Fine Points of Marker-Assisted Pyramiding of Anthocyanin Biosynthesis Regulatory Genes for the Creation of Black-Grained Bread Wheat (*Triticum aestivum* L.) Lines

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Abstract: Enrichment of grains with anthocyanins is considered a feasible approach to improving the nutritional properties of bread wheat. Here, two black-grained substitution lines with either 4B or 4D chromosomes substituted by wheatgrass (*Thinopyrum ponticum*) 4Th were created via marker-assisted combining the anthocyanin biosynthesis regulatory genes *Pp-1*, *Pp3* (*Purple pericarp*), and *Ba1* (*Blue aleurone*) in the genetics background of cv. Saratovskaya 29. The black grains manifested the simultaneous accumulation of anthocyanins in aleurone, and the pericarp layers resulted in the highest total anthocyanin content (TAC), which amounted to approximately the sum of TACs of the purple and blue grains. The lines with substitution 4Th(4B) had long trichomes on the leaves, whereas the lines with substitution 4Th(4D) did not differ from parental cv. Saratovskaya 29. Despite the chromosome substitution having a positive effect on the main spike length, the other yield-related traits (grain weight per main spike, the spike number per plant, and the 1000-grain weight) were decreased in the lines with chromosome substitution. The developed lines together with the parental ones constitute a valuable model for comparative studies and genetic stock for breeding commercial wheat cultivars featuring high levels of anthocyanins in grains.

Keywords: *Triticum aestivum*; colored grain; anthocyanin synthesis; marker-assisted selection; multicolor FISH

1. Introduction

Anthocyanins are vegetable pigment glycosides of a polyphenolic nature that have a positive effect on human health. Due to their antioxidant, anti-inflammatory, hypoglycemic, antimutagenic, antidiabetic, anticancer, and neuroprotective properties, these compounds are currently regarded as desirable components of functional foods and as possible therapeutic substances for medicine [1,2]. In addition to berries and fruits, anthocyanins can accumulate in grains of cereals including the most common species: bread wheat (*Triticum aestivum* L., 2n = 6x = 42, BBAADD) [3], which can have a purple or blue color for its grains owing to the accumulation of certain anthocyanin pigments in the pericarp or aleurone layer [4]. At the same time, widespread red grains of wheat contain polymeric proanthocyanidins that are synthesized in the seed coat [5]. Purple and blue grains are characterized by different profiles of phenolic compounds, including anthocyanins, and non-phenolic chemicals [6,7]. The most abundant anthocyanins in purple grains are cyanidin-based compounds, among which cyanidin-3-glucoside, cyanidin-3-rutinoside, and peonidin-3-glucoside are present, whereas in blue grains, delphinidin-based compounds represent the majority of the anthocyanins: delphinidin-3 glucoside is dominant, followed by delphinidin-3-rutinoside [6,8–10].
Biological activity assays revealed that individual anthocyanins have distinct effects on physiological processes in animals and humans. It has been reported that the antioxidant activity of these compounds depends on the structural features, such as the number of hydroxyl and methyl groups and the nature of glycosylation [11]. Among anthocyanins, the highest antioxidant activity and inhibitory effect on lipid peroxidation was featured by derivatives of delphinidin, followed by cyanidin, pelargonidin, malvidin, and others [12]. The greatest inhibitory effect on low-density lipoprotein oxidation was demonstrated by cyanidin and cyanidin-3-glucoside compared to other anthocyanins [13].

In wheat, the highest antioxidant activity has been detected in black-grained plants that accumulate anthocyanins in the aleurone layer and in the pericarp simultaneously [14]. The black flour extract, having the largest anthocyanin content of the colored wheat grains, exhibited maximum antimicrobial activity against Staphylococcus aureus (MTCC 1934), Pseudomonas aeruginosa (MTCC 1434), Escherichia coli, and Candida albicans (MTCC 227) [15]. Therefore, breeding for the anthocyanin-rich content in grains is considered a promising approach to develop wheat varieties that have not only a high antioxidant level but also diverse biological activities.

Currently, the target genes for the breeding of anthocyanin-rich wheat cultivars are well known. Two complementary Pp (Purple pericarp) genes Pp-1 and Pp3, which have been mapped to chromosomes of homeologous group 7 and chromosome 2A, respectively [4,16], stimulate the biosynthesis of anthocyanins and their accumulation in pericarp cells. The Pp-1 genes encode transcription factors containing the R2R3-MYB regulatory domain. Their dominant alleles are widespread among diploid, tetraploid, and hexaploid wheat species [17–19]. In comparison, the dominant allele of the Pp3 gene, which encodes the transcription factor with the bHLH regulatory domain, originates from the tetraploid emmer (T. aephiopicum Jakubz.) and has been transferred to bread wheat by hybridization and selection [20–22].

Ba (Blue aleurone) genes are responsible for the activation of anthocyanin synthesis in the aleurone layer [4]. These genes can be introduced into the bread wheat genome from wild relatives such as blue grained wheatgrass (Thinopyrum ponticum (Podp.) Barkworth and D.R. Dewey (syn. Agropyron elongatum (Host.) Beauv.), 2n = 70) via a substitution of one pair of the homeologous group 4 chromosomes or translocations to these chromosomes [23–27]. By comparative high-throughput RNA sequencing analysis of blue and white grains of wheat, the ThMyc4E gene (encoding the bHLH transcription factor) has been identified and functionally confirmed as a candidate gene for Th. ponticum Ba1 [28].

In the current study, pyramiding of genes Pp and Ba was carried out to obtain wheat lines rich in anthocyanins in the grain. Specific procedural requirements for the development of such lines and their morphological characteristics were studied. These lines will be useful for the competent selection of modern wheat varieties with a high content of anthocyanins in the grain to obtain functional food, which will reduce milling waste in turn as well [29–31].

2. Materials and Methods

2.1. Plant Material

To create a black-grained wheat line accumulating anthocyanins in the aleurone and pericarp layers simultaneously, purple-grained near-isogenic line (NIL) i:S29Pp3Pp-D1PF and blue-grained substitution line s:S29Ba14Th(4D), both of which have been constructed in a spring cv. Saratovskaya 29 (S29) genetic background [27], were used as parental plants. The first line—i:S29Pp3Pp-D1PF—carries dominant alleles of genes Pp3 and Pp-D1, which were inherited from cultivars Purple (P) (k-46990, Australia) and Purple Feed (PF) (k-49426, Canada) as parts of introgression fragments in chromosomes 2A and 7D. This line was developed in the current study through the crossing of two dissected NILs—i:S29PpA1pp-D1Pp3Pp-D1PF (called i:S29Pp3Pp-D1PF here) and i:S29PpA1PP-D1pp3Pp-D1PF (i:S29Pp-D1PF) [32]—with subsequent marker-assisted selection of F2 plants combining the shortest introgressed fragments from cultivars Purple and Purple Feed.
The second parental line—s:S29Ba14Th(4D) [also known as s:S294Th(4D)]—is characterized by the substitution of whole wheat chromosomes 4D by *Th. ponticum* chromosomes 4 (hereafter: 4Th) inherited from winter cv. Meropa and carrying the *Ba1* gene [27].

The plants of the parental genotypes were grown in a greenhouse of the ICG SB RAS (Novosibirsk, Russia) during the vegetation season of 2018/2019 and were crossed with each other. For this purpose, immature green anthers were removed from the flowers of the recipient parental line with spikes appearing half above the flag leaf and isolated with a paper bag. The forced pollination was carried out using mature yellow anthers from individual spikes of the donor parental line plants over two or three days. The resulting F1 seeds were propagated, and among the obtained F2–4 hybrids, plants with dominant alleles of genes *Ba1, Pp-D1,* and *Pp3* were selected by the marker-assisted approach.

2.2. Marker-Assisted Selection

Genomic DNA was extracted from cut leaves of wheat seedlings at the tillering stage following a procedure detailed in ref. [33]. The extracted DNA was genotyped by means of a set of informative wheat microsatellite markers from Gatersleben (WMS or GWM) series [34–36] located on chromosomes 2A, 4B, 4D, and 7D (Table 1). For accurate diagnosis of the dominant alleles of the *Pp3* and *Ba1* genes, molecular intragenic markers were designed and applied. A polymerase chain reaction (PCR) was carried out in a 20 µL mixture containing 100 ng of a DNA template, 67 mM Tris-HCl (pH 8.8), 1.8 mM MgCl2, 0.01% of Tween 20, 18 mM (NH4)2SO4, 0.2 mM each dNTP, 0.25 µM direct and reverse specific primers, and 1 U of *Taq* DNA polymerase. The PCR was run on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the protocol described in ref. [34] and a touchdown protocol for diagnostic markers [37]. The PCR products were separated by electrophoresis in a 2% agarose gel (LE Agarose, Lonza Rockland, Inc., Rockland, ME, USA) or in 5% high-resolution agarose gel made of HyAgarose™ HR Agarose (ACTGene, Inc., Piscataway, NJ, USA) for 2–5 h and then they were analyzed under UV light using a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table 1. Molecular markers used for DNA analysis of the bread wheat lines.

| Marker       | Chromosome | Annealing Temperature, °C | Length of PCR Products, bp |
|--------------|------------|---------------------------|-----------------------------|
|              |            |                           | S29P or S29PF/S29 4Th       |                              |
| *Pp3*_diagnostic | 2A         | 65–56                     | ~405/~420                   |
| *Xgwm0328*  | 2A         | 55                        | 195/183                     |
| *Xgwm0044*  | 7D         | 60                        | 173–175/171                 |
| *Xgwm0111*  | 7D         | 55                        | 198/221                     |
| *Xgwm0676*  | 7D         | 50                        | ~190/119                    |
| *Xgwm0066*  | 4B         | 60                        | 150                         |
| *Xgwm0251*  | 4B         | 55                        | ~210                        |
| *Wms0375*   | 4B         | 65–56                     | ~190                        |
| *Xgwm0888*  | 4B         | 60                        | 197                         |
| *Xgwm0910*  | 4B         | 55                        | 148                         |
| *Xgwm0624*  | 4D         | 55                        | 120                         |
| *Xgwm1163*  | 4D         | 55                        | ~140                        |
| *Xgwm1397*  | 4D         | 55                        | ~150                        |
| *Xgwm1706*  | 4D         | 55                        | ~130                        |
| *Xgwm3156*  | 4D         | 55                        | ~380                        |
Table 1. Cont.

| Marker      | Chromosome | Annealing Temperature, °C | Length of PCR Products, bp |
|-------------|------------|---------------------------|---------------------------|
|             |            |                           | S29^P or S29^PF/S29       | 4Th                       |
| Xgwm4001 *  | 4D         | 55                        | ~250                      | ~220                      |
| Xgwm4736 *  | 4D         | 55                        | ~250                      | -                         |
| ThMyc4E     | 4Th        | 65–56                     | -                         | ~200                      |

Note: * the sequence of the primers is available upon request from Dr. M. Röder (IPK Gatersleben), and the structure of the other markers can be found in the GrainGenes database (https://wheat.pw.usda.gov/GG3/, accessed on 1 October 2022).

2.3. The Design of Diagnostic PCR Markers

To choose diagnostic PCR markers for the Pp3 gene, nucleotide sequences of the dominant allele TaPpb1a (MG066455) and the recessive allele TaPpb1b (MG066456) of the gene were found in the NCBI database (https://www.ncbi.nlm.nih.gov/, accessed on 1 August 2019) by a homology search, with the nucleotide sequence of the Pp3 gene (KJ747954) as a query. These sequences were aligned in the MultAlin software (http://multalin.toulouse.inra.fr/multalin, accessed on 1 August 2019), and differences in the promoter region of the gene were revealed. Using the PrimerQuest™ Tool (Integrated DNA Technologies, Inc., Coralville, IA, USA, https://eu.idtdna.com/pages, accessed on 1 August 2019), a Pp3-diagnostic marker, which is detected by forward Pp3_Fd: 5′-TAGTGCCGTCTAACTGGTGA-3′ and reverse Pp3_Rd: 5′-ACGACGCCTAAGGAAACAC-3′ primers, was designed. These primers flank the polymorphic region and amplify PCR products of different lengths that allows for distinction of the dominant and recessive alleles (Figure S1).

To choose PCR marker primers that would amplify a fragment of the Th. ponticum Ba1/ThMyc4E gene and not amplify its orthologous sequences from the common-wheat genome, multiple alignment involving the nucleotide sequence of ThMyc4E (KX914905) and wheat orthologous sequences TraesCS4B02G397400 and TraesCS4D02G224600 found in the Ensembl Plants database (https://plants.ensembl.org/index.html, accessed on 1 August 2019) was performed. Substitutions in wheatgrass nucleotide sequences relative to wheat ones were identified. Specificity of the ThMyc4E-specific primers (ThMyc4E_F: 5′-GAAACAACAGGACCGAGCAG-3′ and ThMyc4E_R: 5′-CTTGATGGCGTCAAACACTT-3′) to the Th. ponticum Ba1/ThMyc4E gene was ensured by means of the 3′ end of the primers, which was completely identical to wheatgrass nucleotide sequences and differed from the wheat orthologs (Figure S2).

2.4. Karyotyping

Karyotypes of lines and parental forms were examined by fluorescence in situ hybridization (FISH) with probes based on cloned DNA repeats. Clone pAs1 contains a 1 kbp DNA fragment derived from Aegilops tauschii Coss. and belonging to the Afi family [38], and clone pSc119.2 contains a 120 bp sequence isolated from Secale cereale L. [39]. Hybridization with pSc119.2 and pAs1 enabled us to identify the individual chromosomes of the B and D subgenomes of wheat [40]; the pSc119.2 probe is mainly localized to T. aestivum B-subgenome chromosomes, whereas pAs1 is localized to D-subgenome chromosomes. In addition, individual Th. ponticum chromosomes can be identified with the help of a pattern of hybridization with probes pSc119.2 and pAs1 [25,27]. Preparations of mitotic chromosomes were made from root tips as described before [41]. At least 10 metaphase cells per slide were analyzed. Two to five slides were examined for each wheat line and parental form. Our FISH protocol was borrowed from ref. [42], with some modifications [43].

2.5. Analysis of the Total Anthocyanin Content (TAC)

Ripe grains of each line were crushed to prepare whole-grain flour samples using an L0M-1 laboratory grain mill (Zernotechnika, Moscow, Russia). For the total-anthocyanin extraction, 1 g of the sample material was homogenized in 10 mL of a 1% HCl solution...
in methanol, shaken, and incubated at 4 °C for 12 h. After centrifugation at 12,000 rpm for 15 min at 4 °C, the samples were measured on a SmartSpec TM Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 530 and 700 nm. The corrected value ($A_{530} - A_{700}$) was used to calculate the mass concentration in micrograms of cyanidin-3-glycoside (Cy-3-Glu, the main representative of dietary anthocyanins from fruits and berries) per gram of dry weight of the samples (DW) according to the protocol described in ref. [44]. Three biological replicates were performed. The significance of differences in TAC between the samples was assessed using the Mann–Whitney $U$-test.

2.6. Histological Analysis of Grains
To study the localization of anthocyanin pigments in the seed’s envelopes, the seeds of the newly created and parental lines were collected at the dough stage of spike development and frozen at −20 °C. Cytological analysis, including cryosection preparation and assessment of the visible pigments, was performed according to previously developed protocols without modifications on identical equipment [45].

2.7. Phenotyping
The new lines and their parental cultivars were grown in an experimental field of the ICG Breeding Genetic Complex (Novosibirsk, N54.847102 and E83.127422). The lines were grown in one place in 5 rows of 1 m-long plots, with 50 plants in each row. For phenotypic comparison between the obtained lines, 10 plants of each genotype were taken. The following parameters were evaluated: plant height, the spike number per plant, the grain number and grain weight per plant, main spike length, the number of grains, grain weight per main spike, and 1000-grain weight (TGW).

2.8. Statistics
A statistical analysis was carried out in STATISTICA v. 10 software (StatSoft, Inc., Tulsa, OK, USA). The statistical significance of the differences in yield-related parameters was assessed by the Mann–Whitney $U$ test, with $p < 0.05$ indicating significance. The non-parametric Kruskal–Wallis $H$ test (one-way analysis of variance [ANOVA] on ranks) and the Fisher $F$ test (two-way ANOVA) were performed for determining the influence of factors the “grain color” and “chromosome substitution” on the yield-related parameters of the wheat lines. Post hoc multiple pairwise comparisons of the groups of specimens were carried out by the Duncan test. Correlations between parameters were evaluated by means of Spearman’s rank correlation coefficients.

3. Results
3.1. The Crossing Scheme and Marker-Assisted Selection
The pyramiding of genes $Pp$ and $Ba$ to construct black-grained wheat lines enriched with anthocyanins was implemented in a two-stage crossing scheme (Figure 1). In the first stage, NILs $i$:S29$Pp^3$ and $i$:S29$Pp-D1^{PF}$ were crossed to obtain a purple-grained wheat line with minimized introgressive regions on chromosomes 2A and 7D carrying dominant alleles of genes $Pp^3$ and $Pp-D1$, respectively. Genomic DNA was isolated from the resulting $F_2$ plants, 48 in total, and marker-assisted selection was conducted using microsatellite (SSR) markers located on chromosomes 2A ($Xgwm0328$ and $Xgwm0444$ and $Xgwm0111$) and the $Pp^3$-diagnostic marker. The plants whose coleoptiles had a red color (because of the dominant allele(s) of the $Pp-D1^{PF}$ gene) were planted and their genomic DNA was isolated. The specimens that carried PCR products of SSR markers linked to the $Pp^3$ gene on chromosomes 2A, and to the $Pp-D1^{PF}$ gene on chromosomes 7D corresponding to lines $i$:S29$Pp^3$ and $i$:S29$Pp-D1^{PF}$, respectively, were selected. The coleoptile and grain color homogeneity of $F_3$ family plants were scored, and one of the family members in which these traits did not segregate was chosen and named $i$:S29$Pp^3Pp-D1^{PF}$.
Figure 1. The crossing scheme and marker-assisted selection (MAS) of the homozygous near-isogenic and substitution purple-, blue-, and black-grained lines in the genetic background of cv. Saratovskaya 29. Where: obtaining the isogenic purple-grained line with minimal recombinant regions in the chromosomes (stage I), obtaining the black-grained lines (stage II). An outline of wheat chromosomes with anthocyanin biosynthesis regulatory genes and intragenic (Pp3-diagnostic and ThMyc4E-specific) and microsatellite markers is shown.
At the second stage of the crossing scheme, newly developed line i:S29Pp3Pp-D1PF was crossed with blue-grained substitution line s:S29Ba14Th(4D). The hybrid F1 plants, nine in total, self-pollinated and showed segregation of white and blue grains in one spike. The darkest F2 grains harvested from them, 38 in total, were propagated further. Genomic DNA was isolated from the grown F3 plants and genotyped by intragenic Pp3-diagnostic and ThMyc4E-specific markers (Figure S3). Three black-grained plants were selected. They were characterized by the red coleoptile color (due to the dominant allele(s) of PpD1), were homozygous for dominant alleles of the Pp3 gene, and amplified wheatgrass ThMyc4E gene products. Twenty F4 grains from each plant, 60 in total, were sown. Homozygosity for Pp-D1PF was tested via genotyping of the resultant F4 plants by means of SSR marker Xgwm0111 linked to Pp-D1PF (Figure S3). All the F4 plants carried homozygous Pp-D1PF. When the grain color homogeneity of the three F4 families was scored, there was no segregation of the grain color in any family, but segregation by leaf trichome morphology was noted.

Among the hybrids’ F2–3 progenies, there were plants with parental-like short trichomes (hairs) on the leaves and plants possessing a new phenotype featuring hard, long trichomes not characteristic of any parental lines (Figure 1). Because wheatgrass 4Th may substitute chromosomes 4B or 4D [26,27], we hypothesized that the observed differences in trichome morphology can be attributed to substitutions of different wheat chromosomes. To test this assumption, DNA genotyping of the F3 plants was carried out by means of microsatellite markers polymorphic between parental lines i:S29Pp3Pp-D1PF and s:S29Ba14Th(4D) located on chromosomes 4B and 4D (Table 1). As an example, microsatellite genotyping results with some of the markers are given in Figure S4. All the samples with hard trichomes yielded amplicons of ThMyc4E gene fragments and amplicons of the microsatellite markers only from wheat chromosome 4D (but not 4B), implying a substitution of the pair of wheat chromosomes 4B by the pair of wheatgrass chromosomes 4Th [substitution 4Th(4B)]. These plants carried homozygous dominant alleles of genes Pp3Pp and Pp-D1PF and are characterized by the black-grained phenotype. Homogeneity of grain color and trichome morphology in F4 plants (60 in total) were scored next; traits segregation was absent, and the resulting plants were named substitution line s:S29Ba14Th(4B)Pp3Pp-D1PF.

Other F3 black-grained plants had rare, long trichomes among soft, short, parental-like hairs. They had amplicons of ThMyc4E gene fragments and microsatellite markers only from chromosome 4B (not 4D), indicating a substitution of the pair of wheat chromosomes 4D by the pair of wheatgrass chromosomes 4Th [4Th(4D)] (Figure S4). These plants possessed a stable parental-like soft trichome phenotype and a black color of grains in the F4 generation and were designated as substitution line s:S29Ba14Th(4D)Pp3Pp-D1PF.

In addition to the obtained black-grained lines, blue-grained lines s:S29Ba14Th(4B)Pp-D1PF and s:S29Ba14Th(4B) with hard, long trichomes on the leaves were clearly defined and selected from the progenys of the F2–3 hybrids. Chromosome substitutions in these lines were confirmed by microsatellite genotyping. Both lines lacked dominant alleles of the Pp3 gene but were differed from each other by coleoptile pigmentation. The former line had intense red coleoptiles due to the presence of dominant alleles of the Pp-D1PF gene, whereas the latter one had a pale red color of coleoptiles owing to the Pp-A1 allele inherited from S29. The characteristics of the parental and constructed lines are summarized in Table 2.

### 3.2. Karyotypic Examination of the Lines

To confirm the substitution of wheat chromosomes 4B and 4D by wheatgrass chromosome 4Th, the selected black-grained substitution lines s:S29Ba14Th(4B)Pp3Pp-D1PF and s:S29Ba14Th(4D)Pp3Pp-D1PF together with parental cultivars S29 and Meropa were examined by FISH (Figure 2). The analysis showed that cv. Meropa and black-grained line s:S29Ba14Th(4D)Pp3Pp-D1PF indeed lack both copies of wheat chromosome 4D, which can be easily distinguished by its unique pAs1 hybridization pattern; instead, they carry a pair of chromosomes with an intercalary pAs1 signal in the short arm close to the cen-
tromere (Figure 2B,C); this feature has previously been identified in blue-grained line s:S29Ba14Th(4D) as *Th. ponticum* chromosomes 4Th [27]. In comparison with these lines, s:S29Ba14Th(4B)Pp3*Pp-D1* was found to lack both copies of wheat chromosome 4B, which can be distinguished by its hybridization pattern with the pSc119.2 probe but contains pairs of *Th. ponticum* chromosomes 4Th (Figure 2D). Thus, the karyotypic analysis confirmed the substitution of wheat chromosome 4D or 4B by wheatgrass chromosome 4Th in the black-grained lines constructed in the current study.

**Table 2.** The set of bread wheat parental and newly created lines with grain pigmentation reflecting various combinations of anthocyanin biosynthesis regulatory genes *Pp-1* and *Pp3* in the pericarp and *Ba1* in the aleurone layer.

| #  | Name                        | *Pp-A1* (7A) | *Pp-D1* (7D) | *Pp3* (2A) | Pericarp Color | Coleoptile Color | Aleurone Color | Chr Substitution | Ref.          |
|----|-----------------------------|--------------|--------------|------------|----------------|-----------------|---------------|-----------------|--------------|
| 1  | Saratovskaya 29 *           | d            | r            | r          | no             | light red       | no            | no              |              |
| 2  | i:S29Pp3*                   | d            | r            | d          | no             | light red       | no            | no              | [32]         |
| 3  | i:S29Pp-D1*                 | d            | d            | r          | no             | dark red        | no            | no              | [32]         |
| 4  | i:S29Pp3*Pp-D1*             | d            | d            | d          | purple         | dark red        | no            | no              | current study |
| 5  | s:S29Ba14Th(4D)             | d            | r            | r          | no             | light red       | blue          | 4Th(4D)         | [27]         |
| 6  | s:S29Ba14Th(4B)             | d            | r            | r          | no             | light red       | blue          | 4Th(4B)         | current study |
| 7  | s:S29Ba14Th(4B)Pp-D1*       | d            | d            | r          | no             | dark red        | blue          | 4Th(4B)         | current study |
| 8  | s:S29Ba14Th(4D)Pp3*Pp-D1*   | d            | d            | d          | purple         | dark red        | blue          | 4Th(4D)         | current study |
| 9  | s:S29Ba14Th(4B)Pp3*Pp-D1*   | d            | d            | d          | Purple         | dark red        | blue          | 4Th(4B)         | current study |

Notes: * background cultivar; *P* the source of the dominant alleles of *Pp3* and *Pp-D1* was *T. aestivum* cv. Purple; *PF* the source of the dominant alleles of *Pp3* and *Pp-D1* was *T. aestivum* cv. Purple Feed; d—dominant allele; r—recessive allele. The coloring background corresponds to the color of grains and coleoptiles.

3.3. Histological Analysis of Grain Pigmentation

Cryosections of seeds of red-grained cv. S29, of purple-grained NIL i:S29Pp3*Pp-D1*; of blue- and black-grained substitution lines s:S29Ba14Th(4D), and of s:S29Ba14Th(4D)Pp3*Pp-D1* were assayed by light microscopy at the hard dough stage of ripening (Figure 3). In S29, the aleurone, seed coat (or testa), and pericarp were clearly visible, but except for a brown strip that we identified as a testa, no pigments in the grain layers were detectable (Figure 3A). In the grain of blue-grained substitution line s:S29Ba14Th(4D), many blue small inclusions in the cells of the aleurone were observed within organelles similar to vacuoles (Figure 3B). The brown-red pigmented structures identified as anthocyanins were found in the pericarp of purple-grained line i:S29Pp3*Pp-D1* in organelles that were similar to vacuoles but looked different from the organelle accumulating the blue pigments in the aleurone (Figure 3C). In black-grained line s:S29Ba14Th(4D)Pp3*Pp-D1*, both blue and brownish-red pigmented structures similar to vacuoles with anthocyanins were visible in the aleurone and pericarp layer (Figure 3D). Simultaneous accumulation of blue and purple pigments in black (dark purple) grains means an increased total content of anthocyanins in the grain as proven by the spectrophotometric assay.
Figure 2. FISH with combinations of probes pSc119.2 and pAs1 specific to the mitotic metaphase chromosomes of *T. aestivum* cv. Saratovskaya 29 (S29) (parental line) (A), cv. Meropa (B), black-grained line s:S29Ba14Th(4B)pP3Pp-D1PF with trichomes on leaves (C), and black-grained line s:S29Ba14Th(4D)pP3Pp-D1PF with leaves like those of cv. S29 (D). pSc119.2 (red) and pAs1 (green) are presented in (A); pSc119.2 (green) and pAs1 (red) are presented in (B–D).

Figure 3. The seeds and cross-sections of the seeds produced by cv. S29 (A) and lines s:S29Ba14Th(4D) (B), s:S29pP3Pp-D1PF (C), and s:S29Ba14Th(4D)pP3Pp-D1PF (D) sampled at the hard-dough stage. al: aleurone layer, te: testa, pe: pericarp. The scale bars for the seeds are 2.5 mm, and for the micrographs, 25 μm.
3.4. The TAC of Whole Grains

The TAC of ripe grains was determined in the series of parental and newly developed lines grown in the fall and spring seasons of 2018–2019 in a greenhouse and in the summer 2019 in the field (Figure 4).

Figure 4. The total anthocyanin content (TAC) of methanol extracts from whole-grain (WG) flour of wheat plants grown in a greenhouse in the autumn and springtime or in the field in the summer. S29: parental cv. Saratovskaya 29, its uncolored sister lines i:S29Pp3Pp-D1 and i:S29pp3Pp-D1PF, purple-grained NIL i:S29Pp3Pp-D1PF, blue-grained substitution lines s:S29Ba14Th(4D) and s:S29Ba14Th(4B)Pp-D1PF, and black-grained lines s:S29Ba14Th(4D)Pp3Pp-D1PF and s:S29Ba14Th(4B)Pp3Pp-D1PF; DW: dry weight. Letters (a, b, c, d, e) indicate statistically significant differences between the lines within one season (U test, \( p < 0.05 \)). Data are presented as the mean ± SD.

In the greenhouse fall vegetation season, the TAC of grains of red-grained parental lines i:S29Pp3Pp, i:S29Pp-D1PF, and cv. S29 was the lowest and ranged from 2.5 to 3.1 \( \mu g/g \). The TAC of blue-grained line s:S29Ba14Th(4B)Pp-D1PF was significantly different from that of blue-grained line s:S29Ba14Th(4D); the TACs of both blue-grained lines were almost twice as high as the TAC of purple-grained NIL i:S29Pp3Pp-D1PF: 149.8 and 138.0 \( \mu g/g \) compared to 70.1 \( \mu g/g \), respectively. The TACs of black (dark purple) grains of lines s:S29Ba14Th(4B)Pp3Pp-D1PF and s:S29Ba14Th(4D)Pp3Pp-D1PF did not differ significantly from each other, showing 289.7 and 297.3 \( \mu g/g \), which was more than the sum of TACs of the blue- and purple-grained lines in this season.

In the greenhouse spring vegetation season, the TAC of grains of the purple-grained NIL was significantly different and was almost three times the level seen after the autumn vegetation season: 218.1 versus 70.1 \( \mu g/g \). On the other hand, the TAC of both blue-grained substitution lines did not differ significantly from that in the same specimens harvested from the fall season: 131.9 and 134.0 \( \mu g/g \). The TAC did not differ significantly between the black-grained lines (360.7 and 389.5 \( \mu g/g \)) and was significantly higher than that of the same specimens harvested from the fall season. The total anthocyanin content of the black-grained lines was approximately the sum of TACs of the blue- and purple-grained lines harvested from the same season.

Wheat plants grown in the summertime in the field showed the lowest anthocyanin content of the grain: 58.9 \( \mu g/g \) in the purple-grained line, 87.6 and 96.4 \( \mu g/g \) in blue-grained lines, and 236.7 and 269.3 \( \mu g/g \) in the black-grained lines. TACs of all the tested lines significantly differed from those of the same lines harvested in the other vegetation
seasons, indicating a strong influence of the environmental factors on the biosynthesis and accumulation of anthocyanins in the wheat grain.

There were some quantitative differences in the TAC of grains of the same specimens harvested from different vegetation seasons. A positive correlation was noted in the TAC between grains harvested from the greenhouse fall and spring seasons ($r_s = 0.83, p = 0.0416$) as well as between grains harvested from the greenhouse fall season and in the field ($r_s = 0.94, p = 0.0048$), whereas no correlation in the TAC was observed between grains harvested from the greenhouse spring season and in the field ($r_s = 0.77, p = 0.0724$).

3.5. Comparative Analysis of Yield-Related Parameters of the Parental and Newly Developed Lines

To detect the possible effects of the genes regulating anthocyanin synthesis in grains and the corresponding transfers of chromosomes (substitutions) on yield-related parameters, a comparative phenotyping analysis was performed on the parental and new lines (Table 3).

Table 3. Characteristics of the lines possessing different combinations of anthocyanin pigments. Data are presented as the mean ± SD.

| Trait/Line       | S29 | iS29 Pp3Pp-D1PF | sS29Ba1 P4Th(4B) | sS29Ba1 P4Th(4D) | sS29Ba1 P4Th(4B) Pp3Pp-D1PF | sS29Ba1 P4Th(4D) Pp3Pp-D1PF |
|------------------|-----|----------------|------------------|------------------|-----------------------------|-----------------------------|
| Plant height, cm | 96.4 ± 4.7<sup>a</sup> | 97.9 ± 5.2<sup>bc</sup> | 95.0 ± 3.7<sup>ab</sup> | 92.1 ± 4.6<sup>a</sup> | 99.6 ± 5.5<sup>bc</sup> | 101.2 ± 5.1<sup>c</sup> |
| Number of spikes per plant | 3.2 ± 1.1<sup>b</sup> | 2.8 ± 0.9<sup>b</sup> | 2.1 ± 0.1<sup>a</sup> | 2.6 ± 1.1<sup>a</sup> | 2.1 ± 0.7<sup>ab</sup> | 2.3 ± 1.3<sup>ab</sup> |
| Main spike length, cm | 7.0 ± 0.7<sup>ab</sup> | 6.7 ± 0.7<sup>a</sup> | 8.3 ± 0.8<sup>cd</sup> | 7.0 ± 0.5<sup>ab</sup> | 8.5 ± 0.7<sup>d</sup> | 7.5 ± 0.8<sup>bc</sup> |
| Number of grains per main spike | 32.1 ± 3.4<sup>ab</sup> | 28.7 ± 4.1<sup>a</sup> | 34.6 ± 5.4<sup>b</sup> | 31.5 ± 4.2<sup>ab</sup> | 33.6 ± 4.2<sup>b</sup> | 32.6 ± 5.7<sup>ab</sup> |
| Number of grains per plant | 78.1 ± 36.1<sup>a</sup> | 66.1 ± 22.3<sup>a</sup> | 78.5 ± 39.3<sup>a</sup> | 86.1 ± 22.2<sup>a</sup> | 85.0 ± 24.9<sup>a</sup> | 85.0 ± 52.0<sup>a</sup> |
| Grain weight per main spike, g | 1.3 ± 0.2<sup>c</sup> | 1.1 ± 0.2<sup>b</sup> | 0.8 ± 0.2<sup>a</sup> | 0.9 ± 0.2<sup>a</sup> | 0.9 ± 0.2<sup>ab</sup> | 0.9 ± 0.2<sup>ab</sup> |
| Grain weight per plant, g | 2.9 ± 1.5<sup>b</sup> | 2.3 ± 0.9<sup>b</sup> | 1.6 ± 0.9<sup>a</sup> | 2.1 ± 0.5<sup>ab</sup> | 2.2 ± 0.8<sup>ab</sup> | 2.1 ± 1.4<sup>ab</sup> |
| TGW, g | 36.8 ± 3.3<sup>c</sup> | 34.1 ± 3.5<sup>c</sup> | 20.5 ± 2.9<sup>a</sup> | 24.1 ± 1.6<sup>b</sup> | 25.7 ± 3.8<sup>b</sup> | 24.3 ± 4.1<sup>b</sup> |

Notes: <sup>a,b,c,d</sup>—different letters within a row indicate statistically significant differences between the lines ($U$ test, $p < 0.05$). The coloring background corresponds to the color of grains.

There were no differences between the lines in the number of grains per plant, while the other tested traits differed significantly between the lines. Plant height varied from 92.1 ± 4.6 cm (mean ± SD) in sS29Ba14Th(4D) to 101.2 ± 5.1 cm in sS29Ba14Th(4D)Pp3Pp-D1PF. Line sS29Ba14Th(4B) was characterized by the lowest number of the spikes per plant (2.0 ± 1.1), the grain weight per main spike (0.8 ± 0.2 g), the grain weight per plant (1.6 ± 0.9 g), and the TGW (20.5 ± 2.9 g); these traits had the highest values in S29 (3.2 ± 1.1, 1.3 ± 0.2 g, 2.9 ± 1.5 g, and 36.8 ± 3.3 g, respectively). Line iS29Pp3Pp-D1PF possessed the smallest main spike length (6.7 ± 0.7 cm), the greatest number of grains per main spike (28.7 ± 4.1 cm), and the number of grains per plant (66.1 ± 22.3), whereas line sS29Ba14Th(4B)Pp3Pp-D1PF possessed the greatest main spike length (8.5 ± 0.7 cm), and lines sS29Ba14Th(4B) and sS29Ba14Th(4D) had the greatest number of grains per main spike (34.6 ± 5.4) and the greatest number of grains per plant (86.1 ± 22.2), respectively.

The effects of the factors “grain color” and “chromosome substitution” on the yield-related traits in the six lines were subjected to one-way ANOVA on ranks (Table S1). Neither “grain color” nor “chromosome substitution” affected the number of grains per spike, the number of grains per plant, and the grain weight per plant. Both of the factors influenced the main spike length, the grain weight per main spike, and the TGW. The plant height and the spike number per plant were affected only by “grain color” and “chromosome substitution”.

Post hoc multiple pairwise comparison of the traits’ values between the groups of specimens combined based on the factor analyzed was conducted using the Duncan test. Four groups of specimens combined based on the grain color, among them red, purple, blue, and black were compared (Table S2).

It was found that the black-grained plants were taller than the blue-grained ones \((p = 0.0011)\) and they did not differ in this parameter from the red- and purple-grained lines \((p = 0.0530\) and \(0.1876\), respectively). The blue- and black-grained series of specimens had longer main spikes in comparison to the red-grained cv. S29 \((p = 0.0206\) and \(0.0006\), respectively) and purple-grained \((p = 0.0012\) and \(0.0001\), respectively) line, but they did not differ from each other \((p = 0.1612)\). The blue- and black-grained series of specimens had the smallest grain weight per main spike as compared to the red- \((p = 0.0001\) for both groups), and purple-grained \((p = 0.0029\) and \(0.0333\) lines and they did not differ from each other \((p = 0.2933)\).

The TGW of the blue-grained lines was the smallest one as compared to red- \((p = 0.0001)\), purple- \((p = 0.0001)\), and black-grained \((p = 0.0472)\) groups of lines.

Three groups of lines combined based on the presence of chromosome substitution that were ‘no chromosome substitution’, ‘4Th(4B)’, and ‘4Th(4D)’, were compared (Table S3).

The group of lines that had chromosome substitution 4Th(4B) had a smaller spike number per plant as compared to the group of lines without substitutions \((p = 0.0088)\) and they did not differ from the group of lines with chromosome substitution 4Th(4D) \((p = 0.2378)\).

The group of lines that had chromosome substitution 4Th(4B) had a longer main spike versus the group of lines that did not carry wheatgrass chromosome 4Th \((p = 0.0001)\) and the lines with chromosome 4D substituted by 4Th \((p = 0.0001)\).

The grain weight per main spike and the TGW were lower in the lines carrying 4B or 4D chromosomes replaced by 4Th compared to the group of lines without substitutions \((p = 0.0001\) or \(0.0002\) and \(p = 0.0001\) for both groups, respectively). However, there were no differences in these parameters between the lines with substitution 4Th(4B) or 4Th(4D) \((p = 0.4943\) and \(0.3025\), respectively). An effect of an interaction between the two factors “grain color x chromosome substitution” on any yield-related traits was not detectable, except for the TGW (Figure S5). There were no differences in the TGW between the blue- and black-grained lines \((p = 0.8805)\) with chromosome 4D substituted by 4Th, whereas such differences between the blue- and black-grained lines were found between the lines with chromosome 4B substituted by 4Th \((p = 0.0017)\), with a significantly lower TGW in the blue-grained line. In the group of blue-grained lines, a lower TGW was observed in the line with chromosome 4B replaced by 4Th, as compared to the line with chromosome 4D substituted by 4Th \((p = 0.0178)\), whereas in the black-grained lines, the effect of chromosome substitutions on the TGW was undetectable \((p = 0.3651)\).

It is worth nothing that the grain weight per plant was not influenced by any of the tested factors or their combinations, although this trait correlated with the spike number per plant \((r_s = 0.6822, p = 0.0000)\) and the grain weight per main spike \((r_s = 0.5971, p = 0.0000)\), both of which were affected by the tested factors (Table S4). TGW was positively correlated with the spike number per plant \((r_s = 0.3633, p = 0.0043)\), the grain weight per main spike \((r_s = 0.7293, p = 0.0000)\), the grain weight per plant \((r_s = 0.4271, p = 0.0007)\), and negatively correlated with the main spike length \((r_s = -0.3345, p = 0.0090)\).

4. Discussion
4.1. Marker-Assisted Breeding of Black-Grained Lines

Thanks to previously obtained data on the chromosomal location and sequencing of the major genes that trigger anthocyanin biosynthesis in wheat grain \((20; 25–27)\), we developed the bread wheat lines with different combinations of alleles at the \(Pp\) and \(B1\) loci in cv. Saratovskaya 29 background.
Here, black-grained wheat substitution lines whose grains accumulate anthocyanins simultaneously in aleurone and pericarp layers were selected from the F$_2$–4 populations obtained after the crossing of a purple-grained line with a blue-grained one.

In the presented scheme, to accelerate the selection of black-grained lines and to increase the accuracy of this procedure, we took advantage of a marker-assisted approach involving intragenic PCR markers chosen for the key anthocyanin biosynthesis regulatory genes $Pp3$ and $Ba1$ and microsatellite markers. This approach allowed not only the detection of the dominant alleles of the target genes but also determination of whether they are in a homozygous or heterozygous state.

To select plants with short introgressions on chromosome 2A carrying the dominant allele $Pp3$, the intragenic polymorphic marker $Pp3$-diagnostic in F$_2$–3 generations on the first and the second steps was applied. Specific oligonucleotide primers were designed in such a way that the PCR products had different length between the dominant and recessive alleles $Pp3$. The data presented in this work indicate that the developed $Pp3$-diagnostic DNA marker can be used to select plants with the dominant allele of the $Pp3$ gene both among hexaploid ($T. aestivum$, $T. spelta$) and tetraploid ($T. aethiopicum$, $T. durum$) samples.

Due to the fact that gene $Pp$-$D1$ could regulate the anthocyanin pigmentation of vegetative and generative organs, plants with short introgressions on chromosome 7D carrying the dominant allele $Pp$-$D1$ were selected both visually by the coleoptile coloration and by microsatellite analysis.

The knowledge of the nucleotide sequence of the $Ba1$ / $ThMyc4E$ gene (28) made it possible to design intragenic primers $ThMyc4E$ for the accurate and fast selection of blue grain wheat accessions. The substituted line s: S29$Th$/4D, obtained earlier after crossing S29 with the winter wheat Meropa (Bulgaria-BG15), carrying the 4Th chromosome instead of the 4D chromosome served as the donor of the trait of the blue color of the grain, where the $ThMyc4E$ gene controlling the blue color of the grain was identified (27). The combined the intragenic $ThMyc4E$ with microsatellite markers localized on chromosomes 4B and 4D, in F$_3$ generation, homozygous plants, in which the pair of chromosomes 4B or 4D was completely replaced by a wheatgrass pair of chromosome 4Th, were selected. Two types of chromosome substitutions were identified: 4Th(4B) and 4Th(4D) in blue- and black-grained lines, which was confirmed by FISH.

As evidenced by histological analysis, the black-grained (dark purple) specimens combine purple anthocyanins in the pericarp and blue anthocyanins in the aleurone independently. Some differences in the appearance of the structures accumulating purple and blue pigments were documented. Purple pigments were detected in vacuole-like structures that fill the whole area of some cells of the pericarp, while the blue pigments were seen in numerous small structures similar to protein storage vacuoles filling aleurone cells. Such accumulation patterns of pigments in different grain layers (one of which is located more deeply than the others) made it difficult to select the plant specimens containing the pigments in both layers, especially when dry seeds were evaluated. Segregation of the pigmentation traits occurred. These phenomena have made the morphological selection of stable blue and purple grain pyramided lines adapted to local climatic conditions a difficult and long procedure [46,47]. The lines and the allele-specific markers designed in this work are currently being used for obtaining new, improved, commercial bread wheat cultivars with high anthocyanins content in grains adapted to the Siberian region of Russia.

4.2. Comparative Evaluation of TACs between the Lines

The combination of genes $Pp$ and $Ba1$ in one genome raised the TAC. Although quantitative differences in the TACs were revealed between the grains harvested from different vegetation seasons, the highest TAC was observed in black-grained lines, thus implying the independent biosynthesis pathways for these compounds.

The TACs of the purple-grained lines from the spring vegetation in the greenhouse turned out to be more than three times the level of the TACs of the same samples harvested from the autumn vegetation in the greenhouse and from the summer harvest in the field.
These large fluctuations in the accumulation of anthocyanins confirm the higher sensitivity of anthocyanin synthesis in cells of the pericarp to environmental changes, while anthocyanin synthesis in the aleurone layer seems to be less sensitive to changes in light and temperature. The TAC fluctuation in blue-grained samples was not observed.

There were no anthocyanins in the red grains of cv. S29. Measurements of these grains were 2.5–3.5 µg/g, probably due to the fact that the peak wave 530 nm for anthocyanins with a spectrophotometer captured the attenuation (a lateral end) of the other compounds, probably the proanthocyanidin wave.

These results are consistent with the available data on the TACs from other authors regarding cultivars and breeding lines rich in anthocyanins (Table S5). Some authors have noted that the grain TAC was affected by the genotype, weather conditions in a given year, the field location, genotype × environment interactions, and abiotic stressors [6,10,48,49]. Breeding lines very often demonstrated a greater TAC than the parental donor lines did [50]. This observation implies the significant impact of the genetic background into which the genes controlling anthocyanin synthesis are introduced. Moreover, storage conditions and the time of the year may influence the TAC of cereal grains [51]. It has been demonstrated that the TAC diminished slightly after 6 months of storage [52].

4.3. The Chromosome Substitutions Affect Leaf Trichomes and Yield-Related Traits

The wheatgrass Ba1 locus is transferred into the wheat genome as a part of the entire chromosome 4Th, which can substitute either wheat chromosome 4B or 4D [26,27]. The replacement of a pair of wheat chromosomes with a pair of homoeologous wheatgrass chromosomes occurs randomly and is a consequence of the unstable behavior of chromosomes during meiosis because of the incorrect division of univalent chromosomes 4Th and 4D [53]. Many studies have shown that chromosomes 4B and 4D are not identical in gene composition [54–56], and therefore their substitutions can affect the traits of hybrids in different ways.

As proof of the concept, here, we clearly observed for the first time (to our knowledge) that a substitution of chromosome 4B or 4D by wheatgrass chromosome 4Th affects leaf trichome morphology differently. Therefore, blue- and black-grained lines with chromosomes 4B substituted by 4Th had hard, long trichomes on their leaf blades that differed from the pubescence represented by dense, soft, short trichomes on the leaves of cv. S29 and of the lines with chromosome 4D substituted by 4Th. It was shown that chromosome 4B of cv. S29 carries the Hl1 (Hairy leaves 1) gene which together with Hl2 (mapped to 7BS) and Hl3 (unknown genome position so far) regulates leaf hair length and density [57–59]. Hl1 is reported to have a dosage effect and reduces the pubescence when present in a single dose (as in the S29 line monosomic for chromosome 4B). The absence of functional Hl1 and Hl3 results in a decrease in both trichome length and number. An increase in the proportion (%) of long leaf hairs and to a lesser extent in the overall number of leaf hairs is observed when the Hl2aesp gene responsible for pubescence in Ae. speltoides is added into the wheat genome carrying Hl1 and Hl3 in a dominant or recessive state [59]. Since the hard leaf phenotype with long trichomes was clearly manifested only in lines that lacked chromosome 4B, but not 4D, the primary reason for the appearance of plants without parental pubescence on their leaf blades was probably the absence of a pair of chromosomes 4B.

In our study, using molecular and FISH cytology analyses, the substitution of the pair of the parental cv. S29 wheat chromosomes 4B by the pair of wheatgrass chromosome 4Th in the genomes of the black- and blue-grained F2-3 progeny plants with the hard leaves phenotype was confirmed. Evidently, the new dominant allele of the Hl1 gene, which regulates the formation of the rare, long trichomes on leaf blades, was located on the wheatgrass chromosome 4Th and transferred to the bread wheat genome. These lines will complement the samples of previously obtained isogenic lines with contrasting pubescence in the cv. S29 genetic background for studying the trait of leaf hairiness of different trichome length and density.
Aside from the visible morphological differences, ANOVA on ranks revealed the effects of the chromosome substitutions on some yield-related traits such as main spike length (positive effect), the spike number per plant (negative effect), grain weight per main spike (negative effect), and the TGW (negative effect), which was apparently related to the lengthening of their growing season.

The blue-grained lines reportedly have either an unchanged or lower yield as compared to the initial varieties or sister lines. For example, lower grain yields and fertility have been detected in blue-grained wheat-wheatgrass chromosome addition lines ($2n = 44$), but not in disomic lines ($2n = 42$) as compared to their non-blue aleurone sister lines [60]. Winter wheat cv. Skorpion with blue grains and featuring chromosome 4A substitution by 4Th has a ~25% lower yield in comparison with check cultivars [61]. A successful transfer of the $Ba1$ gene as part of the long arm of chromosome 4Th from low-yield blue aleurone donor TA3972 into locally adapted Indian high-yield wheat cultivars has also been reported [62]. Besides productivity, the wheatgrass chromosomes substituting wheat ones affect grain quality. Reduced total vitreousness was observed in blue-grained lines with 4B and 4D substituted by 4Th [26,27].

Our comparative analysis of the yield-related traits within the series of lines obtained here indicates that there are differences in the expression of some traits depending on whether 4B or 4D is substituted by 4Th. The main spike length is greater in the lines with substitution 4Th(4B) in comparison to the lines with substitution 4Th(4D). This finding suggests that chromosome 4B carries the gene(s) controlling this trait, while the absence of differences in grain weight per main spike and in the spike number per plant between the lines with different substituted chromosomes implies the main inhibitory effect on these traits is either due to chromosome 4Th itself or due to the presence of these traits’ regulators on the chromosomes 4B and 4D with equal effects.

According to the literature, group 4 chromosomes carry the genomic regions with the largest number of stable quantitative traits loci (QTLs) for yield-related parameters, among which are “green revolution” genes $Rht1$ and $Rht2$ (known also as gibberellic acid insensitive dwarfism genes) located on chromosomes 4BS and 4DS, respectively [63]. In addition, an extended genomic region of a “QTL hot spot” for grain mass has been isolated on chromosome 4B [63]. Candidate gene $TraesCS4B01G037200$ for the number of grains per spike has been found on chromosome 4B, while orthologs of this gene have not been detected on chromosomes 4A and 4D [56]. Chromosome 4B has been shown to have the largest cluster of $TaGLP$ genes among the other group 4 wheat chromosomes; these genes encode germin and germin-like proteins, which participate in development, growth, and stress resistance [64]. On chromosome 4B, there are four hot spot clusters containing 45 QTLs for the regulation of assimilation of K, Ca, and Mg in wheat [65] and QTLs controlling root rot resistance [66]. Chromosome 4D features one of the lowest polymorphisms [54], approximately 20 QTLs associated with fungi resistance and virus resistance genes (Qfhs.jic-4D, Lr22, Lr67, $Yr28$, and $Wsm1$), aluminum and salt resistance genes/QTLs ($Al2L$ and $Kna1$), and genes/QTLs related to adaptation, flowering, and yield ($Vrn2$, $Rht1$, and $QSpn.fcu-4D$), but genes for the genetic control of lipoxygenase activity ($Lpx1$) associated with grain quality have been found here [55].

Considering the differences in the yield-related traits between blue- and black-grained lines independently from the substituted chromosome, only traits the “plant height” and TGW were affected by “grain color” and they were greater in black-grained plant specimens than in blue-grained ones. It can be theorized that the introgressed fragments on wheat chromosome 2A counteract the negative effect of wheatgrass chromosomes 4Th.

Overall, during marker-assisted construction of the black-grained (dark purple) wheat line, it is important to monitor not only the presence of the $Ba1$ gene but also the chromosome replacements of wheatgrass chromosome 4Th. The leaf pubescence trait may serve as a marker of cultivars with soft, short trichomes on their leaves like those of cv. S29.
5. Conclusions

In the current study, wheat black-grained lines accumulating anthocyanin pigments simultaneously in the aleurone and pericarp grain layer were created via pyramiding of anthocyanin synthesis–regulatory genes \( \text{Pp-D1} \) and \( \text{Pp3} \) together with \( \text{Ba1} \). These lines carry \( \text{Th. ponticum} \) chromosome 4Th, which completely replaces bread wheat chromosome 4B or 4D, as confirmed by visual examination, microsatellite analyses, and cytological multicolor FISH of the chromosomes. In addition to parental near-isogenic and substitution lines with different anthocyanin pigments in grains, the new lines constitute a valuable model for comparative studies and genetic stock for breeding new commercial wheat cultivars featuring high levels of anthocyanins in their grains.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12122934/s1, Figure S1: Schematic representation of structural organization of promoters of dominant allele \( \text{TaPpb1a} \) and recessive allele \( \text{TaPpb1b} \) of the \( \text{Pp3} \) gene with sites of annealing of the \( \text{Pp3-diagnostic} \) marker; Figure S2: Multiple alignment of a partial wheatgrass \( \text{ThMyc4E} \) gene sequence with its orthologous sequences \( \text{TraesCS4B02G397400} \) and \( \text{TraesCS4D02G224600} \) from the common-wheat genome; Figure S3: Genotyping of the black-grained \( F_3 \) plants obtained after crossing \( i:S29 \text{Pp3} \text{Pp-D1} \text{PF} \) and \( s:S29 \text{Ba1}4\text{Th}(4D) \); Figure S4: Genotyping of the black-grained \( F_{3.4} \) plants obtained after crossing \( i:S29 \text{Pp3} \text{Pp-D1} \text{PF} \) and \( s:S29 \text{Ba1}4\text{Th}(4D) \); Figure S5: Effects of factors “chromosome substitution” and “grain color” on 1000-grain weight (TGW) assessed in groups of lines compiled based on the grain color or substituted chromosomes, according to two-way ANOVA (Fisher’s F test); Table S1: Effects of various factors on the yield-related traits in the six wheat lines according to the Kruskal–Wallis \( H \) test (i.e., one-way ANOVA on ranks); Table S2: Post hoc comparison of the yield-related traits values between the groups of specimens combining based on the GRAIN COLOR by Duncan test; Table S3: Post hoc comparison of the yield-related traits values between the groups of specimens combining based on the CHROMOSOME SUBSTITUTION by Duncan test; Table S4: Spearman’s rank coefficients of correlation between yield-related parameters; Table S5: The total anthocyanin content (TAC) of whole-grain flour extracts from anthocyanin-rich cultivars and breeding lines created in different countries. References [67,68] are cited in the supplementary materials.

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