

Article

Anatomical and image analysis of grain coloration in rye

Pavel A. Zykin ¹, Elena A. Andreeva ^{1,2*}, Natalia V. Tsvetkova ^{1,2} and Anatoly V. Voylovkov ²

¹ Faculty of Biology, St. Petersburg State University, Universitetskaya nab.7/9, St. Petersburg 199034, Russia; pavel.zykin@gmail.com (P.A.Z.); ntsvetkova@mail.ru (N.V.T)

² Laboratory of Plant Genetics and Biotechnology, Vavilov Institute of General Genetics Russian Academy of Sciences, Gubkina st. 3, Moscow 119991, Russia; voylovkov@vigg.ru

* Correspondence: elena.alex.andreeva@gmail.com; Tel.: +7-921-747-9981

Abstract: In rye, there is a considerable variety of grain color which is determined by the diversity of compounds localized in different parts of the grain (caryopsis) - pericarp, testa, and aleurone. The localization of anthocyanins and proanthocyanidins was analyzed in 26 rye samples with identified anthocyanin genes, along with the analysis of CIE color coordinates. The Grain Scan program [1] was used to analyze images of individual grains. The localization of anthocyanins and proanthocyanidins was studied on longitudinal and cross sections of grains using light microscopy and MALDI-imaging. The violet-grained samples contain anthocyanins in the pericarp, and the green-grained samples contain anthocyanins in the aleurone layer. The green, violet and yellow-grained rye, with the exception of two anthocyaninless mutants vi3 and vi6, shows the presence of proanthocyanidins in the brown-colored testa. Four main color groups of the rye grains (yellow, green, brown, violet) could be differentiated using the color coordinate h° (hue angle). Interspecies and intraspecies variability for the localization of colored flavonoids in cereal grains is discussed.

Keywords: rye, image analysis, grain color, anthocyanins, proanthocyanidins

1. Introduction

Grain color in cereals is determined by the pigments synthesized in the maternal (fruit and seed coats) and hybrid (aleurone layer and starchy endosperm) tissues. Blue-purple anthocyanins, red-brown proanthocyanidins and phlobaphenes accumulate in the upper layers of grains along with yellow glycosides of flavones, flavonols and flavanols. Carotenoids determine the yellow color of the endosperm. In addition to the pigments, colorless copigments, complexes of pigments and copigments with metal ions, pH-value in different tissues and the structure of the surface and the endosperm can affect the preception of grain color by human eyes. According to the study done on a huge number of accessions from Vavilov Institute of Plant Industry collection of rye [2], the grains could be classified by it's color as "green, yellow, white, purple, blue, brown, black and transitional: yellow-green, light green, gray-green, dark green, yellow-brown, light brown". In most open-pollinated varieties there are grains of only three main colors: yellow, green (gray-green) and rarely – brown. Some time ago in Germany and Russia the varieties with constant white, yellow, green, blue and brown color of grain were produced [2]. However in the available literature there is no data available concerning the distribution of pigments in tissues of rye grains with different colors. In the Peterhof genetic collection of rye [3] self-fertile lines with violet (purple), brown, green and yellow coloring of the grain are being reproduced. The genetic nature of anthocyanin pigmentation of these lines [3,4] as well as the composition of anthocyanins were established [5]. The purpose of our study is to analyze the localization of pigments in the tissues of the rye grains and to develop a method of digital evaluation of their color. These tasks reflect the growing interest to cereal flavonoids due to their role in plant environmental adaptation, domestication and possible health benefits and risks of whole-grain consumption.

2. Results

2.1. Color coordinates analysis

Data on the digital evaluation of the grain color are presented in Table 1.

Table 1. CIE color coordinates of grains in rye inbred lines.

Accession #/name ¹	L*	a*	b*	C*	h°
1 <i>vi1</i>	49.09±2.12	3.69±0.54	15.19±0.60	15.64±0.64	76.36±1.83
2 Esto	48.24±2.23	3.83±0.70	15.56±0.82	16.04±0.90	76.23±2.08
3 <i>vi2</i>	45.74±1.96	3.86±0.75	15.46±0.64	15.95±0.73	76.06±2.39
4 <i>vi3</i>	50.14±1.75	3.19±0.48	17.27±0.68	17.56±0.72	79.56±1.39
5 <i>vi4</i>	49.24±2.54	3.06±0.38	14.80±0.52	15.11±0.56	78.35±1.19
6 <i>vi5</i>	48.66±3.18	3.81±0.76	16.16±1.09	16.62±1.18	76.80±2.05
7 <i>vi6</i>	50.73±2.11	3.29±0.83	15.66±1.04	16.02±1.10	78.18±2.64
Mean 1-7	48.83 a ²	3.53 a	15.73 a	16.13 a	77.36 a
8 Ra-2/63	45.10±2.55	3.02±0.57	14.66±1.17	14.98±1.19	78.36±2.03
9 T-23	46.76±1.95	2.93±0.45	13.35±0.85	13.67±0.90	77.64±1.48
10 line 5	46.07±2.22	3.82±0.91	14.74±0.96	15.25±1.05	75.57±3.04
11 GC-12	45.04±2.53	3.06±0.50	14.69±1.16	15.01±1.17	78.22±1.76
Mean 8-11	45.74 a	3.21 a	14.36 ab	14.73 ab	77.45 a
12 GC-37	39.80±4.06	4.70±0.78	14.65±0.82	15.40±0.85	72.24±2.78
13 Rmu1b1	44.44±2.79	4.10±0.62	14.87±0.13	15.43±1.20	74.53±2.40
14 Rmu1b2	38.93±3.47	4.80±0.69	15.40±0.99	16.14±1.08	72.74±1.94
15 <i>vi2Vs</i>	36.81±3.15	5.05±0.80	15.72±0.83	16.53±0.84	72.22±2.44
Mean 12-15	40.00 b	4.66 b	15.16 ab	15.88 ab	72.81 c
16 S10g	48.60±2.27	2.18±0.66	13.10±0.93	13.29±0.98	80.65±2.52
17 P-168	43.99±2.48	2.74±0.74	15.13±1.02	15.38±1.24	79.87±4.65
18 Line 301	47.07±2.05	1.96±0.42	13.51±0.70	13.66±0.73	81.77±1.55
19 Line 87	49.46±2.32	2.02±0.63	14.86±0.99	15.00±1.06	82.36±1.95
20 Rmu1g	49.06±2.32	1.87±0.51	12.65±1.04	12.80±1.06	81.64±2.05
21 Rmu5	46.43±2.09	2.27±0.62	13.03±0.94	13.24±1.01	80.24±2.13
22 Rmu26	45.14±2.68	1.91±0.87	14.28±1.32	14.43±1.40	82.58±2.98
Mean 16-22	47.11 a	2.14 c	13.79 b	13.97 bc	81.30 d
23 V-49	33.53±3.52	5.05±0.94	13.31±1.06	14.26±1.11	69.23±3.52
24 V-5	32.19±2.85	6.01±0.76	12.11±1.14	13.54±1.28	63.54±3.3
25 Rmu12	24.06±2.36	3.02±0.97	8.24±1.00	8.80±1.22	70.36±4.69
26 Rmu13	25.02±3.44	4.61±1.20	9.06±1.55	10.20±1.81	63.30±4.33
Mean 23-26	28.70 c	4.67 b	10.68 c	11.71 c	66.61 e

¹ 1-7 anthocyaninless lines, 8-11 yellow-seeded lines with anthocyanin, 12-15 brown-seeded lines, 16-22 green-seeded lines, 23-26 violet-seeded lines; ²Mean values marked by the same letter in each column are not significantly different at P≤0.05.

Twenty six accessions were divided into five groups in accordance with the color of their grains and genes of anthocyanin pigmentation. These groups were named according to the previous publication [5] as anthocyanin-less (#1-7), yellow-grained (#8-11), brown-grained (#12-15), green-grained (#16-22), and violet-grained (#23-26). The results were analyzed by comparing the means in five groups of lines using ANOVA and Turkey's test at P≤0.05.

L* (Lightness). Anthocyanin-less, yellow-grained and green-grained lines, do not differ for the value of L* - 48.83, 45.74 and 47.11, respectively. This coordinate is lower in brown-grained (40.00) and is the minimal in violet-grained lines (28.70).

a* (greenness-redness). Green-grained lines have the lowest value of a* 2.14, anthocyanin-less and yellow-grained lines have intermediate and an equal values of 3.53 and 3.21 respectively, and

the brown-grained and violet-grained lines have the highest and almost identical a^* of 4.66 and 4.67 correspondingly. Thus this coordinate reflects the reddening of grains from green through yellow to brown and violet.

b^* (blueness-yellowness). Violet-grained lines have the lowest b^* 10.68, green-grained lines have intermediate value 13.79 and grains in anthocyaninless lines have the highest b^* 15.73. Yellow- and brown-grained lines with b^* 14.36 and 15.16, respectively, do not differ from anthocyaninless and green-grained lines. It is interesting to note that this parameter shows a close relation to presence of different anthocyanins in pericarp of violet-seeded lines and aleurone of green-grained lines as well as their absence in this tissues in anthocyaninless lines.

C^* (saturation or color purity). For this coordinate only grains of anthocyaninless 16.13 and violet-grained lines 11.71 differs from each other.

h° (hue, actual color). Coordinate h° to the largest extent reflects the visual division of grains by their color. The yellow grains of the anthocyaninless lines (77.36) do not differ from the grains of yellow-grained lines (77.45), and brown-grained (72.81), while green-grained (81.30) and violet-grained (66.61) lines differ from the yellow ones and each other for the values of this coordinate. Apparently, this agreement may reflect the presence of different pigments – nonidentified in the pericarp of brown grains, presumably delphinidin derivatives in aleurone of green and cyanidin derivatives in the pericarp of violet grains and absence of pigments in yellow grains. Violet-seeded lines are characterized by the greatest intra-group variability in directly measured (L^*, a^*, b^*) and calculated (C^*, h°) parameters. This probably reflects the variability in the composition of anthocyanins content previously found in these lines by HPLC-MS [5].

2.2. Anthocyanin and proanthocyanidin localization in rye grain

Histological slices of yellow, brown, green, and violet grains were studied under a microscope to describe the original color of its upper layers (Figure S1). As is seen from the pictures (Figure S1) violet pigment is concentrated in pericarp of violet grains, blue in aleurone of grains described as green and brownish pigment in pericarp of brown grain. The majority of studied accessions have brown colored testa. But the intensity of its coloration varies to a large extent.

The use of hydrophobic 1-Hexadecene as a mounting media for fresh frozen-cut seeds allows to prevent water soluble components delocalization if microscopy is carried out soon after mounting. The Vanilin-HCl stain revealed proanthocyanidins in testa (Figure 1B) but the product color was similar to the color of residual anthocyanin, requiring comparison of Vanilin-HCl treated (Figure 1B) and untreated (Figure 1A) slices, the stain also lasts only for some hours, requiring immediate microscopy analysis. DMACA stain (Figure 1C, D) revealed the same localization of proanthocyanidins in testa as Vanilin-HCl, but the product is colored blue allowing to discern it from the red colored residual anthocyanin on the same slice (Figure 1D) and it lasts for considerable time making it possible to automatically take multiple pictures for high-resolution panoramic images (Figure 2C).

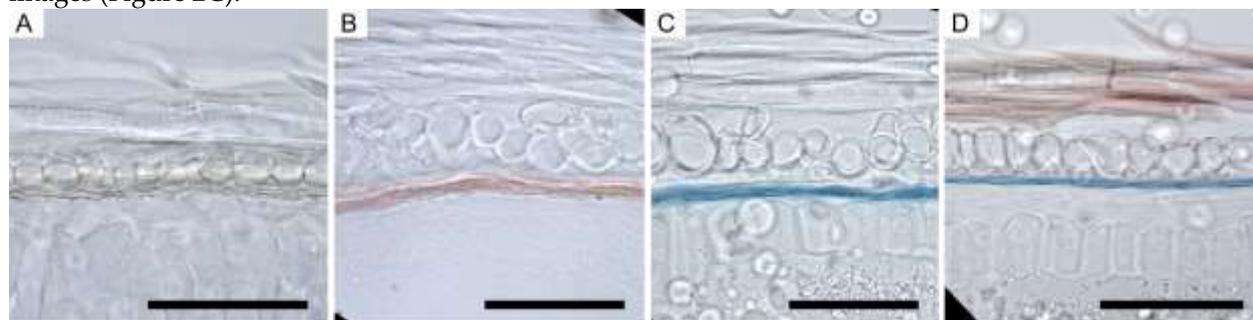


Figure 1. Vanilin-HCl and DMACA staining of proanthocyanidins in slices of rye grains: A. untreated slice of yellow grain, B. Vanilin-HCl treated of yellow grain, C. DMACA staining of yellow grain, D. DMACA staining of violet grain. Scale bar is equal to 100 μm .

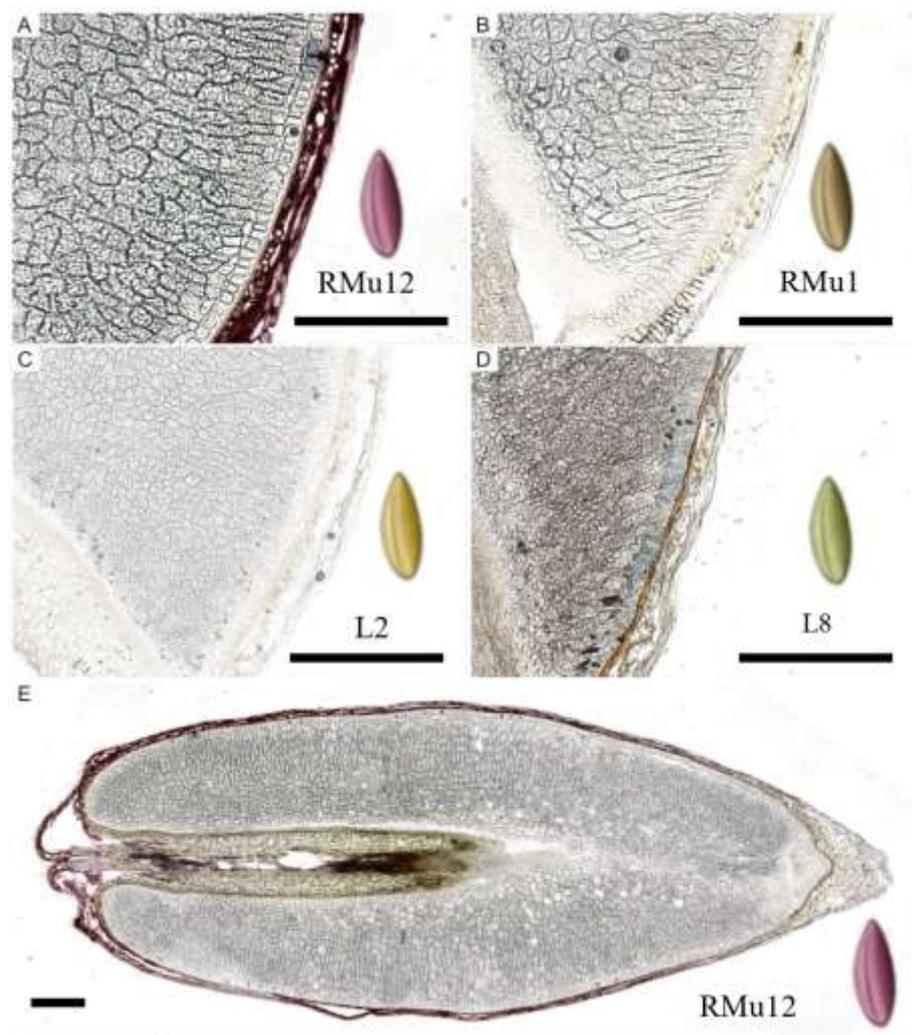


Figure 2. Anthocyanin distribution on histological slices of green and violet grains of rye. A green-grained line L8, B violet-grained line RMu12, C whole grain of line RMu12. Scale bar is equal to 500 μ m.

Violet-grained lines (Figure 2B, C) has anthocyanins in pericarp, mainly peonidin and cyanidin-rutinosides, as identified by HPLC-MS [5], brown colored testa and colorless aleurone MALDI-IMS (Figure 3A-F,H) confirmed the localization of peonidin-rutinoside to the pericarp for molecular ion, M/Z 609.18, as well as MS/MS fragments with M/Z 463.12 and the aglycone fragment M/Z 301.07.

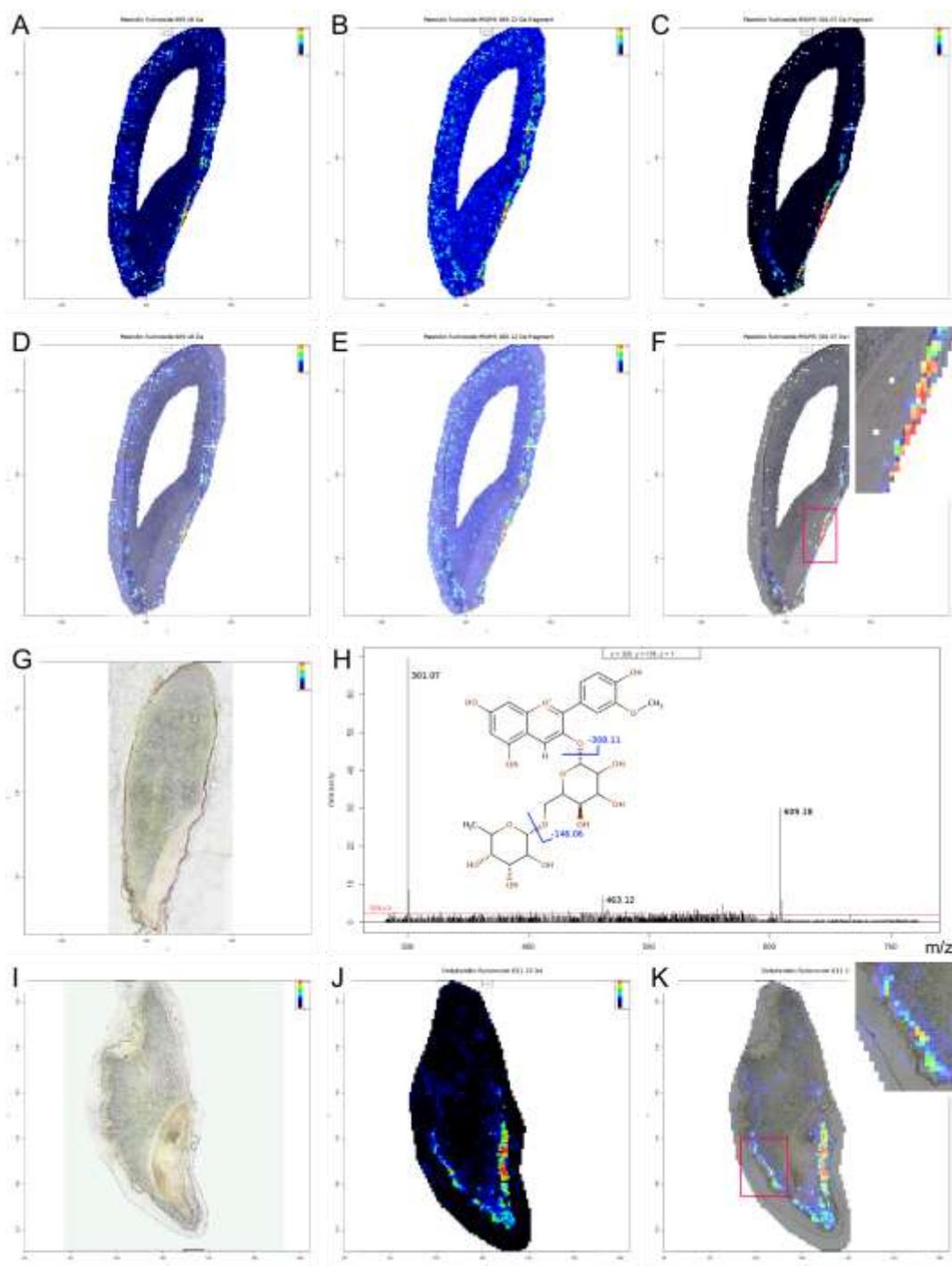


Figure 3. MALDI-imaging analysis of anthocyanins distribution confirmed the presence of anthocyanins in pericarp (violet grains) and aleurone (green grains): A-H violet grain rye line RMu12; I-K green grain rye line L301; A-C, J, K relative intensity of corresponding ions (black – absence, blue – minimal, green – intermediate, red – maximal amount); D-F, K co-registration with histological slice: A, D molecular ion of Peonidin 3-O-Rutinoside with m/z 609.18; B, E fragment ion of Peonidin 3-O-Rutinoside with m/z 463.12 (-146.06 Da); C, F aglycone ion of Peonidin 3-O-Rutinoside with m/z 301.07 (-308.11 Da); G histological slice of violet rye grain of line RMu12 without additional staining; H MS/MS mass spectrum of a single point of image at (x=320, y=139), fragmentation of Peonidin 3-O-Rutinoside, red line denotes S/N=3; J, K molecular ion of Delphinidin 3-O-Rutinoside with m/z 611.16 I histological slice of green rye grain of line L301 without additional staining.

Green-grained lines (Figure S1) has brown colored testa and light blue colored aleurone with main antocyanin identified by HPLC-MS [5] as delphinidin-rutinoside, it's localization to aleuron layer supported by MALDI-IMS only by the presence of its molecular ion, M/Z 611.16 (Figure 3).

DMACA staining detected proanthocyanidins in the majority of lines. Anthocyaninless lines show variability from total absence of stain in line vi3 to weak stain in line vi6, spotted stain in line vi1 and prominent stain in lines vi2, vi4 and vi5. Both methods shows that the blue pigment is more abundant near the embryo and at bottom of the grease.

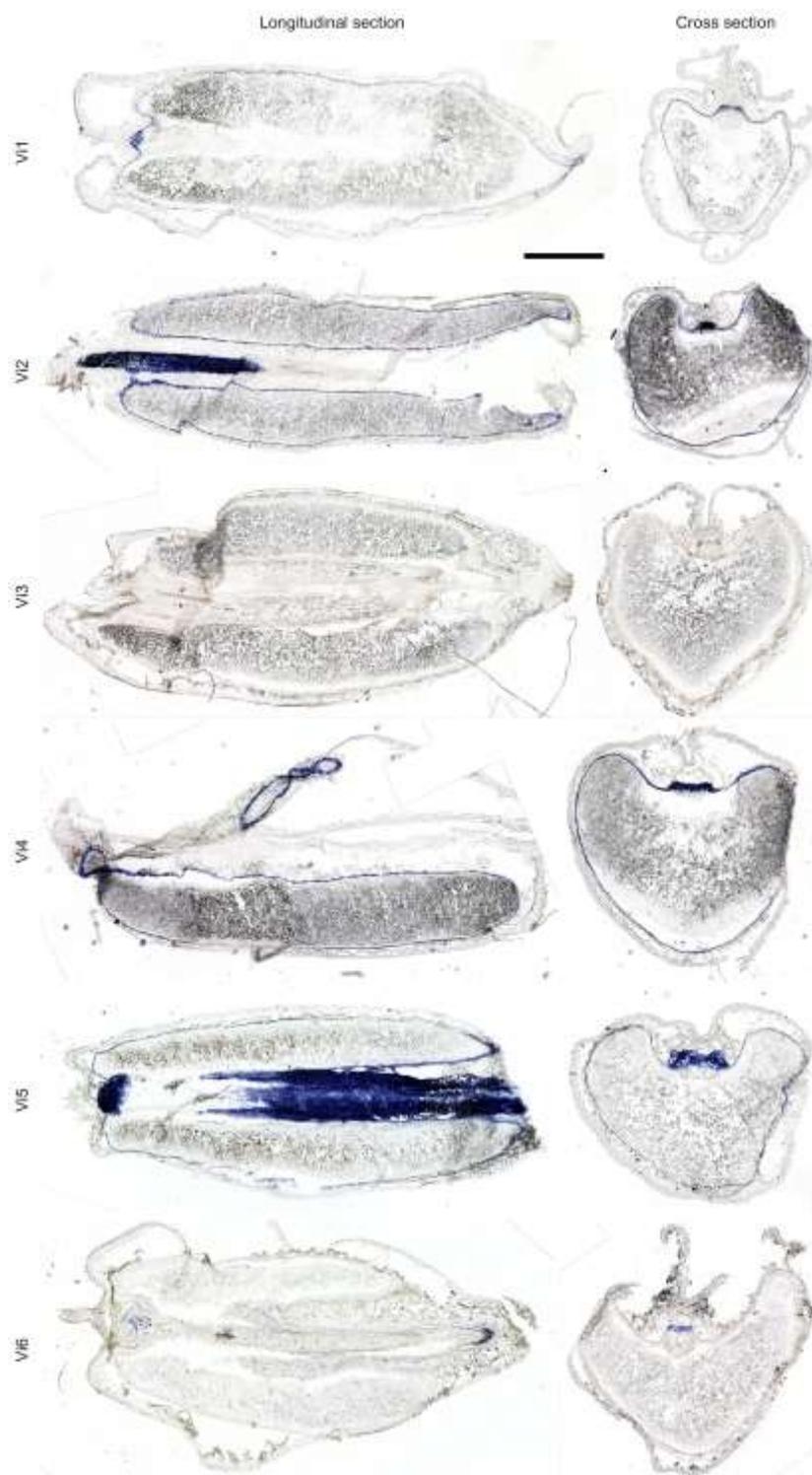


Figure 4. DMACA staining of longitudinal and cross slices of grain in lines carrying anthocyaninless mutations (*vi1...vi6*). Dark blue staining is a marker of proanthocyanidins.

3. Discussion

The main advantage of digital color measurements is their objectivity [6]. Color coordinates are used to describe and classify biological objects and in plants to develop indirect methods for pigment analysis. An example of such a study is two works performed in grape. Berry skin color was studied in 78 grape cultivars belonging to 6 visually identified color groups [20]. The correctness of grouping was confirmed by the analysis of color coordinates. Within the groups there are a slight variation of L^* and b^* , with greater intra-group variation of both a^* and C^* and h^* calculated using a^* . In colored varieties all the parameters were correlated with the total anthocyanin content (TAC), namely, L^* , a^* , b^* , and C^* - negatively but h^* - positively. A specific correlation between individual anthocyanins and color coordinates was also found. In another work [21], new indexes were created based on the coordinates L^* , a^* , b^* . One of these indexes is more correlated with the TAC in berry skin of red winegrapes, while the other is more correlated with each of the five identified anthocyanins. The colorimetric indexes have been proposed for indirect estimating the anthocyanin content.

In cereals the close correlation between the color parameters of grains or flour and pigments content was also established. Anthocyanins (cyanidin-3-O-glucoside, peonidin-3-O-glucoside) in black rice flour were negatively correlated with L^* , b^* , C^* and h^* values, proanthocyanidin in red rice flour was positively correlated with a^* [22]. Grain color analysis of uncolored, red and black rice, shows that white rice varieties have the highest values of L^* , b^* , h^* and the lowest a^* , red rice varieties have the maximum value of a^* and C^* , and black rice is characterized by the minimum values of L^* , b^* and C^* . The correlations between coordinates and TAC calculated for colored and uncolored varieties together have insignificant P values [23]. However, their direction (positive or negative) coincides with the correlations calculated for colored grapes. A negative correlation was established between the anthocyanin content and the values of L^* , a^* and b^* in two hybrid rice populations. In the population without segregation for black color of grains it has average values. In the population segregating for plants with white, red and black grains, the negative association between a^* and TAC has been reduced to an insignificant value [24]. This fact can be explained by the absence of anthocyanins in red and white rice, and the positive influence of proanthocyanidins in red grain on the value of a^* . Results similar to ours in rye were obtained analyzing bread wheat with different coloration of grain [25]. The presence of blue pigment in aleurone, purple – in pericarp, red in testa was established in accordance to previously published data. Black (dark purple) color of grains is explained by the presence of anthocyanins, both in aleurone and pericarp. The five color coordinates for wheat with white (yellow), blue, and purple grain overlap with the values of the coordinates of the rye grains with yellow, green and violet color, respectively. Thus, the average values of h° for these groups in wheat are 73.0, 81.4 and 61.7, and in rye – 77.45, 81.3 and 66.61 respectively. It is significant that these results were obtained using different equipment and software but a device independent color space. Correlations between the concentration of anthocyanins in wheat grain and color coordinates, calculated by us using data from published paper [25], showed consistency with the similar data in grape. Namely, between L^* , a^* , b^* , C^* and TAC a negative correlation was found, and between h^* and TAC- a positive one. The minimum value of a^* in combination with the maximum value of h° was found in “green” rye grain and “blue” wheat grain. The minimum values of L^* , b^* , C^* and h^* are characteristic of “violet” grain in rye, in “purple” grain of wheat this is true only for the values of L^* and h^* . It can be assumed that this similarity is associated with the predominance of delphinidin derivatives in aleurone, and cyanidin derivatives in pericarp of both plants. Some mismatches may reflect the presence of different additional pigments in the grain of studied wheat and rye accessions. It is known that not only the composition but also the structure of individual anthocyanins affects the color coordinates [26]. Significant variation of all coordinates in violet-seeded rye samples (Table 1) may also be based on established differences [5] in anthocyanins composition.

There is a diversity for structure and localization of colored flavonoids in tissues of caryopsis in maize, rice, sorghum, wheat, barley and rye. The inter-species differences might arise in the course of crops evolution as a result of adaptation to environment, domestication and breeding.

Duplications and functional divergence of flavonoid biosynthetic genes and regulatory genes, comprising MYB-bHLH-WD (MBW) transcriptional complex, may lead to origin of specific branches of flavonoids biosynthesis and features in their regulation. In cereals colored flavonoids are synthesized in pericarp, testa and aleurone. Sorghum and corn are the only two cereals that are known to synthesize 3-deoxyanthocyanidins. In sorghum they are almost exclusive type of anthocyanidins but in maize common C-3-hydroxylated anthocyanidins dominate [27]. In both species polymeric phlobaphenes – substances related to 3-deoxyanthocyanidins are present that differ them from the other cereals. Another polymeric flavonoids – proanthocyanidins (PAs) are found at different concentrations and molecular forms in testa of sorghum [28], wheat [29], barley [30] and as evident from our study in rye, but not found at any tissues in maize. In rice PAs are found in pericarp of wild species *Oryza rufipogon* and red-grained varieties of *O. sativa* [31]. PAs are oligomers and polymers of flavan-3-ols (catechins) and flavan-3,4-diols (leucoanthocyanidins) and are colourless compounds by themselves [30] but they may be transformed to insoluble brown or red pigments in plant tissues. Sorghum grains have the highest abundance of proanthocyanidins (tannins) with the highest degree of polymerization among cereals. Tannins are present in pigmented testa layer, their formation is determined by complementary genes *B1* and *B2* [32]. It is shown that *B2* corresponds to gene *Tan1* coding a WD 40 protein [28] and *B1* is a gene of putative bHLH transcription factor *Sb02g006390* [33]. The color of testa is usually brown (*Tp-*) but it may be purple (*tptp*) [34]. The gene *S* controls the spreading of pigments from the testa to the pericarp being localized in cell walls of both tissues [35]. Grain color is not a true indicator of tannins presence in sorghum grains. For their identification the chlorox bleach test was developed. Bleaching dissolves the pericarp and turns tannins in testa black. PAs in barley testa are colorless and for their detection a coloring agents vanillin [30] and DMACA [29] are used. In red wheat as well as in barley the same colorless oligomeric proanthocyanidins (procyanidin B3, prodelfphinidin B3) and flavan-3-ols (+)-catechin are accumulated during grain development. But in wheat as opposed to barley these substances are being converted to insoluble reddish-brown pigment during seed maturation [29] while blue staining by DMACA reagent is similar to its colorless precursors. *R* homeoloci (*Tamyb10* genes) in wheat [36] and their ortholog *Ant28* (*Hvmyb10*) in barley [37] are responsible for PAs synthesis in testa. Rye has long been known to have a brown (pigment) layer in the caryopsis, which is a part of the seed coat [2]. We have shown that the brown color of this layer changes to red or blue in the presence of vanillin or DMACA, respectively. The typical brown layer was found in all studied accessions except for anthocyaninless lines *vi3* and *vi6*. In line *vi3* this layer is not visible at all and in line *vi6* only their traces are found after staining. By analogy with the five anthocyaninless and proanthocyanidin-free mutations in barley (*ant13*, *ant17*, *ant18*, *ant21*, *ant22*) [30] it can be assumed that rye mutations *vi3* and *vi6* affect the biosynthesis of anthocyanins as well as related proanthocyanidins. There is no apparent association between absence of brown layer in *vi3* mutants and measured color coordinates.

Species-specificity for type of pigments and their localization in pericarp and aleurone also is characteristic of discussed cereals. In maize and sorghum the separate branch of flavonoid pathway gives rise flavan-4-ols (apiforol and luteoforol) and their derivatives – 3-deoxyanthocyanidins (apigeninidin and luteolinidin) [35]. 3-deoxyanthocyanidins do not contain the hydroxyl group in the 3-position of the C-ring as majority of natural anthocyanins and are represented mainly in the aglycon form. Flavan-4-ols are also the precursors of insoluble brick red phlobaphene pigments that accumulate in mature pericarp in both species. Their biosynthesis is under control of R2R3 MYB transcription factors encoded by gene *pericarp color1* (*p1*) in maize [38] and their orthologue *yellow seed1* (*y1*) in sorghum [39]. Functional alleles of these genes activate the transcription of structural genes of chalcone synthase, chalcone isomerase, dihydroflavonol reductase and flavonoid 3'-hydroxylase during 3-deoxyflavonoids biosynthesis in maize and sorghum [39,40]. At the same time the structural genes of these enzymes are independently regulated by corresponding sets of MYB and bHLH transcriptional factors in different tissues of seeds and plant in maize [41]. In particular that leads to common anthocyanins formation in pericarp and/or aleurone. Anthocyanins based on cyanidin, pelargonidin, and peonidin were found in both tissues. The color of grain in

sorghum depends on presence and concentration of different 3-deoxyanthocyanidins (orange luteolinidin and yellow apigeninidin), phlobaphenes and tannins in corresponding layers of kernel. In sorghum the gene *R* with unknown function is complementary to MYB-gene *Y* in controlling the pericarp color. Sorghum with red- or black-colored pericarp has dominant alleles of both genes (*R-Y*), yellow pericarp is produced in genotypes *rrY*- and white one in genotypes *rryy* and *R-yy* [35].

Pericarp in rice may contain proanthocyanidins (red rice) or anthocyanins (black rice). The presence of proanthocyanidins in the grain of some varieties of black rice is also reported [42]. That points to biosynthesis of both anthocyanins and proanthocyanidins in pericarp tissues, based on the action of the corresponding transcription factors. In red rice proanthocyanidins are formed in pericarp during ripening of the caryopsis [43]. The formation of red pigments controls two complementary genes *Rc* and *Rd*. The gene *Rc* encodes a bHLH protein, and the gene *Rd* encodes dihydroflavonol-4-reductase (*Dfr*). The simultaneous presence of dominant alleles of these genes (*Rc*-, *Rd*-) leads to the formation of red pigment. In genotypes *rdrd*, *Rc*- a brown unidentified pigment is produced, all the other genotypes (white rice) did not produce pigments [31]. Interaction of the gene *Rd*, later designated as *Kala1* [44] with two other genes, *Kala3* and *Kala4* (another name of known gene *Pb*) leads to production of black rice. Such rice has high content of anthocyanins (mainly cyanidin-3-O-glucoside and peonidin-3-O-glucoside) in pericarp. The gene *Kala3* encodes MYB and the gene *Kala4* – bHLH transcriptional factors. The interaction of these genes leads to the appearance of three phenotypes. Black grain with high content of anthocyanins in the pericarp has genotypes with dominant alleles of all three genes. Plants with dominant alleles of the gene *Kala4* and recessive alleles of the genes *Kala1* and/or *Kala3* have brown grain of different intensity. Homozygosity for the recessive allele of the gene *Kala4* leads to the appearance of white grain, regardless of the genotypes for the other two genes. The nature of brown pigment is not established, but one could assume that this pigment may be similar to pigment found in brown rice with genotype *rdrd*, *Rc*-. In both cases the production of brown pigment depends from the interaction of inactive (weak) alleles of the gene *Dfr* (*Rd*) with bHLH transcriptional factors coded by *Rc* or *Kala4*. These genes regulate several structural genes beyond *Dfr* in anthocyanin and proanthocyanidin biosynthesis that may give rise to the close set of colored compounds originating from the intermediate products. Notice that formation of brown pigment was found in maize aleurone homozygous for inactive alleles of the gene *a1* encoding the same enzyme *Dfr* at the presence of corresponding MYB and bHLH transcriptional factors. The brown pigment is formed also in maize pericarp as result of interaction of recessive allele *a1* with appropriate alleles of MYB gene *P1* controlling phlobaphene synthesis [45].

In the available literature there is no description of the pigments distribution in the tissues of rye caryopsis of different colors. However, the coloration of the different layers of grain coats in yellow, green and brown grains under their anatomical dissection is given [46]. One need note that plant material in this investigation was open pollinating varieties which as a rule include grains of three main colors – yellow, green and brown. In yellow and green grains all four cell layers of the fruit coat were colored yellow. The upper two layers in yellow grains had more intense coloration and were less transparent. In brown grains, they were colored brown and also more intensively than the next two. The grains of all three colors had a brown seed coat (testa), and the aleurone layer was isolated in the form of blue-green transparent flakes, the color intensity of which is highest in green grains. Visual description of the grain coats allowed for a speculative idea about the diversity of rye grain coloration. V. D. Kobylansky presented in the monograph “Rye” [2] a table in which all the diversity of rye grains by color is defined as a result of a combination of color gradations of aleurone (not colored – blue), testa (not colored – red-brown – dark brown) and pericarp (not colored – yellow – purple/violet). The violet color of the rye grain is determined by a dominant gene *Vs* (*Violet seeds*) originating from weedy rye. The gene *Vs* acts similarly to the purple pericarp genes in wheat (*Pp3*), barley (*Pre2/Ant2*) and rice (*Kala4*) encoding bHLH transcription factors. These genes express itself at the background of active alleles of other structural and regulatory genes of anthocyanin biosynthesis and are responsible for anthocyanins accumulation in

the pericarp. The molecular function of gene *Vs* has not been established, but it was localized on chromosome 2R in the fragment orthologous to fragments harboring these genes in related species (unpublished). The qualitative and quantitative composition of anthocyanins in the colored pericarp in discussed cereals including rye varies in large extent [5]. However, the common anthocyanidins in all species, beyond sorghum are cyanidin and its 3-O-methylated derivative peonidin.

Effect of gene interaction leading to appearance of brown grain was found by us in rye at first time. It is genetically similar to formation of brown color in maize and rice grains [45] discusses above. Brown grains have the lines with two genotypes, combining the dominant gene *Vs* with homozygosity for the two anthocyaninless mutations – *vi1* or *vi2*. Genes *Vs*, *vi1* and *vi2* are localized in chromosomes 2R, 7R and 4R respectively (not published), their function is unknown. Brown grain contain no anthocyanins [5], but its pericarp is colored by chemical agents for proanthocyanidins. It is believed that brown grain studied by us may differ genetically and biochemically from brown grain inherent to rye open-pollinating varieties.

Anthocyanins synthesis in aleurone is not common for all discussed cereal crops. Sorghum and rice, unlike other cereals, is not reported to have anthocyanins in aleurone. The color of grains in wheat and barley with anthocyanins in aleurone is described as blue, in rye it is described more frequently as green or gray-green. On slices of the green grains in our study aleurone appear as blue. The characteristic anthocyanidin in aleurone of these three crops is delphinidin [47]. Green-grained rye lines studied by us have cyanidine rutinoside apart from delphinidin rutinoside [5]. There is maize accessions with blue color of grain but they are not capable of delphinidin biosynthesis. In blue maize cyanidin 3-glucoside is the most abundant, followed by pelargonidin and peonidin 3-glucoside [48]. The genes of blue aleurone were transferred into bread wheat genome as chromosomal segment from related wild species [49]. In barley, the combination of five structural and regulatory genes [50] is responsible for the synthesis of anthocyanins in aleurone. In accordance to recent data [47] there is a cluster of three genes *MbHF35* on chromosome 4H. This cluster includes two genes *HvMYB4H* and *HvMYC4H* encoding transcriptional factors and structural gene of flavonoid 3', 5'-hydroxylase (*HvF35H*). Namely activity of this enzyme allows for the synthesis of delphinidin-based anthocyanins providing blue color of aleurone in barley. Barley, wheat and rye are the only species which may carry functional alleles of blue aleurone. In rye this cluster is located in 7R chromosome that agrees with evolutionary translocation between ancestor chromosomes 4 and 7. In accordance to rye genetics [3] the presence of anthocyanins in green-grained lines of rye is connected with unmapped gene *C*. Thus this gene may be one of genes of the trigenic cluster.

4. Materials and Methods

4.1. Plant material.

Twenty six studied rye accessions were divided into five groups which differs by grain color and anthocyanin pigmentation of the plant (Table 1). Accession #2 in the table 1 is an open-pollinating variety *Esto* and the others are inbred lines that have been used previously for study of anthocyanin content and composition in the grain [5]. In anthocyaninless rye (#1-7) the anthocyanin pigmentation is absent throughout the plant due to homozygosity for the recessive spontaneous mutations *vi1* (#1 and 2), *vi2*, *vi3*, *vi4*, *vi5* and *vi6* (#3, 4, 5, 6 and 7, respectively). Grains of these lines are described as yellow. Four lines with anthocyanin pigmentation of the other plant tissues (#8, 9, 10, 11) have yellow grain as well. In these lines, the yellow color of the grains is attributable to homozygosity for recessive alleles of the genes *Vs* and *C* [3]. In accordance to rye genetics the dominant allele *C* leads to green grain (#16-22), and *Vs* – to violet one (#23-26). Brown grain is due to the combination of the allele *Vs* with the homozygosity for recessive mutations *vi1* (#12-14) or *vi2* (#15).

4.2. Color coordinates analysis.

Images of grain were obtained using Epson Perfection V19 scanner (Epson, Japan). The scanner was pre-calibrated with the Munsell Color Checker Mini (Munsell Color, MI) calibration target according to the description of the GrainScan program used for image analysis [1]. When scanning, the resolution was set to 300 dpi. For each grain, three color coordinates were obtained in the three-dimensional space CIE $L^*a^*b^*$. The color coordinates (L^* , a^* and b^*) calculated in this space are device independent and correspond to human visual perception [1,6]. The L^* (Lightness) coordinate takes values from 0 (black) to 100 (white). Intermediate values describe the brightness of colors defined by the combination of a^* and b^* coordinates. The a^* and b^* axes on a plane perpendicular to the L^* axis at any given point reflect the color change from green ($-a^*$) to red ($+a^*$) and from blue ($-b^*$) to yellow ($+b^*$). These coordinates can be used to describe an object (point) of any color. Based on the values of coordinates a^* and b^* , the coordinates c^* and h^* are calculated to describe the color in the space CIE $L^*c^*h^*$, displaying graphically as sphere. Vertical L^* axis is the same Lightness ranging from 0 to 100. Chroma c^* (saturation or color purity) is calculated as $(a^{*2} + b^{*2})^{1/2}$. This coordinate is 0 at any point on the L^* axis and reaches a maximum in a circle corresponding to bright, saturated colors. Tone (actual color) h^* (hue angle) is calculated in degrees (h°) or radians as arctangent b^*/a^* , taking values from 0 (red) through 90 (yellow), 180 (green), 270 (blue) returning to 0. Other values of this coordinate describe all intermediate colors. On the average about 100 grains were scanned individually in each of 26 samples. The means of the coordinates for each color group were compared by ANOVA using PAST v.4 [7]. Significant differences between means are established by Tukey's range test at $p < 0.05$ [8].

4.3. *Histological procedures and microscopy of pigment distribution.*

Grains at the soft dough stage (15-20 days after pollination, depending on variety) were frozen in OCT compound (Sakura Finetek Inc., USA) and sectioned 25 μm on Leica CM-3050S cryomicrotome (Leica, Wetzlar, Germany) at -20°C . For anthocyanin localization, due to high water solubility of anthocyanins at this stage of seed development, the slices were put directly on cold slides and mounted under a coverslip with a cold ($+6^\circ\text{C}$) 1-Hexadecene (Sigma, USA). The coverglass was affixed and sealed with a nail-polish. For proanthocyanidin localization the slices were thaw-mounted on poly-l-lysine coated slides and allowed to dry at $+37^\circ\text{C}$ for 2 hours. Two types of proanthocyanidin specific stains were used: Vanilin-HCl [9,10], and DMACA [11]. Vanilin-HCl stain was done by incubation in absolute ethanol with 50 mg/ml of Vaniline (Sigma-Aldrich, USA) for 30 seconds immediately followed by application of drop of concentrated HCl, coverslipping and microscopy. DMACA stain was done by 20 minute incubation in fresh solution of 0.01% (w/v) of 4-Dimethylaminocinnamaldehyde (Sigma-Aldrich, Germany) and 0.8% (v/v) concentrated HCl in absolute ethanol, followed by three washes of ethanol and coverslipping with Hoyer's medium [12]. Microscopy was done on Leica DM5500 microscope (Leica microsystems, Germany) controlled by the MicroManager v2.0 [13] software, with a 20x 0.7 NA and 40x 0.85 NA objectives. Montage of multiple fields of view, covering the whole slice, was automatically acquired by MicroManager v2.0 and panoramic stitching was done in Fiji [14].

4.4. *MALDI-imaging of anthocyanins localization.*

Grains at the soft dough stage (15-20 days after pollination) were cryosectioned according to Kawamoto technique [15]. In short, grains were immersed in 0.5% Carboxymethylcellulose (Sigma-Aldrich, Germany) followed by rapid freezing in n-Hexane at -80°C . Frozen blocks were mounted on holders and sectioned 12 μm on Leica CM-3050S cryomicrotome (Leica microsystems, Germany) at -20°C , captured on Cryofilm type 2C (Sections-lab, Japan) and allowed to freeze-drying in cryomicrotome's chamber for 4 hours to prevent water-soluble anthocyanin diffusion and delocalization. Slices captured on Cryofilm were glued by conducting carbon tape (Ted-Pella, USA, 16073-2) onto ITO-coated conducting slides (Bruker, Germany). White marker (Edding 750, Germany) was used for fiducial points, optical images of slides were acquired with Bio-Rad GS-800 scanner (Bio-rad, USA), at 700 dpi. As an external control and calibration standard the bilberry anthocyanin extract [16] (Y0001059, Bilberry dry extract, Sigma-Aldrich, USA) was spotted near fiducial points. Slices were

coated in automated coater ImagePrep (Bruker, Germany, version 2.0.1), with the use of a standard inbuilt «DHB_nsh04» protocol with DHB matrix (40 mg/ml, Bruker, Germany) dissolved in methanol/deionised water 70/30 v/v with the addition of 0.1% TFA (Sigma-Aldrich, USA). The spectra were acquired on MALDI-TOF Ultraflex extreme (Bruker, Germany) with Bruker FlexControl v. 3.3 and FlexImaging v. 3.0. Laser intensity was set to 60%, laser spot size was set to “minimal”, raster size to 50 mkm, 250 shorts per raster point and random walk inside the point was used. Real laser spot size measured after matrix ablation was about 20 mkm. High resolution reflectron mode for positive ions with the extraction delay of 80 ns and mass range of 230-1200 Da was used. For tandem mass spectrometry the isolation window was set to +/- 2Da, laser induced dissociation with 100% laser was used for fragmentation. Optical images were co-registered in FlexImaging by aligning fiducial points. The data was converted from proprietary Bruker format to imzML format by proteowizard's msconvert.exe utility [17] and imzMLConverter_1.3 [18]. Cardinal package for R v2.8 [19] was used for visualisation with normalization by total ion current, smoothing with SGolay using 10 Da window, base line subtraction by Median using 100 Da block size and peak detection using Limpic algorithm.

5. Conclusions

We showed that GrainScan software method [1] is suitable for the objective color measurement of rye grains. Four main color groups in rye (yellow, green, brown, violet) were properly differentiated in accordance to value of h^* . It is believed that color coordinates may be used as such or through indexes construction for sophisticated analysis of intra-group color variation. We schedule to perform such analysis using additional set of inbred lines, F3 progenies from hybrids segregating for grain color and open-pollinating rye varieties, polymorphic for grain color.

Histological analysis of proanthocyanidin localization in anthocyanin-less mutant lines revealed proanthocyanidin-deficient *vi3* line. The localization of anthocyanins in the pericarp of violet-grained samples and in the aleurone layer of the green-grained samples was defined by MALDI-imaging analysis.

The future work will include the studying of pigments and uncolored flavonoids in newly discovered color groups with emphasis on the pigment identification in brown grain and mapping the candidate genes for rye genes of grain coloration.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Histological characterization of grains of different color.

Author Contributions: Pavel A. Zykin and Anatoly V. Voylokov designed and coordinated the study; Pavel A. Zykin and Elena A. Andreeva performed the histological analysis, imaging mass spectrometry and analyzed the data; Natalia V. Tsvetkova produced and contributed plant material; Pavel A. Zykin and Anatoly V. Voylokov wrote different parts of the paper; all authors approved the final version of the manuscript.

Funding: This research was funded by RFBR, grant number 19-016-00205, and state budget (project “Genetics and breeding of rye on the base of natural hereditary diversity” – AAAA-A16-116111610177-3).

Acknowledgments: Part of the research was done with the use of “Center for Molecular and Cell Technologies”, Research Park, St. Petersburg State University.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Whan, A.P.; Smith, A.B.; Cavanagh, C.R.; Ral, J.-P.F.; Shaw, L.M.; Howitt, C.A.; Bischof, L. GrainScan: a low cost, fast method for grain size and colour measurements. *Plant Methods* 2014, 10, 23, doi:10.1186/1746-4811-10-23.
2. Kobylansky V.D.; Korzun A.E.; Katerova A.G.; Lapikov N.; Solodukhina O.V. Rye. In *Cultural flora of the USSR*; 1; Agropromizdat: Leningrad, 1989; Vol. 2, p. 368.
3. Smirnov, V.G.; Sosnikhina, S.P. *Genetika rzhii (Genetics of Rye)*; Leningradskiy gosudarstvenny universitet: Leningrad, USSR, 1984;

4. Voylokov, A.V.; Lykholay, A.N.; Smirnov, V.G. Genetic control of anthocyanin coloration in rye. *Russian Journal of Genetics: Applied Research* 2015, 5, 262–267, doi:10.1134/S207905971503020X.
5. Zykin, P.; Andreeva, E.; Lykholay, A.; Tsvetkova, N.; Voylokov, A. Anthocyanin Composition and Content in Rye Plants with Different Grain Color. *Molecules* 2018, 23, 948, doi:10.3390/molecules23040948.
6. McGuire, R.G. Reporting of Objective Color Measurements. *HortScience* 1992, 27, 1254–1255, doi:10.21273/HORTSCI.27.12.1254.
7. Hammer, O.; Harper, D.A.T.; Ryan, P.D. PAST: Paleontological Statistics Software Package for Education and Data Analysis. 2001, 9.
8. Tukey, J.W. Comparing Individual Means in the Analysis of Variance. *Biometrics* 1949, 5, 99–114, doi:10.2307/3001913.
9. Aastrup, S.; Outtrup, H.; Erdal, K. Location of the proanthocyanidins in the barley grain. *Carlsberg Res. Commun.* 1984, 49, 105–109, doi:10.1007/BF02913969.
10. Peterson, R.L.; Peterson, C.A.; Melville, L.H. Teaching Plant Anatomy Through Creative Laboratory Exercises; NRC Research Press, 2008; ISBN 978-0-660-19798-2.
11. Abeynayake, S.W.; Panter, S.; Mouradov, A.; Spangenberg, G. A high-resolution method for the localization of proanthocyanidins in plant tissues. *Plant Methods* 2011, 7, 13, doi:10.1186/1746-4811-7-Hoyer's Medium. *Cold Spring Harbor Protocols* 2011, 2011, pdb.rec12429, doi:10.1101/pdb.rec12429.
12. Edelstein, A.D.; Tsuchida, M.A.; Amodaj, N.; Pinkard, H.; Vale, R.D.; Stuurman, N. Advanced methods of microscope control using μ Manager software. *Journal of Biological Methods* 2014, 1, e10, doi:10.14440/jbm.2014.36.
13. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: an open-source platform for biological-image analysis. *Nature Methods* 2012, 9, 676–682, doi:10.1038/nmeth.2019.
14. Kawamoto, T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Archives of Histology and Cytology* 2003, 66, 123–143, doi:10.1679/aohc.66.123.
15. BILBERRY DRY EXTRACT HRSbatch 2 2017.
16. Chambers, M.C.; Maclean, B.; Burke, R.; Amodei, D.; Ruderman, D.L.; Neumann, S.; Gatto, L.; Fischer, B.; Pratt, B.; Egertson, J.; et al. A cross-platform toolkit for mass spectrometry and proteomics. *Nature Biotechnology* 2012, 30, 918–920, doi:10.1038/nbt.2377.
17. Race, A.M.; Styles, I.B.; Bunch, J. Inclusive sharing of mass spectrometry imaging data requires a converter for all. *Journal of Proteomics* 2012, 75, 5111–5112, doi:10.1016/j.jprot.2012.05.035.
18. Bemis, K.D.; Harry, A.; Eberlin, L.S.; Ferreira, C.; van de Ven, S.M.; Mallick, P.; Stolowitz, M.; Vitek, O. Cardinal: an R package for statistical analysis of mass spectrometry-based imaging experiments. *Bioinformatics* 2015, 31, 2418–2420, doi:10.1093/bioinformatics/btv146.
19. Liang, Z.; Sang, M.; Fan, P.; Wu, B.; Wang, L.; Yang, S.; Li, S. CIELAB Coordinates in Response to Berry Skin Anthocyanins and Their Composition in Vitis. *Journal of Food Science* 2011, 76, C490–C497, doi:10.1111/j.1750-3841.2011.02095.x.
20. Rolle, L.; Guidoni, S. Color and anthocyanin evaluation of red winegrapes by CIE L*, a*, b* parameters. *OENO One* 2007, 41, 193, doi:10.20870/oeno-one.2007.41.4.838.
21. Shao, Y.; Hu, Z.; Yu, Y.; Mou, R.; Zhu, Z.; Beta, T. Phenolic acids, anthocyanins, proanthocyanidins, antioxidant activity, minerals and their correlations in non-pigmented, red, and black rice. *Food Chemistry* 2018, 239, 733–741, doi:10.1016/j.foodchem.2017.07.009.
22. Phaiwan Pramaj; Sudarat Jiamyangyuen Chemometric classification of pigmented rice varieties based on antioxidative properties in relation to color. *Songklanakarin Journal of Science and Technology (SJST)* 2016, 38, 5, doi:10.14456/SJST-PSU.2016.62.
23. Ham, T.-H.; Kwon, S.W.; Ryu, S.-N.; Koh, H.-J. Correlation Analysis between Grain Color and Cyanidin-3-glucoside Content of Rice Grain in Segregate Population. *Plant Breeding and Biotechnology* 2015, 3, 160–166, doi:10.9787/PBB.2015.3.2.160.
24. Garg, M.; Chawla, M.; Chunduri, V.; Kumar, R.; Sharma, S.; Sharma, N.K.; Kaur, N.; Kumar, A.; Munde, J.K.; Saini, M.K.; et al. Transfer of grain colors to elite wheat cultivars and their characterization. *Journal of Cereal Science* 2016, 71, 138–144, doi:10.1016/j.jcs.2016.08.004.

25. Han, F.-L.; Zhang, W.-N.; Pan, Q.-H.; Zheng, C.-R.; Chen, H.-Y.; Duan, C.-Q. Principal Component Regression Analysis of the Relation Between CIELAB Color and Monomeric Anthocyanins in Young Cabernet Sauvignon Wines. *Molecules* 2008, 13, 2859–2870, doi:10.3390/molecules13112859.
26. Xiong, Y.; Zhang, P.; Warner, R.D.; Fang, Z. 3-Deoxyanthocyanidin Colorant: Nature, Health, Synthesis, and Food Applications. *Comprehensive Reviews in Food Science and Food Safety* 2019, 18, 1533–1549, doi:10.1111/1541-4337.12476.
27. Wu, Y.; Li, X.; Xiang, W.; Zhu, C.; Lin, Z.; Wu, Y.; Li, J.; Pandravada, S.; Ridder, D.D.; Bai, G.; et al. Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin1. *Proceedings of the National Academy of Sciences* 2012, 109, 10281–10286, doi:10.1073/pnas.1201700109.
28. Kohyama, N.; Chono, M.; Nakagawa, H.; Matsuo, Y.; Ono, H.; Matsunaka, H. Flavonoid compounds related to seed coat color of wheat. *Bioscience, Biotechnology, and Biochemistry* 2017, 81, 2112–2118, doi:10.1080/09168451.2017.1373589.
29. Jende-Strid, B. Genetic Control of Flavonoid Biosynthesis in Barley. *Hereditas* 1993, 119, 187–204, doi:https://doi.org/10.1111/j.1601-5223.1993.00187.x.
30. Furukawa, T.; Maekawa, M.; Oki, T.; Suda, I.; Iida, S.; Shimada, H.; Takamura, I.; Kadowaki, K. The Rc and Rd genes are involved in proanthocyanidin synthesis in rice pericarp: Rc and Rd genes for red rice seed. *The Plant Journal* 2006, 49, 91–102, doi:10.1111/j.1365-3113X.2006.02958.x.
31. Dykes, L.; Rooney, L.W. Sorghum and millet phenols and antioxidants. *Journal of Cereal Science* 2006, 44, 236–251, doi:10.1016/j.jcs.2006.06.007.
32. Morris, G.P.; Rhodes, D.H.; Brenton, Z.; Ramu, P.; Thayil, V.M.; Deshpande, S.; Hash, C.T.; Acharya, C.; Mitchell, S.E.; Buckler, E.S.; et al. Dissecting Genome-Wide Association Signals for Loss-of-Function Phenotypes in Sorghum Flavonoid Pigmentation Traits. *G3 & Genes | Genomes | Genetics* 2013, 3, 2085–2094, doi:10.1534/g3.113.008417.
33. Casady, A.J. Inheritance of purple testa in sorghum. *Journal of Heredity* 1975, 66, 180–180, doi:10.1093/oxfordjournals.jhered.a108609.
34. Xiong, Y.; Zhang, P.; Warner, R.D.; Fang, Z. Sorghum Grain: From Genotype, Nutrition, and Phenolic Profile to Its Health Benefits and Food Applications. *Comprehensive Reviews in Food Science and Food Safety* 2019, 18, 2025–2046, doi:10.1111/1541-4337.12506.
35. Himi, E.; Maekawa, M.; Miura, H.; Noda, K. Development of PCR markers for Tamyb10 related to R-1, red grain color gene in wheat. *Theor Appl Genet* 2011, 122, 1561–1576, doi:10.1007/s00122-011-1555-2.
36. Himi, E.; Yamashita, Y.; Haruyama, N.; Yanagisawa, T.; Maekawa, M.; Taketa, S. Ant28 gene for proanthocyanidin synthesis encoding the R2R3 MYB domain protein (Hvmyb10) highly affects grain dormancy in barley. *Euphytica* 2012, 188, 141–151, doi:10.1007/s10681-011-0552-5.
37. Grotewold, E.; Drummond, B.J.; Bowen, B.; Peterson, T. The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell* 1994, 76, 543–553, doi:10.1016/0092-8674(94)90117-1.
38. Boddu, J.; Jiang, C.; Sangar, V.; Olson, T.; Peterson, T.; Chopra, S. Comparative Structural and Functional Characterization of Sorghum and Maize Duplications Containing Orthologous Myb Transcription Regulators of 3-Deoxyflavonoid Biosynthesis. *Plant Molecular Biology* 2006, 60, 185–199, doi:10.1007/s11103-005-3568-1.
39. Sharma, M.; Cortes-Cruz, M.; Ahern, K.R.; McMullen, M.; Brutnell, T.P.; Chopra, S. Identification of the Pr1 Gene Product Completes the Anthocyanin Biosynthesis Pathway of Maize. *Genetics* 2011, 188, 69–79, doi:10.1534/genetics.110.126136.
40. Petroni, K.; Tonelli, C. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science* 2011, 181, 219–229, doi:10.1016/j.plantsci.2011.05.009.
41. Mbanjo, E.G.N.; Kretschmar, T.; Jones, H.; Ereful, N.; Blanchard, C.; Boyd, L.A.; Sreenivasulu, N. The Genetic Basis and Nutritional Benefits of Pigmented Rice Grain. *Frontiers in Genetics* 2020, 11, doi:10.3389/fgene.2020.00229.
42. Han, L.; Zhang, H.-Y.; Xu, P.-Z.; Li, Y.; Wang, X.-D.; Wu, X.-J. Genetic Analysis and Histological Study of Red Seed in Rice. *Acta Genetica Sinica* 2006, 33, 559–564, doi:10.1016/S0379-4172(06)60085-1.
43. Maeda, H.; Yamaguchi, T.; Omoteno, M.; Takarada, T.; Fujita, K.; Murata, K.; Iyama, Y.; Kojima, Y.; Morikawa, M.; Ozaki, H.; et al. Genetic dissection of black grain rice by the development of a near isogenic line. *Breeding Science* 2014, 64, 134–141, doi:10.1270/jsbbs.64.134.

44. Coe, E.H.; Neuffer, M.G.; Hoisington, D.A. The Genetics of Corn. In *Corn and Corn Improvement*; John Wiley & Sons, Ltd, 1988; pp. 81–258 ISBN 978-0-89118-212-2.
45. Lubarsky L.N. *A rye (biologo-technological properties of grain)*; Hleboizdat: Moscow, 1956;
46. Jia, Y.; Selva, C.; Zhang, Y.; Li, B.; McFawn, L.A.; Broughton, S.; Zhang, X.; Westcott, S.; Wang, P.; Tan, C.; et al. Uncovering the evolutionary origin of blue anthocyanins in cereal grains. *The Plant Journal* 2020, 101, 1057–1074, doi:<https://doi.org/10.1111/tpj.14557>.
47. Nankar, A.N.; Dungan, B.; Paz, N.; Sudasinghe, N.; Schaub, T.; Holguin, F.O.; Pratt, R.C. Quantitative and qualitative evaluation of kernel anthocyanins from southwestern United States blue corn. *Journal of the Science of Food and Agriculture* 2016, 96, 4542–4552, doi:<https://doi.org/10.1002/jsfa.7671>.
48. Havrlentová, M.; Pšenáková, I.; Žofajová, A.; Rückschloss, L.; Kraic, J. Anthocyanins in Wheat Seed – A Mini Review. *Nova Biotechnologica et Chimica* 2014, 13, 1–12, doi:10.2478/nbec-2014-0001.
49. Strygina, K.V.; Börner, A.; Khlestkina, E.K. Identification and characterization of regulatory network components for anthocyanin synthesis in barley aleurone. *BMC Plant Biology* 2017, 17, doi:10.1186/s12870-017-1122-3.