frequency distribution observed was nearly of a normal Poisson's normal distribution, presenting a slight tendency to classes of low flavonoids content.

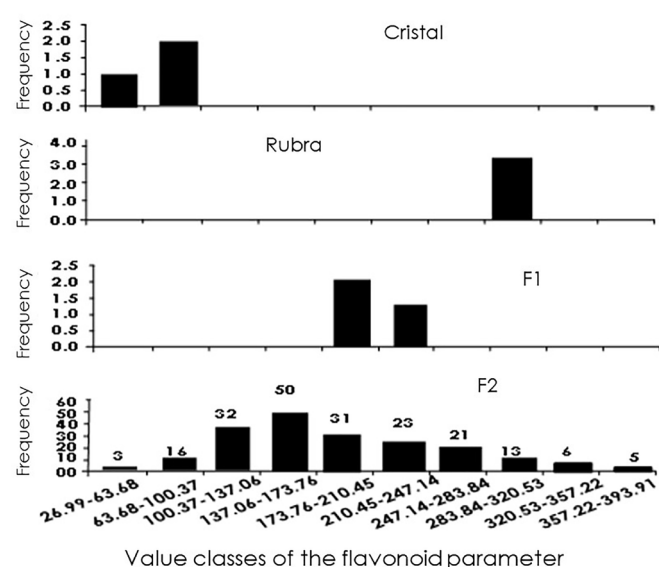


Figure 6. Distribuição de frequência para teores de flavonoides em alface do cruzamento "Cristal x Rubra", parentais, gerações F1 e F2.

Among the F2 genotypes evaluated, 19 was identified as superior to Rubra, which indicates transgressive segregation for this character. Genotypes with either low or high anthocyanin content not necessarily are the same that have low or high flavonoid content. On this sense, more genes influences flavonoids than the anthocyanin class, which explains the difference between the observed frequencies, probably are influencing the flavonoids class. This character presented the higher variances and an intermediate heritability estimates among the studied characters (Table 6).

In relation to heritability and variances, most of characteristics presents high heritability (Table 6). On the other hand, variances allows recognizing genotypic variance, which agree with heritability data. Genotypic variance between the genotypes of segregant population was found to be expressive (Table 6).

The strongest correlations (± 0.82) with anthocyanin was of the parameters h_{ob} and a^* , but with a negative

value for h_{ab} . Therefore, as higher the anthocyanin content, more red is the lettuce plant (Table 7).

The parameter a^* presented a strong and positive correlation with the anthocyanin content (0.820).

Since the values of axis of a^* and b^* are closely linked, as foreseen, the coefficient between this parameters shows a strong and negative (-0.962) correlation (Table 7).

Table 6. Variances and broad-sense heritability of studied variables on the F2 segregating population (Rubra x Cristal).

Variable	Phenotypic Variance	Genotypic Variance	Environmental Variance	Heritability
L*	23.77	22.57	1.2	94.95
h_{ab}	280.82	279.73	1.08	99.61
a^*	15.16	15.03	0.127	99.1
b^*	36.55	1.64	34.9	95.4
Anthocyanins	982.45	539.74	442.7	54.93
Flavonoids	5801.39	4815.01	986.38	82.99

Table 7. Spearman's correlations (r) between the studied variables.

Variables		r	
L	X	a^*	-0.946
h_{ab}	X	a^*	-0.987
L	X	Anthocyanin	-0.780
h_{ab}	X	Anthocyanin	-0.821
a^*	X	Anthocyanin	0.820
b^*	X	Anthocyanin	-0.784
a^*	X	b^*	-0.962
L	X	Flavonoid	-0.578
h_{ab}	X	Flavonoid	-0.575
a^*	X	Flavonoid	0.578
b^*	X	Flavonoid	-0.576
Anthocyanin	X	Flavonoid	0.746

In addition, anthocyanin content affected the luminosity of leaves, as shown by a strong and negative correlation between the two variables. Thus, as higher the anthocyanin content is, more dark is the leaf. By the relation between the anthocyanin and flavonoids content, it is possible to affirm that had a discrepancy in relation to the observed frequency in the population (Figures 5 and 6). The correlation between this two variables (Table 7) was high (0.74), considering that anthocyanin are present on the flavonoids class.

Conclusions

The anthocyanin content in lettuce leaves is under control by more than one gene, with partial dominance from the genes that confers highest contents.

The characteristics studied present high heritability, which allows efficient selection of interesting genotypes.

Intense red color is an alternative to identify lettuce genotypes with high anthocyanin content in breeding programs.

Transgressive segregation in the F2 population allows selecting superior individuals for the studied physicochemical characteristics.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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