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Marker-Assisted Development of a Blue-Grained Substitution Line Carrying the *Thinopyrum ponticum* Chromosome 4Th(4D) in the Spring Bread Wheat Saratovskaya 29 Background

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Abstract: There is growing interest in cereals with anthocyanins in grain as a source of natural biologically active compounds beneficial for human health. In bread wheat, anthocyanins accumulate in the pericarp, under control of *Pp* genes, and in the aleurone layer, under control of *Ba*. Breeding anthocyanin-rich wheat cultivars is possible through the transfer of genes from genetic stocks to the desired cultivars. A blue-grained substitution line, s:S29*4Th(4D)* (BC_7 progeny), of the bread wheat cultivar Saratovskaya 29 (S29) carrying the *Thinopyrum ponticum* (Podp.) chromosome 4Th was developed. The 4Th/4D substitution was confirmed with chromosome C-banding and multicolor FISH, as well as by microsatellite analysis. Total anthocyanin content in the bran fraction of the new blue-grained line was 475.7 μg/g compared to 355.6 μg/g of the control purple-grained near-isogenic line, i:S29*Pp-A1Pp-D1Pp3*^{P}, and a total absence in S29. Although the developed line carries entire chromosome substitution, its 1000 grains weight, milling parameters, and dough physical properties did not differ or decreased slightly comparison to S29. These results support that the developed substitution line can be of interest in breeding programs to increase the anthocyanin production in commercial varieties.

Keywords: *Triticum aestivum*; anthocyanin synthesis; microsatellites; C-banding; multicolor FISH; grain quality

1. Introduction

Bread wheat (*Triticum aestivum* L., *2n = 6x* = 42, BBAADD) is the most important and widespread cereal crop. It not only provides humans and animals with energy and main nutrients but also contains phytochemicals with health-promoting potential, such as dietary fiber, phenols, tocopherols, carotenoids, and anthocyanins [1]. These compounds are considered components of functional foods that help reduce the risk of many diseases. Due to the wide variety of health-promoting effects demonstrated by anthocyanins, such as antioxidative, anti-inflammatory, anticancer, antiaging, lipid-profile regulation, obesity and diabetes prevention, hyperglycemia, and retinal protection, there is currently increasing interest in the production of anthocyanin-rich cereal crops [2–4]. In wheat, purple- and blue-colored grains are caused by anthocyanins with different structures. In purple

grains, the most abundant anthocyanins are cyanidin-based compounds, whereas, in blue grains, it is delphinidin-based anthocyanins [5–7]. These compounds accumulate in the pericarp, under control of the complementary genes *Pp*, *purple pericarp*, and in the aleurone layer under control of the *Ba* gene, *blue aleurone*, reviewed by [8]. Neither blue nor purple grain pigmentation were found in hexaploid wheat. The *Pp* genes were introduced to commercial wheat cultivars from the tetraploid Ethiopian wheats [8]. The complementary genes *Pp-1* and *Pp3* were mapped to the short arms of homologous chromosome 7 and the long arm of chromosome 2, respectively [9–12]. The *Pp-1* and *Pp3* genes encodes for transcription factors belonging to the R2R3-MYB and bHLH families, respectively, which together trigger anthocyanin biosynthesis in pericarp tissues [13,14].

Three incompletely dominant *Ba* genes that regulate anthocyanin biosynthesis in the aleurone layer were discovered in hexaploid blue-grained wheats [8]. These genes were transferred into cultivated wheat varieties from distant relatives. Zheng et al. [15] described the dominant *Ba1* gene as originating from *Thinopyrum ponticum* (Podp.) Barkworth and D.R. Dewey (decaploid tall wheatgrass; $2n = 10x = 70$, StStStStEeEeEbEbExEx; syn. *Agropyron elongatum* Host., *Elytrigia pontica* Podp., Holub). The gene was mapped to the long arm of chromosome 4Th [15]. One pair of wheat homologous chromosomes 4B or 4D can be substituted with the *Thinopyrum* chromosome with the dominant *Ba1* locus [15,16]. Using a set of wheat-*Th. ponticum* 4Th translocation lines, Liu et al. [17] mapped the *Th. ponticum Ba1* gene to the 4ThL-6 bin with FL 0.75–0.89. With the use of comparative RNA-seq analysis of blue- and white-grained wheat genotypes, the *ThMyc4E* gene was isolated and functionally verified as a candidate gene for *Ba1* [18]. It encodes a bHLH transcription factor, regulating anthocyanin synthesis in the aleurone layer of grain. Nonfunctional orthologues of the *ThMyc4E* gene were predicted in bread wheat homologous group 4 chromosomes by additional studies [19].

The incompletely dominant gene *Ba2* was mapped close to the centromere of chromosome $4A_{bo}L$ in *T. boeoticum* Boiss. ($2n = 2x = 14$, AA; syn. *T. monococcum* L. ssp. *aegilopoides*) [20,21]. As previously described by Zeven [8], in blue-grained wheat, one pair of *T. aestivum* chromosomes 4A was substituted with a pair of *T. boeoticum* chromosomes $4A_{bo}$.

The third gene, *BaThb*, which originates from *Th. bessarabicum* (Savul. and Rayss) A. Löve ($2n = 2x = 14$, EbEb = JJ), was described and localized on 4JL between the centromere and a breakpoint at FL0.52 [22]. The *BaThb* gene from *Th. bessarabicum* and *Ba1* from *Th. ponticum* were thought to have a common origin [23]. According to Zhang et al. [24], *Th. bessarabicum* was the probable donor species that contributed to the Eb genome of many polyploid wheatgrasses, including *Th. ponticum*.

Although there are differences in quantity and composition of anthocyanins between purple- and blue-grained wheat genotypes, studies showed that the wheat bran fraction of any colored wheat grain is a good source of natural antioxidants [25,26]. Breeding of wheat cultivars with high anthocyanin content is an important step in producing functional food, such as anthocyanin-rich bread, biscuits, and pastries, which are expected to represent a new direction in the food industry [27,28]. Saturating modern bread wheat cultivars with anthocyanins is possible through the transfer of genes that control anthocyanin pigmentation from genetic stocks into the desired cultivars. The development and characterization of the donor lines with respect to yield and grain quality is an actual task in breeding programs aimed at the development of anthocyanin-rich wheat cultivars.

A set of near-isogenic lines (NILs) with different combinations of regulatory genes controlling anthocyanin biosynthesis, *Pp-A1*, *Pp-D1*, and *Pp3*, were previously created in the genetic background of cv. Saratovskaya 29 (S29) [12]. The aim of the current study was to perform marker-assisted development of the blue-grained spring wheat line in the same S29 background and comparative characterization with respect to chromosomal composition, yield, grain quality, anthocyanin content, and antioxidant activity.

2. Materials and Methods

2.1. Plant Materials and Backcross Program

A winter bread wheat cultivar Meropa (Bulgaria: BG15) with blue grains was kindly provided by Prof. Ivan Panayotov (Institute of Wheat and Sunflower 'Dobroudja', General Toshevo, Bulgaria) and was used as a donor of the *Ba1* locus. A Russian spring bread wheat cultivar Saratovskaya 29 (S29) served as the recurrent recipient. The scheme of the backcross (BC) program aimed at obtaining the spring blue-grained line is illustrated in Figure 1. Consecutive rounds of hybridizations, beginning in April 2014, were performed in greenhouses and experimental fields of the ICG SB RAS. The strong xenia effect of the *Ba1* gene affects the color of hybrid grains [8], allowing visual selection of blue-grained genotypes after each round of backcrossing. Homozygous plants were selected among offspring obtained by self-pollination of the BC_7 plants (the BC_7F_2 generation). The near-isogenic line (NIL) i:S29*Pp-A1Pp-D1Pp3*^{P}, which has a purple grain color, was used as a control for studying anthocyanin content and antioxidant activity [12].

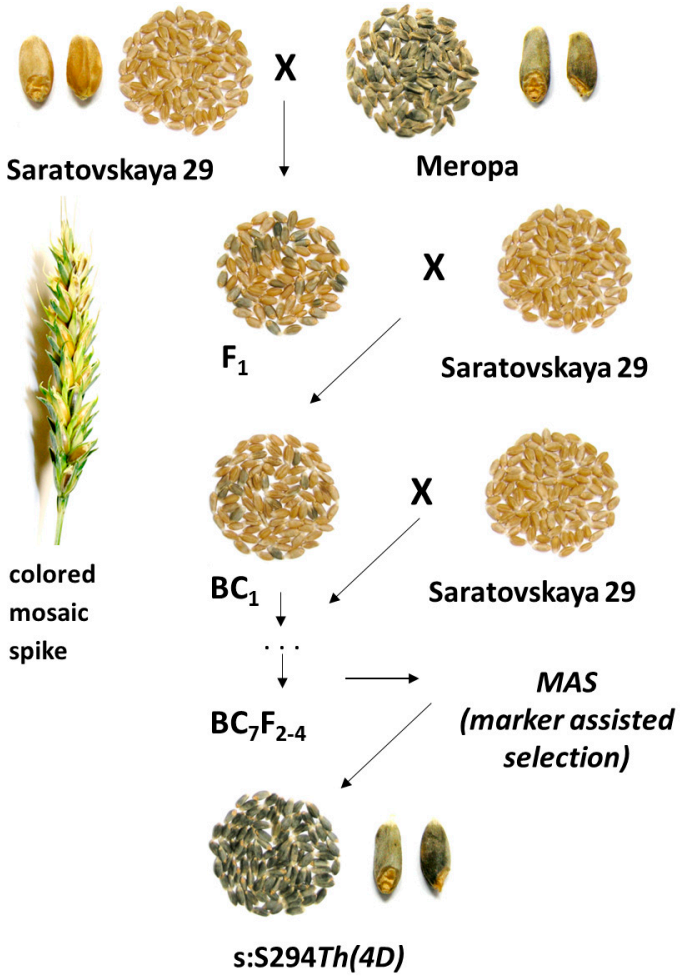

Figure 1. Crossing scheme used to obtain the homozygote blue-grained substitution line from the spring wheat cv. Saratovskaya 29 as a recipient and the blue-grained winter wheat cv. Meropa as a donor.

2.2. DNA Extraction and Microsatellite Analysis

DNA was extracted from the first leaf harvested from each segregant and parental cultivars, following a procedure described by Plaschke et al. [29]. A set of informative simple sequence repeat (SSR) markers chosen from the Gatersleben wheat microsatellite (GWM) series [30,31] was assembled

for marker-assisted selection purposes (Table 1 and Figure A1). The conditions for PCRs were as described in Röder et al. [30]. Resulting PCR products were separated in 5% high-resolution agarose gel HyAgarose™ HR Agarose (ACTGene, Inc., Piscataway, NJ, USA) and photographed in UV light, using the Molecular Imager® Gel Doc™ XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Cytological Examination

To characterize karyotypes of the new blue-grained line, standard C-banding technique was performed in accordance with a previously published protocol [32]. Chromosomes were classified according to the standard genetic nomenclature [33,34]. Detailed protocols of material pretreatment, fixation and chromosomal preparation for fluorescence in situ hybridization (FISH) are given in Badaeva et al. [35]. Chromosomes were counter-stained with DAPI (4′,6-diamidino-2-phenylindole). Plasmid clones pSc119.2 [36], pAs1 [37], and pAesp_SAT86 [38] were labeled with FITC (fluorescein-12-dUTP, Roche, Germany) and biotin (biotin-16-dUTP, Roche, Germany), respectively, using Nick Translation Mix (Roche, Germany). In addition, we used the oligo probe pTa535-1 [39,40] end-labeled with 6-carboxytetramethylrhodamine (TAMRA). Probes labeled with fluorescein were detected by using antifluorescein/Oregon green®, rabbit IgG fraction, Alexa Fluor® 488 conjugated antibody (Molecular Probes, Inc., Eugene, OR, USA). Biotin was detected with streptavidin-Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Metaphase cells were examined, using a Zeiss Imager D-1 microscope equipped with AxioCam HRm black-and-white digital camera and AxioVision software, version 4.6.

2.4. Analysis of Total Anthocyanin Content and Antioxidant Activity

Mature grains were crushed for whole-meal grain samples either using a laboratory grain mill LZM-1 (Zernotechnika, Moscow, Russia) or a grinding mill, separating bran from flour.

For extraction of total anthocyanins, 1 g of sample material was homogenized in 10 mL of 1% HCl solution in methanol or in a 40% ethanol solution. In the first case, the mixture was incubated at 4 °C for 12 h. In the second, it was kept in a boiling-water bath for 30 min. Absorbance of the supernatants obtained by centrifugation of the mixtures at 12,000 rpm for 15 min at 4 °C was measured on a SmartSpec TM Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 530 and 700 nm. The corrected absorbance value ($A=A_{530}-A_{700}$) was converted into the anthocyanin mass concentration and expressed as micrograms of cyanidin 3-glucoside (Cy-3-Glu) equivalents per gram of sample material dry weight (DW), using the method described by Abdel-Aal and Hucl [41]. Three biological repeats were run for each sample.

Total antioxidant activity (TAA) in the bran was measured using an amperometric method described by Yashin et al. [42]. Extracts were obtained by adding 20 mL of 1% of HCl in a 40% ethanol solution to 1 g of the sample material, followed by mixing and incubating for 30 min in a boiling-water bath and then filtering through 'Green Ribbon' filter paper. Antioxidant activity was estimated by using a Blizar antioxidant activity analyzer (Interlab, Moscow, Russia), according to the manufacturer's instructions. Resultant solutions were diluted 20 times to the corresponding standard dilution curve. Gallic acid (mg/L) was used as a reference substance. The average of three successive measurements was used for analysis. The significance of differences between the samples in terms of the studied parameters was estimated using the Mann–Whitney *U*-test. Spearman's rank correlation coefficient (r_s) between total anthocyanin content (TAC) and antioxidant activity of the ethanol extracts from brain fractions was calculated using Statistica v. 6.1 (StatSoft, Inc., Tulsa, OK, USA).

2.5. Analysis of the Grain Quality

Grains of the blue-colored line and parental cv. S29 were assayed for technological parameters by using the methods recommended in Russia for crop-variety testing [43], with modifications for small samples of grain. Grains for the tests were harvested after greenhouse spring and autumn vegetation seasons in 2017. Thousand-grain weight (TGW) was determined by using the express method by weighing 100 grains. Total virtuousness (TV, %) was determined visually after cutting 100 grains.

Row gluten content (RGC, %) was determined by hand-washing 1 g of whole meal. Particle size was defined using a PSH-4 device. Physical properties of flour and dough were studied by using Chopin alveograph profiling. To measure properties such as flour strength (W, Joule $\times 10^{-4}$), tenacity (P, mm), extensibility (L, mm), and P/L ratio, an alveograph with a 50 g mixer was used (method ISO-5530-4-91). Data are expressed as the mean ±SD. Student's *t*-test was applied to infer statistically significant differences, with $p \leq 0.05$ considered significant.

3. Results

3.1. Marker-Assisted Development of the Blue-Grained Line

After pollination of the spring bread wheat cv. S29 plants with the blue-grained Meropa's pollen, blue hybrid grains were obtained. F_1 hybrids were backcrossed with cv. S29. Offspring of the first (BC_1) and subsequently obtained generations exhibited mosaic ears with respect to grain color (Figure 1). Only blue grains were chosen for subsequent BCs. SSR marker control was applied for selection accuracy. As the *Ba* gene was introduced into wheat genomes in composition of the alien orthologous chromosome substituting for the homologous group 4 chromosomes, microsatellites mapped to these chromosomes were chosen for genotyping the parental cultivars and their progeny (Table 1 and Figure A1). Among the microsatellites tested, only *Xgwm1163* and *Xgwm1397* mapped to 4D amplified PCR-products of different length on gDNA of the parental cultivars and were heterozygous in the blue-grained offspring, whereas the other markers from the same chromosome, *Xgwm0624*, *Xgwm3156*, *Xgwm4001*, *Xgwm4083*, *Xgwm4736*, and *Xgwm1706*, did not amplify gDNA of cv. Meropa and S29, respectively. The absence of PCR products on gDNA of cv. Meropa comparing with S29 confirmed the long-distance origin of the chromosome carrying the *Ba* gene controlling blue color in wheat grains.

Table 1. Microsatellite markers polymorphic between Saratovskaya 29 and Meropa cultivars used in marker-assisted selection of the wheat blue-grained line.

Chromosome	Markers
2A	*Xgwm: 0294, 0312, 0636*
2D	*Xgwm: 0261*
4A	*Xgwm: 0610, 1091*
4B	*Xgwm: 0066, 0251, 0910*
4D	*Xgwm: 0624, 1163, 1397, 1706, 3156, 4001, 4083, 4736*
5A	*Xgwm: 0291*
5B	*Xgwm: 0540*
5D	*Xgwm: 0174*
7A	*Xgwm: 0060, 0282, 0631, 0748*
7B	*Xgwm: 0046, 0537*
7D	*Xgwm: 0044, 0111, 0885, 1044*

Markers from chromosomes 4A and 4B were polymorphic between parental cultivars. In blue-grained hybrids, alleles of the markers corresponded to S29. Selection of plants homozygous for Meropa's *Xgwm1397* and *Xgwm1163* alleles was performed in the BC_7F_2 progeny (Figure A2). Genotyping of selected plants using other SSR markers did not reveal any microsatellite alleles corresponding to wheatgrass or Meropa in genomes of the obtained blue-grain line.

3.2. Cytological Examination of the Developed Line

Blue-grained BC_4F_2 seeds were examined, using C-banding and FISH. C-banding analysis showed that the new developed line lacked both copies of wheat chromosome 4D; instead, it contained a pair of small heterochromatin-poor chromosomes with a small intercalary C-band in the short arm, close to the centromere. This pair of chromosomes was identified as *Th. ponticum* chromosomes 4Th (Figure 2). FISH analysis confirmed the loss of wheat chromosome 4D and showed that the alien

chromosomes carried small, overlapping pAs1 and pTa-535 signals in the short arm, coinciding with the C-band position. In contrast to wheat chromosome 4D, chromosome 4Th did not contain any sites with the pAesp_SAT86 (pTa-713) repeat (Figure 2). Thus, cytological analysis confirmed the substitution of wheat chromosome 4D with wheatgrass chromosome 4Th in the blue-grained BC_4 offspring. The substitution line developed was designed as s:S29*4Th(4D)*.

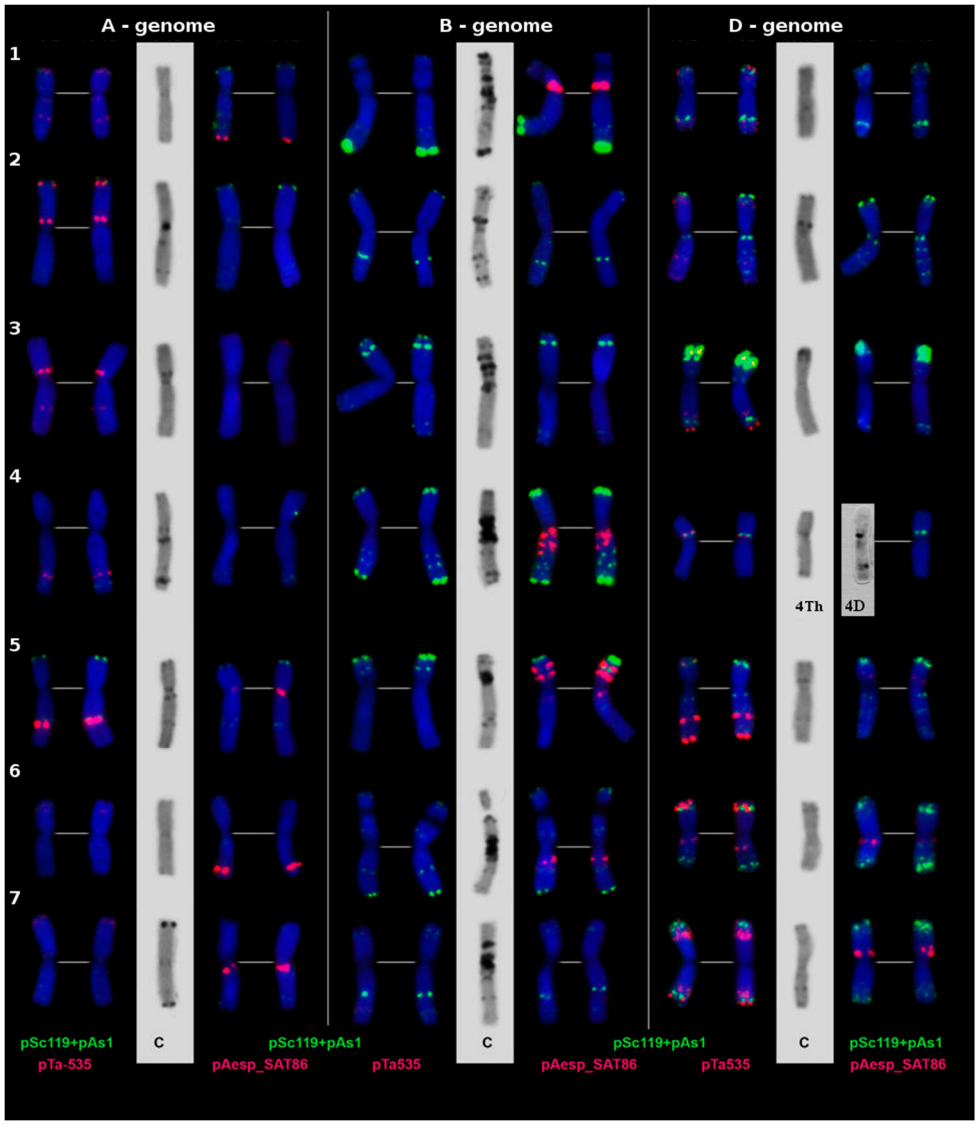

Figure 2. C-banding of chromosomes of the progeny BC_4 line (in white columns) and FISH with combinations of probes pSc119.2 and pAs1 and probes pTa-535 and pAesp_SAT86, which are green and red, respectively. The C-banding pattern of the wheatgrass chromosome 4Th is compared to the pattern of bread wheat chromosome 4D.

3.3. Total Anthocyanin Content in Whole Grains and Brans

TAC of methanol extracts from whole grain and bran fractions was determined for cv. S29, NIL i:S29*Pp-A1Pp-D1Pp3*^{P} with purple pericarp and for the created blue-grained line, s:S29*4Th(4D)* (Figure 3). There were no anthocyanins in the grains of S29. In brans of the both NIL and substitution line, TAC was more than twice as high as the whole grain flour, consisting of 355.6 and 475.7 μg/g, compared to 117.2 and 204.5 μg/g, respectively. Another extraction method using boiled 1% HCl in a 40% ethanol solution applied for the bran fraction increased TAC in the developed blue-grained

substitution line but not in the NIL. Measurements were 329.4 µg/g for i:S29*Pp-A1Pp-D1Pp3*^{P} and 607.4 µg/g for s:S29*4Th(4D)* (Figure A3).

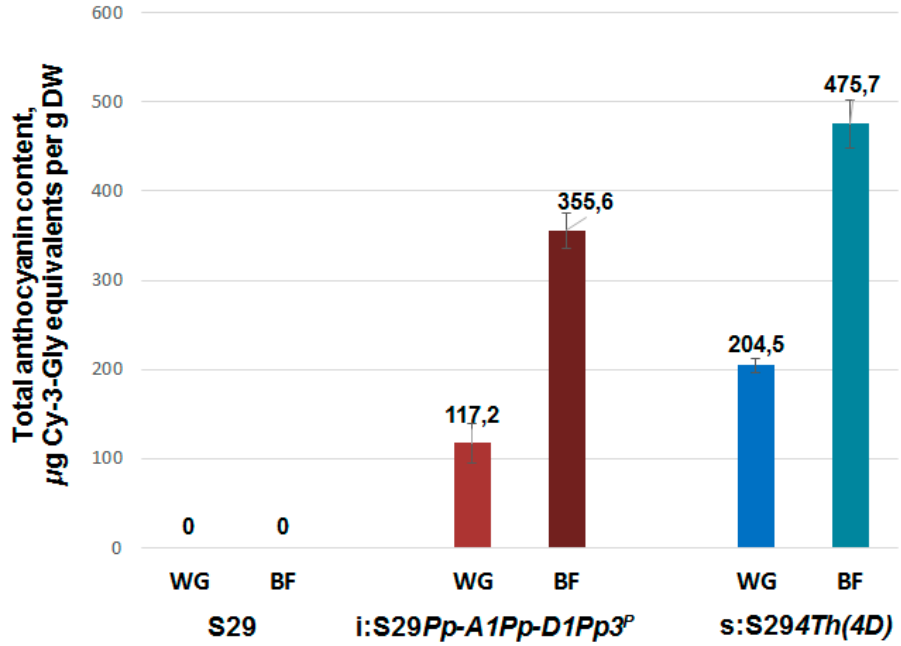

Figure 3. Total anthocyanin content of methanol extracts from whole grain (WG) flour and bran fraction (BF) of parental cultivar Saratovskaya 29 and its purple-grained near-isogenic i:S29*Pp-A1Pp-D1Pp3*^{P} and blue-grained substitution s:S29*4Th(4D)* lines. DW—dry weight.

3.4. Antioxidant Activity of Bran Extracts

TAAs of bran extracts of the new blue-grained substitution line, s:S29*4Th(4D)*, purple-grained NIL, i:S29*Pp-A1Pp-D1Pp3*^{P}, and S29 were not significantly different at 1.062, 1.664, and 1.246 mg/g, respectively (Figure 4). There was no correlation between TAC and TAA in bran fraction extracts ($r_s = -0.45$, $p = 0.22$).

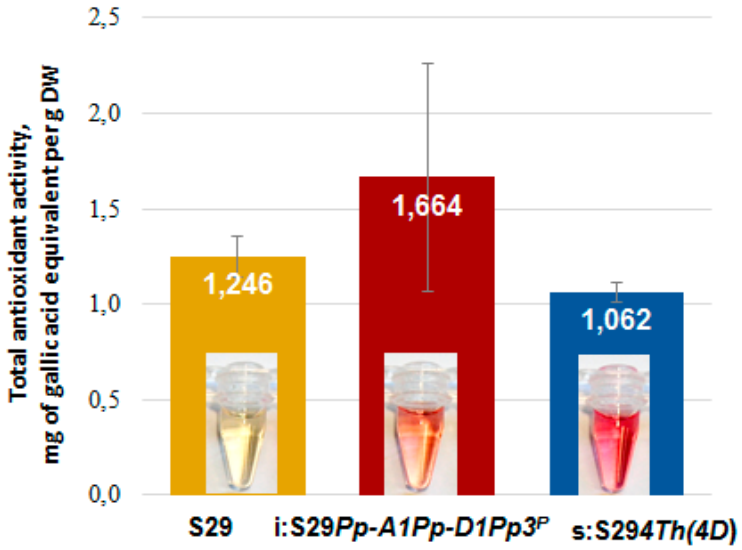

Figure 4. Total antioxidant activity (gallic acid equivalent, mg/g) of supernatants from bran fraction of parental cv. Saratovskaya29, its purple-grained near-isogenic i:S29*Pp-A1Pp-D1Pp3*^{P} and blue-grained substitution s:S29*4Th(4D)* lines after boiling 30 min in 1% HCl solution in 40% ethanol as an eluent.

3.5. Analysis of Grain Quality and Other Parameters

Grain quality and milling parameters of the developed substitution line, s:S29*4Th(4D)*, were assessed and compared with S29 (Table 2). Among the parameters tested, statistically significant differences were noted for flour particle specific area and flour strength, with the higher values in s:S29*4Th(4D)* and S29, respectively, whereas other parameters did not differ significantly between genotypes including 1000 grains weight. In addition to the aforementioned differences, substitution of a pair of wheat chromosomes 4D with a pair of tall wheatgrass chromosomes 4Th extended the terms of earing and ripening to 10 days (visual observation).

Table 2. Milling parameters and physical properties of dough from grains of Saratovskaya 29 (S29) and substitution line, s:S29*4Th(4D)*, with the blue aleurone layer.

Parameters	S29	s:S29*4Th(4D)*
1000 grains weight, g	25.7 ± 0.6	26.9 ± 4.6
Grain vitreousness, %	67.3 ± 15.2	60.8 ± 9.5
Mean flour particle diameter, μm	19.9 ± 1.6	20.8 ± 1.8
Flour particle specific area, cm^2/g	1795.5 ± 166.2	1942.5 ± 163.3 *
Raw gluten content in grain, %	36.8 ± 0.7	38.1 ± 1.9
Flour strength, e.a.	573.0 ± 84,9	414.0 ± 101.8 *
Dough tenacity, mm	163.5 ± 0.7	143.0 ± 26.9
Dough extensibility, mm	80.5 ± 9.2	78.0 ± 12.7
Test balance	2.0 ± 0.2	1.9 ± 0.7

* differences between S29 and s:S29*4Th(4D)* are statistically significant at $p \leq 0.05$ (*t*-test).

4. Discussion

Due to the health benefits of anthocyanins and accompanying polyphenols, there is increasing demand for anthocyanin-rich wheat cultivars that can be used in the production of whole-meal or bran-enriched bakery products. At the moment, bread wheat cultivars with high levels of anthocyanins accumulating in grain have been registered in some countries [44–46]. For the development of anthocyanin-rich cultivars, appropriate donors are required. The *Pp* gene, controlling anthocyanin biosynthesis in the grain pericarp, was previously transferred from the tetraploid wheat *T. aephiopicum* into bread wheat [8]. In the case of the blue color, more distant relatives, such as *Th. ponticum* and *Th. bessarabicum*, were used as donors of the *Ba* genes. These genes are usually transferred into the wheat genome as additional chromosomes or as alien chromosomes substituting for wheat chromosomes in the homologous group 4 [16,22,47–49], whereas there are great difficulties in obtaining stable wheat recombinant chromosomes carrying the *Ba* gene. Recently, based on the line Blue58 having chromosome substitution 4Th(4D), a set of blue-grained translocation lines with *Th. ponticum* chromosomal segments were produced by ^{60}Co-γ ray irradiation treatment [17]. These lines represent valuable sources of the *Ba* gene where the effect of linked genomic segments on growth, yield, and quality parameters of flour is expected to be reduced as much as possible.

In the current study, the blue-grained line was developed in the spring bread wheat cv. Saratovskaya 29 background by seven rounds of backcrosses. The obtained line carries a pair of chromosomes 4D entirely substituted with 4Th, as demonstrated by C-banding, FISH, and microsatellite genotyping. The chromosome substitution effect on TAC, TAA, yield, and flour technological parameters was evaluated by comparative study of the developed line and its parental cv. S29 (Figures 3 and 4, Table 2).

The chromosome substitution led to increasing TAC in the bran fraction of the developed line, up to 475.7 μg/g, which was higher than in the purple-grained line (355.6 μg/g). These results are in good agreement with previously obtained data of the TAC from colored wheat grains [5,6,26,50,51]. Although TAC was higher in the blue-grained s:S29*4Th(4D)* line compared to the purple-grained NIL and red-grained parental cv. S29, which has the polymeric proanthocyanidins in the seed

coat, measured TAA was not different between the tested lines and was not correlation with TAC (Figure 4). Currently, the high capacity of isolated anthocyanins, anthocyanin powder, whole grain, bran, and white flour to act against free radicals and lipid peroxidation is under discussion. The antioxidant properties of anthocyanins are reportedly dependent upon their structure, and there are differences between cyanidin-containing anthocyanins and delphinidin-based anthocyanins [25]. In addition, the sugar moiety attached to the anthocyanins aglycones influences the free-radical scavenging capacity of anthocyanins, with higher ability shown in rutinoside-containing anthocyanins compared to glucoside-containing pigments. Other components of the bran fraction, such as phenolic acids or polymeric proanthocyanidins from testa, can also contribute to TAA [25,26].

The developed blue-grained s:S29*4Th(4D)* line was compared to the parental cv. S29 with respect to yield and technological parameters of flour (Table 2). As was demonstrated, substitution of the entire S29 chromosome 4D with the *Th. ponticum* chromosome 4Th did not affect spike productivity or grain quality, with the exception of flour-particle specific area (plus effect) and flour strength (minus effect). Total vitreousness was decreased in absolute value compared to S29 but remained similar to high-quality wheats (no less than 60%). Significantly decreased total vitreousness was also reported earlier for the line s:S29*4B(4Th)*, having substitution of S29 chromosome 4B with 4Th from cv. Rescue [16]. Data indicated that chromosomes 4B and 4D of S29 carry genes associated with grain-quality parameters, and their removal from the wheat genome decreased grain quality. Recently, quantitative trait loci associated with dough strength, tenacity, extensibility, and P/L ratio, underlining the unique physical properties of flour and dough of high-quality S29, were revealed in the proximal region of chromosome 4DL [52]. Although the milling parameters and technological properties of dough from grains of the new line, s:S29*4Th(4D)*, were slightly decreased, they remained similar to the high-quality wheat cv. S29. In respect to yield, the published data showed that the blue-grained lines had either not changed or decreased yield in comparison to the initial varieties or sister lines. Lower grain yield with lower fertility were detected in the blue-grained wheat-wheatgrass chromosome addition lines ($2n$ = 44) compared to their non-blue aleurone sister lines, while these defects were not found in the blue-grained disomic lines ($2n$ = 42) [47]. The winter wheat cv. Skorpion with blue grain, carrying substitution of 4A chromosome with *Th. ponticum* 4Th was about 25% lower yield in comparison to check cultivars [45]. The successful transfer of the *Ba1* gene in composition of the long arm of chromosome 4Th from low-yielding blue aleurone donor TA3972 to locally adapted Indian high-yielding wheat cultivars has also been reported. Yield for some of the obtained blue-grained lines did not differ significantly from elite recipient cultivars [48].

5. Conclusions

In the current study, a blue-grained substitution line, s:S29*4Th(4D)* (BC_7 progeny), was developed in the cultivar Saratovskaya 29 background. Although the new line carries the *Thinopyrum ponticum* chromosome 4Th, which completely replaced the bread wheat chromosome 4D, the yield and quality parameters of the line do not change significantly in comparison to the recurrent parental cultivar. These results support that the developed substitution line can be of interest in breeding programs, to increase the anthocyanin production in commercial varieties.

Author Contributions: Investigation and formal analysis, E.G., E.B., R.Y., and L.S.; marker-assisted development, E.G.; cytological examination, E.B.; analysis of antioxidant activity, R.Y.; analysis of grain quality, L.S.; data curation, writing—original draft preparation, E.G.; writing—review and editing, O.S.; visualization, E.G. and E.B.; conceptualization, methodology, and supervision E.K.; project administration and funding acquisition, E.K. and O.S.

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Appendix A

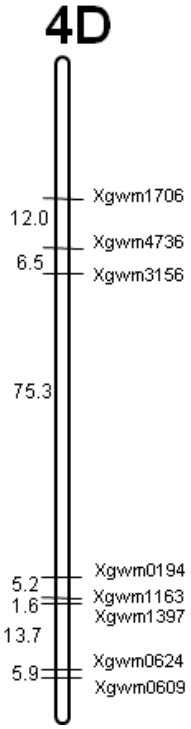

Figure A1. Schematic representation of positions of SSR-markers mapped to chromosome 4D, chosen for marker-assisted development of the blue-grained wheat line.

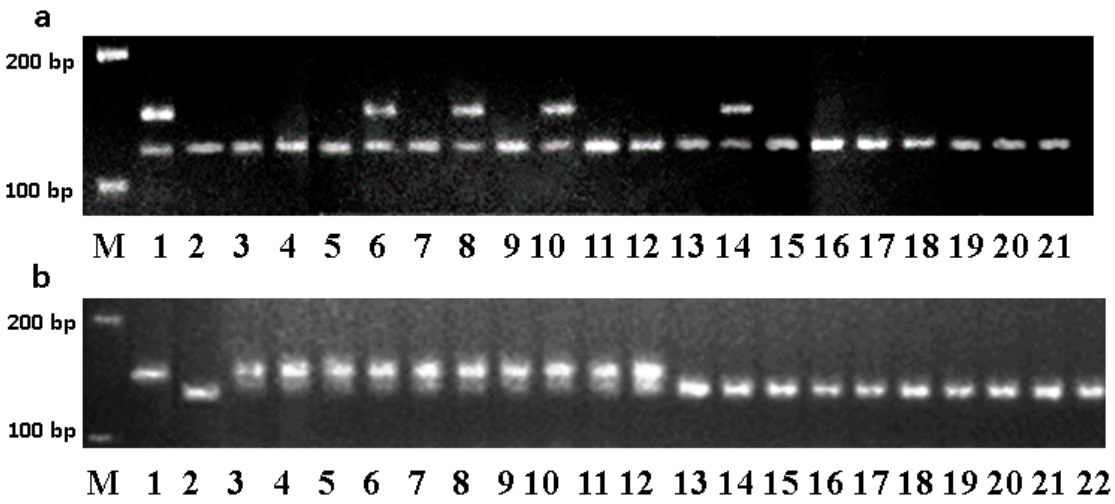

Figure A2. Electropherogram of PCR products obtained by using primers for the microsatellite locus *Xgwm1163* on DNA of cv. Saratovskaya 29 DNA (lane 1), Meropa (2), and blue-grained BC_7F_2 heterozygous (6, 8, 10, 14) and homozygous (3–5, 7, 9, 11–13, 15–21) progeny (**a**). Electropherogram of PCR products obtained using primers for the microsatellite locus *Xgwm1397* on DNA of cv. Saratovskaya 29 (lane 1), Meropa (2), and blue-grained BC_7F_3 heterozygous (3–12) and homozygous (13–22) descendants of the selected plants from BC_7F_2 (**b**).

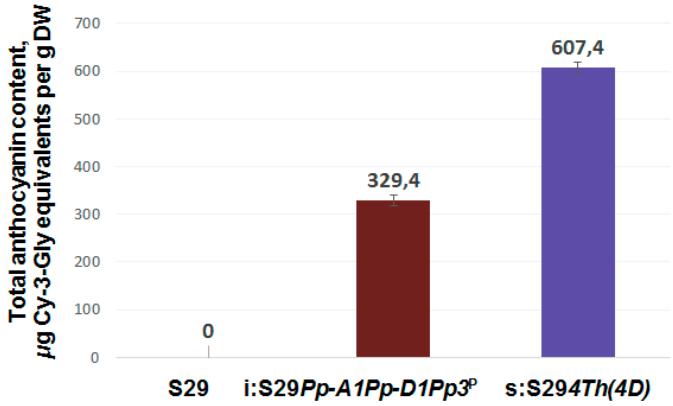

Figure A3. Total anthocyanin content in boiled ethanol extracts from wheat brans of parental cv. Saratovskaya 29 (S29) and its purple-grained near-isogenic i:S29*Pp-A1Pp-D1Pp3*^{P} and blue-grained substitution s:S29*4Th(4D)* lines. DW—dry weight. Different letters indicate statistically significant differences between the lines (*U*-test, $p \leq 0.05$).

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