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Origin of black glume color in *Triticum aestivum* lines with introgressions from wheat wild relatives

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ABSTRACT

Dark glume color in Triticinae is a much-studied morphological character. However, pigments that cause the color, the molecular basis of their biosynthesis, and mechanisms of its regulation are still unknown. Dark glumes is a common trait in *Triticum aestivum* lines with alien introgressions. To elucidate the molecular basis of this character, we analyzed the total phenolic content (TPC) in the dark glumed introgression lines and their parents. TPC in the dark ILs was found to be higher than their parents and light-glumed lines. All the genotypes were screened for the expression of eight genes encoding the flavonoid biosynthesis pathway enzymes. Expression of three flavonoid genes in the pathway was changed in the introgression lines as compared to their parents. The expression of *F3'H* and *FNS* was absent in the lines with introgressions from *Aegilops* spp. The expression of three genes, *F3'H*, *FLS*, and *FNS*, was found to be altered in the light glumed lines with introgressions from *A. muticum*. No expression of *F3'H* was detected in the *T.aestivum* / *Am. muticum* lines with light and dark plants, and the other genes of the pathway were found to be expressed at the parental levels. No correlation between the expression profiles and the phenotype was found in the *T. aestivum* / *A. muticum* lines indicating other undetected changes in these lines.

Keywords: glume color, spike morphology, amphidiploids, wheat

INTRODUCTION

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Brown or black glume color is a morphological character of many grasses, like maize, barley,

rice, and wheats. In maize, the brown (or red) glume pigmentation is provided by the phlobaphenes, which are polymers of flavan-4-ols. Accumulation of phlobaphenes in maize glumes is activated by the *P1* gene encoding the MYB family transcription factor (Grotewold et al. 1994). This transcription factor activates expression of five genes: *CHS*, *CHI*, *DFR*, *F3'H*, and *FNS*, whose products are the flavonoid biosynthesis pathway enzymes (Vermerris and Nicholson 2006) (Sharma et al. 2012)(Falcone Ferreyra et al. 2015)(Morohashi et al. 2012). Similarly, the black glume color is caused by phlobaphenes in barley, whose accumulation is activated by the *Blp1* gene (Jia et al. 2017).

In Triticinae, dark glume pigmentation is a well-known easily scored character, consequently it is used for classification of *Triticum* varieties (Dorofeev et al. 1979). In bread wheat, it is controlled by three homoeologous red glume genes located on the group 1 chromosomes – *Rg-A1*, *Rg-B1*, and *Rg-D1* (Khlestkina et al. 2006). The molecular nature of the *Rg* genes products has not been identified yet, and the compounds that provide glume pigmentation have not been characterized.

It may be assumed, that glume pigmentation in wheat, like in the related grasses maize and barley, results from the accumulation of phlobaphenes, or other flavonoid pigments, like anthocyanins or proanthocyanidins, which are also known to color different organs in grasses (Quattrocchio et al 2006). Then, *Rg* genes may upregulate the genes that encode the enzymes from the flavonoid biosynthesis pathway, and their expression should differ in light glumed and dark glumed plants.

Introgression lines (ILs) *Triticum aestivum* / *Aegilops sharonensis*, *T. aestivum* / *Ae. umbellulata*, and *T. aestivum* / *Amblyopyrum muticum* were developed from crossing the genome-substitution amphidiploids Aurosis, Aurolata and Aurotica as a male parent with the winter cultivar of *T. aestivum* Aurora as a female parent (Zhiron and Ternovskaya 1984; Antonyuk and Ternovska, 2001; Antonyuk et al. 2009). Some of the lines have dark glumes, which is more intense than glume color of their parents, *T. aestivum* Aurora, and the genome-substitution amphidiploids Aurosis (AABBS^{sh}S^{sh}), Aurolata (AABB^{UU}), and Aurotica (AABB^{TT}) (Zhiron and Ternovskaya 1984; Antonyuk et al. 2013). As no data about biochemistry and molecular biology of glume pigmentation in Triticinae is available, we aimed to find out the nature of pigments that provide dark glume color, and to detect variation in the expression of the structural genes from the flavonoid biosynthesis pathway in the ILs.

MATERIALS AND METHODS

Plant materials

For this study, we used common wheat *Triticum aestivum* L. cultivar Aurora (2n = 6x = 42, AABBDD), three genome substitution amphidiploids – Aurolata (2n = 6x = 42, AABB^{UU}) with two genomes AABB from Aurora and the UU genome from *Aegilops umbellulata* Zhuk., Aurosis (2n=6x=42, AABBS^{sh}S^{sh}) with the S^{sh}S^{sh} genome from *Ae. sharonensis* Eig, and Aurotica (2n=6x=42, AABB^{TT}) with the TT genome from *Amblyopyrum muticum* (Zhiron and Ternovskaya 1984; Antonyuk et al. 2013).

Hexaploid wheat lines with introgressions from the *Aegilops* species were obtained by crossing one of the amphidiploids with the cultivar Aurora, followed by two or four backcrosses to Aurora until the recovery of plant self-fertility. Among fertile progenies, stable_hexaploid lines were selected (Zhiron and Ternovskaya 1984; Antonyuk and Ternovska, 2001; Antonyuk et al. 2009) and were maintained by self-pollination under bags to ensure no cross pollination. The introgression wheat lines employed in this The introgression wheat lines employed in this study were the following: Aurosis derivatives res 122-2 and res 133-1, as well as Aurolata derivatives res 191, res 193, res 201, and f04 1352 lata (Table 1)

Table 1. Plant material

Line designation	Phenotype	Number of plants studied	Line designation	Phenotype	Number of plants studied
Aurora	light glumes	15	Tica 30 bl1/l	light glumes	6
Aurosis	light brown glumes	15	Tica 30 bl2/l	light glumes	6
Aurolata	light brown glumes	15	Tica 42 bl/l	light glumes	6
Aurotica	light glumes	15	Tica 8 bl/l	light glumes	6
res 122-2	black glumes	15	Tica 30 bl2/br	light brown glumes	6
res 133-1	black glumes	15	Tica 8 bl/br	light brown glumes	6
res 191	black glumes	15	Tica 30 bl1/bl	brown glumes	6
res 193	black glumes	15	Tica 30 bl2/bl	brown glumes	6
res 201	black glumes	15	Tica 42 bl/bl	brown glumes	6
lata f04 1352	black glumes	15	Tica 8 bl/bl	brown glumes	6

The development of *T. aestivum* / *A. muticum* lines was described in Iefimenko et al (2018). The F7 generation of the introgression lines employed in this study are presented in Table 1. Numbers in lines designations indicate the F2 plants from which this line originated. Introgression lines and their parental lines (the cultivar Aurora and the genome-substitution amphidiploids Aurosis, Aurolata and Aurotica) were visually evaluated for glume color (light, light brown, brown and black) every year when they were grown in the field. Lines with a stable color phenotype were selected for the study. In 2017, the selected lines were grown in triplicate complete blocks (total area 0.06 hectares) under the same lighting and moisture conditions (Kyiv, Ukraine). To determine TPC two or five plants (Table 1) from each replication were taken from the middle of a one-meter row, (there were 11-18 plants per row). Variation in total phenolic content (TPC) for each sample is shown as average ± standard deviation.

Extraction of phenolic compounds and evaluation of their content in the glumes

Extraction of phenolic compounds was conducted with methanol, acidified with 1% HCl, as described in (Himi and Taketa 2015). In brief, 40 mg of the dry glumes and lemmas were ground with liquid nitrogen and extracted for 15 h with 0.8 mL of methanol-HCl. The extract was purified twice by adding the equal volume of 24:1 chloroform-isoamyl alcohol followed by 13 000 rpm centrifugation.

Determination of the total phenolic content (TPC) was conducted with Folin-Ciocalteu reagent (Sigma-Aldrich, USA) as described in (Ainsworth and Gillespie 2007). TPC was estimated from the gallic acid calibrating curve using a corresponding regression equation, and expressed in the units of

nanogram of gallic acid equivalent per gram of dry tissue (ng GAE / g).

RNA extraction

Tissue samples were collected from the studied plant material 10 days after fertilization. Lemmas and glumes were taken to provide the tissue specificity of the expression detection. Total RNA was extracted according to Peng et al (2014) with minor modifications. The stage of high-molecular weight RNAs separation was omitted.

Reverse transcription and end-point detection quantitative PCR

Reverse transcription reaction was conducted using oligo(dT) priming with RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Lithuania) in accordance with the manufacturer's instruction. After this, cDNA was amplified in PCR with the reaction conditions described previously (Nolan et al. 2006). Amplification products were separated in 2% agarose gel on sodium borate (SB) buffer with OGeneRuler Ultra Low Range DNA Ladder (Thermo Fisher Scientific, Lithuania) as a marker. Visualization of products was performed using ethidium bromide staining (Sambrook et al. 1989).

Primer design and data analysis

Primers (Table 2) to the coding parts of the genes were designed with the Primer-BLAST service (Ye et al. 2012). Sequences of the genes were found in the GenBank database: *TaCHS* (AY286097.1), *TaCHI* (AB187026.1), *TaF3H* (AB223024.1), *TaDFR* (AB162138.1), *TaANS* (AB247917.1), *TaFLS* (KJ193852), *TaFNS* (AK333567.1), and Plant Transcription Factor Database: *TaF3'H* (TRIAE_CS42_6BS_TGACv1_513245_AA1635810). Primers for actin (Actin_F ACCTTCAGTTGCCAGCAAT, Actin_R CAGAGTCGAGCACAATACCAGTTG) used as reference gene were taken from (Debernardi et al. 2017)

Table 2. Primer sequences

Gene	Gene ID	Primer name	Primer sequence	Product length
CHS	AY286097.1	TaCHS-F	GCGCCTGCGTACTCTTCATC	104
		TaCHS-R	AAAGCCGAAGAGAACACCCC	
CHI	AB187026.1	TaCHI-F	ACAAGGTGACGGAGAAGTGC	146
		TaCHI-R	GGGGGAGTGGGTGAAGAGTA	
F3H	AB223024.1	TaF3H-F	CGGCCACCAAGTCTCTCAAC	150
		TaF3H-R	CTCCTCCCTGCTACACACG	
DFR	AB162138.1	TaDFR-F	AAGGGCCTCATTCTCTCGG	157
		TaDFR-R	CCCATGCAAATTCCGAACACA	
ANS	AB247917.1	TaANS-F	GCCCGTCACCAAGACCTTC	146
		TaANS-R	CCTCCACCTCCTGGGTCTC	
F3'H	TRIAE_CS42_6BS_TGACv1_513245_AA1635810	TaF3'H-1F	ACGCGATCATCAAGGAGACC	138
		TaF3'H-1R	CGATTCCCCACACGTTCACT	
FLS	KJ193852	TaFLS-F	CTATGAGCAACGGGAGGCTAC	157

		TaFLS-R	CTTGTA CT TGGCCGGGA ACT	
FNS	AK333567.1	TaFNS-F	ACTTCCAGTACCTGCCGTTC	160
		TaFNS-R	TCGGCCTCCTCCATGTCTAT	

Quantification of the band DNA content was performed in the Gel Analyzer program (<http://www.gelanalyzer.com/>) with the calibration to the per band DNA quantity in the ladder used ($r > 0.9$). The data on gene expression were normalized to the reference gene. Statistical analysis was performed using Shapiro-Wilk test for testing the distribution for normality (Shapiro and Martin, 1965), analysis of variance (ANOVA) (Samuels et al.2012) for testing H0 hypothesis, Dunnett’s test for statistical check of difference between groups in the ANOVA complex (Dunn 1961, Dunnett 1964).

RESULTS

Total phenolic content differs in the introgression lines with dark and light glumes

The results of the long-term visual assessment show that Aurora and Aurotica have light glumes, Aurosis and Aurolata have light brown glumes. Lines res 122-2, res 133-1, res 191, res 193, res 201, and f04 1352 lata have black glumes, their color is more intense than that in Aurosis and Aurolata. The lines derived from Aurotica have three gradations of glume color – light glumes (the parental phenotype, lines Tica 30 bl1/l, Tica 30 bl2/l, Tica 42 bl/1, and Tica 8 bl/l), light brown glumes (lines Tica 30 bl2/br and Tica 8 bl/br), and brown glumes (lines Tica 30 bl1/bl, Tica 30 bl2/bl, Tica 42 bl/bl, Tica 8 bl/bl) (Table 1). Phenotypes with respect to glume color : light, brown, and light brown are presented in Fig.1., the latter two contrasting to their light glumed parents, the genome-substitution amphidiploid Aurotica and the bread wheat variety Aurora. Considering that other grasses species have dark glumes pigmented by the phenolic compounds, phlobaphenes, which belong to the flavonoids (Grotewold et al. 1994, Jia et al. 2017), we analyzed the content of phenolic compounds (TPC) in glumes of the lines and parent genotypes. We found that TPC value in the dark colored IL res 122-2, derived from the Aurosis and Aurora crossing, was ~3-fold greater compared to both Aurora and Aurosis–on average 381.0 ± 13.0 ng GAE/g compared to 138.6 ± 5.1 ng GAE/g in Aurora and 157.3 ± 10.5 ng GAE/g in Aurosis (Fig. 2). An increase in the total phenolic content was also observed in the black glumed ILs derived from Aurolata, res 193 (387.5 ± 44.0 ng GAE/g), res 191 (432.0 ± 29.1 ng GAE/g), and f04 1352 lata (385.1 ± 87.4 ng GAE/g). The TPC in the lines was three times higher than that in their parents with the average value in Aurora 138.6 ± 5.1 ng GAE/g and Aurolata 104.3 ± 7.4 ng GAE/g (Fig. 3).

In the Aurotica lines TPC was evaluated in three phenotypic classes.: Plants with brown glumes had an average of 294.5 ± 26.9 ng GAE/g of phenolic compounds. Plants with light brown glumes averaged 150.9 ± 18.6 ng GAE /g, and the plants with light glumes averaged 113.1 ± 14.3 ng GAE /g. Phenolic content in glumes of the brown-colored plants was 2.6 times higher than that in the light glumed plants Aurora and Aurosis. (Fig. 4). The dark glumed ILs, derived from Aurosis, Aurolata, and Aurotica, have significantly higher content of phenolic compounds in the glumes compared to their parents and light glumed lines. These results suggest that black or brown (dark) color of glumes in the ILs is provided by phenolic compounds.

lines with introgressions from *Ae. sharonensis* and *Ae. umbellulata*

As we found that dark color of glumes in the ILs is associated with high content of phenolic compounds, we further evaluated expression of eight genes that encode enzymes of the flavonoid biosynthesis pathway – chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), flavonoid 3'-hydroxylase (F3'H), flavone synthase (FNS), flavonol synthase (FLS), flavanone 3-hydroxylase (F3H), and anthocyanidin synthase (ANS). High phenolic content in the ILs was expected to be caused by the changes in the expression of some of these genes compared to the parents.

Four parental genotypes, the *T. aestivum* cultivar Aurora, and three amphidiploids, Aurosis, Aurolata, and Aurotica, were found to express seven of the studied genes, *CHS*, *CHI*, *DFR*, *F3'H*, *FNS*, *FLS*, and *F3H*, in their glumes. The levels of expression of all these transcripts were found to be similar among the genotypes. The *ANS* gene was not found to be expressed neither in glumes of the parents, nor in the derived ILs. So, we concluded that anthocyanidins and anthocyanins, whose synthesis requires ANS, do not contribute to the formation of the dark glume color of the plants studied.

Introgression lines res 122-2 and res 133-1, derived from the cross between Aurora and Aurosis, showed expression of five transcripts, *CHS*, *CHI*, *DFR*, *F3H*, and *FLS*, at levels identical to their parents. The similar observation was made for the Aurolata-derived lines res 201 and f04 1352 lata, the above-mentioned five genes were found to be expressed at the parental levels.

Two of the studied transcripts, *F3'H* and *FNS*, showed variation in the expression levels between the parents and the lines. Expression of the *F3'H* transcripts was absent in all plants of the Aurosis-derived lines, res 122-2 and res 133-1, and expression of the *FNS* gene was absent in the res 133-1 line, while in res 122-2 its level was lower than the parental one ($t = 6.9$, $p < 0.01$) (Fig. 5).

In the Aurolata-derived f04 1352 lata line, both *F3'H* and *FNS* genes showed no expression (Fig. 6). Three other Aurolata lines, res 201, res 191, and res 193, had expression of these two genes at the lower levels compared to that in the parents ($t = 4.4$, $p < 0.01$ for *F3'H*, and $t = 2.5$, $p < 0.05$ for *FNS*).

Expression of three flavonoid biosynthesis pathway genes is altered in the lines with introgressions from *A. muticum*

Similarly to the black glumed lines with introgressions from *Ae. sharonensis* and from *Ae. umbellulata*, the *A. muticum* ILs showed no difference in the expression of four flavonoid biosynthesis pathway genes, *CHS*, *CHI*, *DFR*, and *F3H*, compared to their parents. The variation between expression levels in the parents and the lines was found for three genes encoding F3'H, FNS, and FLS. The pattern of the expression variation did not follow the phenotype differences; as the discovered changes appeared in all plants of certain lines regardless their phenotype.

The *F3'H* transcript expression was found to be absent in all studied *A. muticum* ILs with the exception of three plants: one from the Tica 8 l light glumed line, and two others with contrasting phenotypes, Tica 30 bl1/l and Tica 30 bl1/bl (Fig. 7, Fig. 8).

The *FNS* transcript expression was absent in the light glumed ILs Tica 8 l, Tica 42 l, and Tica 30 l (Fig. 7). Lines Tica 8 bl, Tica 30 bl2, and Tica 14 bl had parental levels of *FNS*, and two lines, Tica

42 bl and Tica 30 bl1, had no expression of this transcript (Fig. 8). Plants in Tica 8 bl, Tica 30 bl2, and Tica 14 bl lines have different color of glumes, so presence of *FNS* expression did not follow phenotype, and it was inherent to all plants of the certain line regardless the individual phenotype.

The variation in the *FLS* transcript expression correlated almost perfectly with the described variation in the *FNS* transcript. *FLS* was not expressed in glumes of the majority of plants from the light glumed lines Tica 42 l, Tica 30 l, Tica 14 l (except one plant), and Tica 8 l (except one plant) (Fig. 7). On the contrary, the lines Tica 8 bl, Tica 30 bl2, and Tica 14 bl had the parental expression level of *FLS*, and its presence was not related to the plant phenotype (Fig. 8). The exceptions from the correlated *FLS* and *FNS* expression pattern were Tica 30 bl1/l and Tica 14 l. The dark-glumed line Tica 30 bl1/ showed the parental level of *FLS* expression but lacked the *FNS* product; in the white glumed line Tica 14 l, *FLS* expression was not detected but the *FNS* expression was present.

Two lines, Tica 42 bl and Tica 30 bl1, did not follow the general pattern of the *FNS* and *FLS* expression. The expression profiles of the dark and the light plants in the Tica 42 bl line were similar to the light glumed stable lines: products of *FLS* and *FNS* were absent. In the T30 bl1 line, the phenotype-specific pattern of *FLS* expression was observed: the light glumed T30 bl1/l had *FLS* expression, while the black glumed T30 bl1/bl had no *FLS* expression.

DISCUSSION

Dark glume color in the introgression lines is provided by phenolic compounds

High content of phenolic compounds in the dark glumed ILs supports the hypothesis that the pigments, causing the brown and black color of glumes, belong to the phenolics.

Among phenolic compounds, flavonoids and their polymers compose the largest group of plant pigments. Flavonoids are produced in plants via a common flavonoid biosynthesis pathway (Fig. 9). This pathway gives several classes of flavonoid compounds, such as flavonoles, flavones, leucoanthocyanidins, and flavan-4-ols (Quattrocchio et al 2006). Flavonoid pigments include: chalcones, aurones, anthocyanidins, and anthocyanins, providing yellow, red, blue, and orange color in flowers; anthocyanins, accumulation of which is known to produce purple color of different organs in grasses (e.g., glume, coleoptile, and leaf sheath); proanthocyanidins, which are responsible for dark coloration of seed pericarp; phlobaphenes, that give brown color to the pericarp and glumes in maize, barley, and sorghum (Vermerris and Nicholson 2006)(Davies 2004).

As we found that the *ANS* gene is not expressed in the glumes of the dark ILs, anthocyanidins and anthocyanins are not formed in their glumes, and so they cannot contribute to the formation of the dark glume color. After all, we believe that among the known phenolic compounds, phlobaphenes are the most likely candidates for the glume pigments in our plant material, because they are known to color glumes in other grasses (Vermerris and Nicholson 2006)(Jia et al. 2017).

Four of the studied genes, *CHS*, *CHI*, *DFR*, and *F3'H*, are involved in biosynthesis of the phlobaphenes monomers, luteoforol and apiferol (Vermerris and Nicholson 2006) (Fig. 9). In maize, expression of these genes as well as *FLS* gene are jointly upregulated by a single transcription factor encoded by the *PI* gene (Quattrocchio et al 2006). If the cluster expression regulation in Triticinae is similar to maize, then we expect to see higher expression of the five flavonoid pathway genes in the

dark glumed ILs. However, in our study the parents with light and light brown glumes and the dark glumed lines had equal expression levels of the *CHS*, *CHI*, and *DFR* transcripts. Moreover, *F3'H* and *FLS* expression was missing in the dark lines. From this we conclude that, firstly, the regulation of the flavonoid biosynthesis gene expression in wheats differs from that in maize, as *F3'H* and *FLS* genes can be downregulated separately. Secondly, accumulation of the pigments in glumes of the dark lines is not caused by increased expression of the flavonoid biosynthesis genes but is associated with the absence of expression of some of them. There may be undetected changes in the flavonoid biosynthesis pathway leading to the increased synthesis of the pigments. It is possible –that the last step of phlobaphenes formation, the non-enzymatic polymerization, is affected. However, the mechanism of this reaction is still unknown (Vermerris and Nicholson 2006; Landoni et al. 2020).

The absence of *F3'H* and *FNS* expression in the dark glumed *T. aestivum* / *Aegilops* spp. introgression lines increases production of the phenolic pigments.

The light glumed bread wheat variety Aurora, the amphidiploid Aurotica (AABBTT), and light brown amphidiploids Aurosis (AABBS^{shS^h}) and Aurolata (AABBUU) can synthesize all classes of flavonoids: dihydroflavonols, flavonols, leucoanthocyanidins, flavones, and flavan-4ols, as all of the studied flavonoid biosynthesis genes are expressed in their glumes. However, the parents are not able to synthesize the compounds that provide black pigmentation of glume in their derived ILs. No different branches in the flavonoid pathway appear in the lines compared to their parents. So, it is possible that changes in the transcriptome of the dark glumed lines do not result in the emergence of the new compounds but rewire the flux of the metabolites in the pathway. This may lead to the greater accumulation of the pigments or their precursors.

In the dark glumed lines derived from Aurora x Aurosis and Aurora x Aurolata crossings, we found the interruption in the synthesis of certain flavonoids– caused by the absence of expression of two genes, *F3'H* and *FNS*. The synthesis of dihydroquercetin– and derived compounds quercetin and leucocyanidin is disrupted in the absence of *F3'H*. However, the synthesis of other dihydroflavonols, as well as derived flavonols and leucoanthocyanidins, remains normal. The absence of the *F3'H* transcripts expression also disables synthesis of luteoforol, one of the phlobaphenes monomers, because its precursor, eriodictyol, is not formed from naringenin. It would be logical to assume that blocking the monomer biosynthesis should reduce the content of phlobaphenes in glumes of the ILs, which contradicts our observation. The second variation of the ILs expression profile, the absence of *FNS* expression, may be the key change in the flavonoid pathway that could have led to the accumulation of phlobaphenes. Naringenin is the node of the flavonoid pathway, and it serves as a substrate for *F3'H*, *F3H*, *FNS*, and *DFR* enzymes. If one of the enzymes is absent, the naringenin pool becomes more accessible to the other enzymes. This condition results in the redistribution of the flux through the flavonoid biosynthesis pathway, which can make the *DFR* catalyzed conversion of naringenin to apiferol more favorable (Quattrocchio et al 2006). Consistent with this, the accumulation of phlobaphenes in glumes of the ILs may result from the intensified synthesis of the phlobaphenes-monomer apiferol. The intensification may be caused by the favorable conditions due to the lack of *FNS* expression.

***muticum* introgression lines**

In the ILs derived from Aurotica x Aurora crossings, the loss of *F3'H*, *FNS*, and *FLS* expression is associated with the parental light glumed phenotype. The expression of the *F3'H* and *FNS* transcripts was found to be absent in the dark glumed Aurosis and Aurolata lines, and additional loss of *FLS* seems to return the flavonoid biosynthesis pathway to the parental mode.

It is likely that the loss of *FLS* expression leads to the disruption of dihydroflavonols to flavonols–conversion. Accumulation of dihydroflavonols makes them more accessible substrates, particularly for the DFR enzyme that reduces them to leucoanthocyanidins. As dihydroflavonols are the most preferable substrate for DFR, this condition makes the other DFR catalyzed reaction, conversion of naringenin to phlobaphenes monomer, apiferol, infrequent. Thus, the additional loss of *FLS* expression in the light glumed lines redistributes the flow in the flavonoid biosynthesis pathway, which results in the decreased phlobaphenes–monomers biosynthesis.

The absence of the single *F3'H* transcripts expression is sufficient for the dark glumed phenotype development in the *T. aestivum* / *A. muticum* introgression lines

The expression profile of Tica 30 bl2, Tica 8 bl, and Tica 14 bl lines with introgressions from *A. muticum* is similar to the parental one with the exception of one gene, *F3'H*, whose expression is absent in the lines (Fig. 9). The lines do not show a phenotype-specific expression pattern, as both light glumed and dark glumed plants have the similar expression profile. Since only some of the plants with the absence of *F3'H* expression show a dark glumed phenotype, there must be some other factors that led to the changes of flavonoid metabolic flux. As no variations were found in the expression of the *CHS*, *CHI*, and *DFR* transcripts involved in the biosynthesis of phlobaphenes monomers, we can expect that intensification of phlobaphenes biosynthesis is achieved due to the changes in the non-enzymatic polymerization step.

Similarly, the polymerization step alteration can explain why two Aurotica lines, Tica 42 bl and Tica 30 bl1, with both light glumed and dark glumed plants have expression profiles similar to the light glumed stable lines. Otherwise, some changes in the expression of the flavonoid biosynthesis genes, which were not examined in this study, like *F3'5'H*, *LAR*, and *ANR*, could contribute to the flavonoid pathway rewiring. Additional studies may shed the light on the mechanisms underlying the development the dark glume color.

Compliance with Ethical Standards

Anastasiia Navalikhina declares that she has no conflict of interest.

George Fedak declares that he has no conflict of interest

Maksym Antonyuk declares that he has no conflict of interest.

Tamara Ternovska declares that she has no conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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FIGURE CAPTIONS

Fig. 1 Three phenotypes of the lines with introgressions from *A. muticum*: light (left), brown (middle), and light brown (right) glume color.

Fig. 2 Total phenolic content in glumes of the IL res 122-2 and its parents, the *T. aestivum* cultivar Aurora and the genome-substitution amphidiploid Aurosis. The Dunnet's test value (t) for comparisons with Aurora is given above. The asterisk indicates the statistical significance of the difference.

Fig. 3 Total phenolic content in glumes of the ILs res 193, res 191, f04 1352 lata, and their parents, the *T. aestivum* cultivar Aurora and the genome-substitution amphidiploid Aurolata. The Dunnet's test value (t) for comparisons with Aurora are given above. The asterisk indicates the statistical significance of the difference.

Fig. 4 Total phenolic content in glumes of the *T. aestivum* / *A. muticum* ILs with light, light brown, and brown glume color, and their parents, the *T. aestivum* cultivar and the genome-substitution amphidiploid Aurotica. The Dunnet's test value (t) for comparisons with Aurora are given above. The asterisk indicates the statistical significance of the difference.

Fig. 5 Expression of the flavonoid biosynthesis pathway transcripts *F3'H* and *FNS* in the dark glumed ILs res 122-2 and res 133-1, and their parents, the *T. aestivum* cultivar (Au) and the genome-substitution amphidiploid Aurosis (sis).

Fig. 6 Expression of the flavonoid biosynthesis pathway transcripts *F3'H* and *FNS* in the dark glumed ILs f04 1352 lata and res 201, and their parents, the *T. aestivum* cultivar (Au) and the genome-substitution amphidiploid Aurolata (lata).

Fig. 7 Expression of the flavonoid biosynthesis pathway transcripts *F3'H*, *FNS*, and *FLS* in the light glumed ILs, and their parents, the *T. aestivum* cultivar (Au) and the genome-substitution amphidiploid Aurotica (tica).

Fig. 8 Expression of the flavonoid biosynthesis pathway transcripts *F3'H*, *FNS*, and *FLS* in the ILs segregating for the glume color, and their parents, the *T. aestivum* cultivar (Au) and the genome-substitution amphidiploid Aurotica (tica).

Fig. 9 Biosynthesis of major flavonoid compounds in grasses. From (Vermerris and Nicholson 2006) and (Yang et al. 2017) with modifications

Fig. 1

Fig. 2

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