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Original scientific article

STUDY OF CAROTENOID CONTENT IN DURUM WHEAT

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The purpose of the study is to determine the variability and stability of traits related to the amount of yellow pigments in the grain, semolina and the finished pasta product. In addition the polymorphism in one of the phytoene synthase gene on chromosome 7A related to carotenoids synthesis was detected. In the investigation were included 10 durum wheat genotypes – cultivars and breeding lines of different origin – Bulgaria - Field Crops Institute – Chirpan and Dobroudja Agricultural Institute – General Toshevo, Europe, CYMIT-Mexico. All genotypes were grown in field conditions in competitive variety trials in three replications in harvesting years 2014/2016. The results obtained were processed statistically via basic statistics (TIBCO statistica 13.3.0 software package). Great variability was found between genotypes on the study traits. The breeding lines: M-431 (10.71 ppm in grain, 10.42 ppm in semolina and 8.96 ppm in pasta) and TD-97 (9.83 ppm in grain, 9.58 ppm in semolina and 8.52 ppm in pasta) and the Bulgarian standard cultivar Predel (9.25 ppm in grain, 8.87 ppm in semolina and 7.27 ppm in pasta) are characterized by the highest yellow pigment content in grain, semolina and pasta. Both lines are also distinguished by the lowest total pigment losses (2.6%) in milling grain to semolina and in processing to pasta product (11 – 11.4 %), while line TD-97 has the lowest total pigment losses (13.6%). Furthermore the genotypes characterized by the highest yellow pigment content (M-431 and TD-97) have an acceptable stability and can be used in the hybridization program to improve the color potential of Bulgarian durum wheat varieties. The Psy-A1 codominant marker, based on a 37 bp insertion at the 5' end of intron 2 of the PsyA1 gene amplifies two fragments. The Psy-A1a allele with a 194 bp

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fragment in length was observed in all 7 studied cultivars and lines of durum wheat. The 231-bp Psy-A1b allele is found in 3 genotypes. The obtained results could not be associated with phenotypic differences in the yellow pigment content in the durum wheat genotypes studied.

Key words: durum wheat, yellow pigments, phytoene synthase

INTRODUCTION

Durum wheat is used mainly for the production of pasta. The color of durum wheat products is an important marketing characteristics related to the consumer choice of pasta. High yellow pigment content in grain enhances not only pasta quality but their nutritive value as well. The yellow color is due to the carotenoid (yellow) pigment content (YPC) in the whole grain. The color of the grain and the end products results from phenotypic variations in the pigments of grain, which depend on genetic factors, growing conditions, and technological processes (FICCO *et al*., 2014; TANEVA *et al.*, 2019). BRAATEN *et al*. (1962), RHARRABTI *et al.* (2003) report significant effect of genotype and weak effect of the environment on the variation of yellow pigment content. The information on the effect of interaction between genotype and the environment on the phenotype manifestation of that trait is contradictory (LEE *et al*., 1976; CLARKE *et al*., 2006).

A significant part of carotenoid pigments are lost during grain grinding to semolina (approximately 8%) or during processing to pasta (approximately 16%). The color of ready pasta results from the balance between the accumulation of yellow pigments (carotenoids) in the grain and their oxidative decomposition under the effect of lypoxygenase activity (LOX) during processing (BORRELLI *et al*., 1999). These complicated interactions must be considered in breeding programs for creation of cultivars with higher pigment content.

In recent years the stability of quality parameters has become an important requirement of the processing industry due to the great variation by years in the yield and the parameters responsible for the grain harvest quality influenced by climate change as well (RHARRABTI *et al*., 2003).

Small differences in the variation of genes responsible for basic quality parameters, incl. yellow pigment content in modern durum wheat cultivars are a significant restriction to their breeding improvement (BLANCO *et al*, 1994). Genetic diversity is an important resource in breeding, especially under the changing climatic conditions. According to some authors (LAIDO *et al*., 2013; DAVID *et al*., 2014) contemporary plant breeding programs aimed at reducing plant height and creation of cultivars meeting the high quality standards has resulted in strong and constant decrease of genetic diversity in modern durum wheat cultivars. When studying variation and interactions between yield and some quality parameters in Turkish local durum wheat samples AKCURA (2009) found out that variation in semolina color is greater in local samples than in modern durum wheat cultivars.

All that necessitates breeding programmes to include a great number of genotypes of different environmental and geographic origin. The study of available genetic diversity and incorporating it in hybridization programmes is important for the further improvement of durum wheat.

In recent years, genetic analyses have been deepened by the use of molecular markers that allow the identification of genomic regions controlling the concentration of pigments in wheat grain. Using different approaches and DNA marker systems, it has been found that YPC inheritance is complex because loci (QTLs) with minimal effect are found in almost all durum wheat chromosomes (FICCO *et al*., 2014). Several studies are focused on the identification of candidate genes involved in YPC control. One of the best studied genes is the gene that directs metabolism to carotenoid synthesis and encodes a phytoene synthase (PSY) enzyme. Phytoene synthase (PSY) catalyzes the dimerization of two molecules of geranyl-geranyl pyrophosphate to phytoene and is considered to be an enzyme limiting the rate of accumulation of carotenoids in seeds (LINDGREN *et al*., 2003). Nearly 50 different alleles have been identified in different wheat species in the *Psy-A1* and *Psy-B1* loci. The genes encoding PSY have been assigned to the groups of 7 chromosomes by POZNIAK *et al*. (2007), in particular the *Psy-B1* locus, co-segregating with the QTL of chromosome 7B, which demonstrates an association between the position of this gene and part of the phenotypic variation for endosperm color. The identified *Psy-A1* alleles of this gene located on chromosome 7A are due to polymorphisms that lead to the appearance of two *Psy-A1* haplotypes, which explains 20-28% of the phenotypic variation of YPC in different environments (HE *et al*., 2008). On the basis of this gene, a codominant marker YP7A was created, which was used for associative analysis of the amount of yellow pigments in the grain of about 200 genotypes of common wheat (HE *et al*., 2008).

Conducting in-depth studies to determine the variation of yellow pigment content in grain and their loss after grinding and processing to the final product is important in connection with the establishment of a proper breeding strategy for improving the quality of the Bulgarian durum wheat cultivars. The finding of molecular markers, closely linked to genes and/or quantitative trait loci (QTL) that control the grain color creates new opportunities for increasing the pigment content in new cultivars using markerassisted selection (FICCO *et al*., 2014).

The objective of the present study is to determine the variation and stability of traits associated with the yellow pigment content in the grain-semolina-pasta chain and detection of polymorphism in one of the genes related to carotenoid synthesis in a sample of 10 durum wheat genotypes of different origin.

MATERIALS AND METHODS

The study includes 10 durum wheat genotypes - cultivars and breeding lines of different origins - Bulgaria - Institute of Field Crops – Chirpan and Dobroudja Agricultural Institute – General Toshevo, Europe, CYMIT - Mexico (Table 1). All genotypes were grown under field conditions in a competitive cultivar trial in four replications in the 2014/2016 harvest years.

The experimental milling of the grain to semolina was carried out with a QC-109 Labor Mim laboratory mill adapted for semolina milling, complete with a laboratory plansifter with a set of sieves for sifting the semolina (PETROVA, 1993a). Grain moisture was determined according to BDS EN ISO 712: 2010. Yellow pigment content, ppm d.b. was determined according to BDS EN ISO 11052: 2006. The principle of the method is extraction of pigments with water-saturated n-butanol and spectrophotometric reading of the optical density of the clear filtrate at 440 nm wave length with Specol 11, Carl Zeiss. The pigment calculation is based on a calibration curve constructed with pure β-carotene used as a standard.

Table 1. Origin of genotypes

Country	Genotype
Bulgaria, Institute of Field Crops - Chirpan	Predel
Bulgaria, Dobroudja Agricultural Institute – General Toshevo	Severina
Austria	Superdur
Germany	TD-97
Bulgaria, Institute of Field Crops - Chirpan	M-287
Bulgaria, Institute of Field Crops - Chirpan	M-376
Bulgaria, Institute of Field Crops - Chirpan	M-431
Bulgaria, Institute of Field Crops - Chirpan	D-8326
Mexico, CYMIT	D-8367
Mexico, CYMIT	D-8370

Molecular analysis of the genotypes using STS (functional) markers was performed to study the polymorphism in one of the phytoene synthase genes on chromosome 7A, showing an association with the carotene content in 6x wheat (HE *et al.*, 2008). To isolate genomic DNA, leaves of second-leaf plants grown under greenhouse conditions were used. The collected leaf material was ground into fine powder in mortars with liquid nitrogen and separated in 100-150 mg in 1.5 ml tubes. The ground material was stored at -70 °C until DNA analysis was performed. Genomic DNA was isolated according to the protocol of MURRAY and THOMPSON (1980) with minor modifications. The concentration and quality of the isolated genomic DNA was determined by electrophoresis in a 0.8% agarose gel containing 0.5% ethidium bromide. λ-phage DNA at a concentration of 50 ng/µl was used as a standard. PCR was performed in a volume of 20 µL containing 100 ng of gDNA, 1x PCR My Taq HS master mix, 5 pM from each primer (forward - F and reverse - R) to amplify alleles of the phytoene synthase gene with localization on 7A (*Psy-A1a* and *Psy-A1b*).

Psy-A1(7A)_F1 F: GGACCTTGCTGATGACCGAG
Psy-A1(7A)_R1 R: TGACGGTCTGAAGTGAGAATGA

PCR was conducted on Verity (Applied Biosystems) device and the basic steps for amplifying the *Psy-A1a and Psy-A1b* alleles comprise initial denaturation of gDNA at 94 °C for 4 m, followed by 30 cycles, each of these comprising: denaturation at 94 °C for 30 s, hybridization of primers at 65 °C for 30 s and synthesis at 72 °C for 30 s. The last step of elongation is 5 m at 72 °C.

The amplified products were separated in 2% agarose gels to visualize the efficiency and correctness of amplification. Subsequently, PCR products were analyzed on a 6% polyacrylamide gel (19:1) in 1x Tris-borate buffer at 100 V for 2 h. As a marker for determining the length of the alleles obtained, a ladder consisting of fragments differing in length of 100 bp (Ladder 100 bp) was used. Prior to conducting PAGE electrophoresis 3 µl of each PCR product was mixed with 2 µl of H2O and 1 µl of Gel Red.

After electrophoresis is complete, the gel is documented with a photo-documentation system.

RESULTS AND DISCUSSION

The mean results from the three years of study presented in Table 2 show a significant diversity of the studied genotypes by yellow pigment content and pigment losses in the grain-semolina-pasta chain. Yellow pigment content in grain over a three-year average ranged from 6.25 to 10.71 ppm and in semolina - from 5.66 to 10.42 ppm. Four genotypes (Predel, Superdur, TD-97 and M-431) have yellow pigment content in the grain above 9-10 ppm, in semolina - above 8-10 ppm and products with relatively intense yellow color (7-9 ppm) are obtained from them. The breeding lines: M-431 (10.71 ppm in grain, 10.42 ppm in semolina and 8.96 ppm in pasta) and TD-97 (9.83 ppm in grain, 9.58 ppm in semolina and 8.52 ppm in pasta) and the Bulgarian standard cultivar Predel (9.25 ppm in grain, 8.87 ppm in semolina and 7.27 ppm in pasta) are characterized by the highest yellow pigment content in grain, semolina and pasta.

Loss of yellow pigments in milling grain to semolina averaged for the three years of study is within the range from 2.6 to 9.3%, and in processing semolina to pasta it is considerably higher from 11.0 to 18.8% (Table 2). Total pigment losses vary within the range 13.6-23.9%. The lines with the highest yellow pigment content in grain: M-431 and TD-97 are characterized by the lowest pigment losses in milling grain to semolina (2.6%). In processing to pasta product line TD-97 (11%) has again the lowest pigment losses, as well as line M-287 (11.4%) d.b. and cultivar Superdur (11.5%). Line TD-97 is also characterized by the lowest total pigment losses (13.6%), followed by cultivar Superdur (15.1%) and line M-431 (16.9%).

Table 2. Mean values of the traits yellow pigment content in grain, semolina and pasts disc, pigment losses and variatrion coefficients in durum wheat genotypes for the period 2014-2016

Genotype	Yellow pigments in grain, ppm	Yellow pigments in semolina, ppm	Yellow pigments in disc, ppm	Losses in milling, % compared to grain	Losses in pasta products, % compared to semolina	Total pigment losses, %	CV %
Predel	9,25	8,87	7,27	4,0	18,3	22,3	7,83
Severina	6,25	5,66	4,84	9,3	14,6	23,9	7,9
Superdur	9,09	8,76	7,75	3,6	11,5	15,1	11,53
TD-97	9,83	9,58	8,52	2,6	11,0	13,6	7,16
M-287	6,27	5,67	5,03	9,3	11,4	20,7	16,13
M-376	7,95	7,55	6,54	5,1	13,4	18,5	9,22
M-431	10,71	10,42	8,96	2,6	14,3	16,9	9,73
D-8326	8,67	8,29	6,74	4,4	18,8	23,2	5,52
D-8367	8,38	7,96	6,75	5,0	15,2	20,2	8,86
D-8370	8,91	8,38	7,04	5,9	15,9	21,7	13,44
Mean x	8,53	8,11	6,94	5,18	14,44	19,61	9,73
Min/Max	6,25-10,71	5,66-10,42	4,84-8,96	2,6-9,3	11,0-18,8	13,6-23,9	5,52-16,13
Variance	2,01	2,32	1,74	5,81	7,5	12,16	
Std. dev.	1,42	1,52	1,32	2,41	2,74	3,49	
CV	16,62	18,78	18,99	46,54	18,96	17,78	

The best potential line is M-431, in which yellow pigment content in the grain-semolina-pasta chain average for the three years is 10.71-10.42-8.96 ppm and total pigment losses of 16.9%. Line TD-97 follows it with yellow pigment content of 9.83-9.58-8.52 ppm and pigment losses of 13.6%.

Our results show that during the processing of semolina to pasta, a higher percentage of pigments is lost than when milling the grain to semolina. In milling the grain, the mean pigment losses of all genotypes averaged 5.18% over three years, while in processing to pasta these losses were 14.44%. Similar results have been reported by BORRELLI *et al*. (1999). These authors believe that increased lipoxygenase activity is a major factor associated with the loss of colour in finished pasta. Therefore, they suggest that the indicators high yellow pigment content in grain and low lipoxygenase activity in semolina to be used as selection criteria for improving the colour of pasta.

The stability of the studied genotypes by yellow pigment content in grain, estimated by the coefficients of variation calculated on the basis of the average results from the replications and the years of cultivation are presented in Table 2, too. Coefficients of variation (CV%) for that trait range from 5.52% at line D-8326 to 16.13% in line M-287. The most stable line D-8326 has relatively high yellow pigment content in the grain – 8.67 ppm, and the most unstable line M-287 - low – 6.27 ppm. The M-431 breeding line characterized by the highest yellow pigment content (10.71 ppm) has a medium high coefficient of variation of 9.73%, i.e. acceptable stability.

The standard cultivar Predel is of medium high stability (CV – 7.83%). The foreign line TD-97 is also characterized by medium-high stability (CV-7.16%) and exceeds the standard cultivar Predel by this trait. All other cultivars - Bulgarian and foreign have lower and less stable values of the yellow pigment content indicator compared to the standard cultivar Predel.

The data demonstrated a relatively large variation in studied genotype sample in yellow pigment content and pigment losses along the grain-semolina-pasta chain. Genotypes high in yellow pigments in the grain, semolina and pasta and with relatively low pigment losses have been identified, which can be used in the hybridization program to improve the color potential of Bulgarian durum wheat varieties. The genotypes characterized by the highest yellow pigment content (M-431 and TD-97) have an acceptable stability. All this together with high heritability and genetic advance for yellow pigments content in the grain found in our previous study (TANEVA *et al*., 2019) will further facilitate breeding work regarding this important quality trait.

It has been reported that some of the phytoene synthase gene alleles on the 7th homology group are associated with a higher carotene content in hexaploid wheat (HE *et al*., 2008; HE *et al*., 2009) thus affecting the quality of the final product. Our results obtained from the analysis of polymorphism in one of the phytoene synthase genes on chromosome 7A cannot be associated with the phenotypic differences in the yellow pigment content of the studied genotypes. Molecular analysis of the 10 durum wheat genotypes was performed to study polymorphism in one of the phytoene synthase genes on chromosome 7A, showing an association with the carotene content in hexaploid wheat.Phytoene synthase gene amplification was performed on the 7A chromosome - *Psy-A1* (7A) with STS primers amplifying an area localized in intron 2 of this gene. The primers used were designed to allow detection of two *Psy-A1a* and *Psy-A1b* alleles differing in length due to the presence/absence of 37 bp insertion in this region of the gene. The

PCR products obtained were tested on a 2% agarose gel and the presence of a fragment of about 200 bp in length was detected in all 10 durum wheat genotypes analyzed (Figure 1).

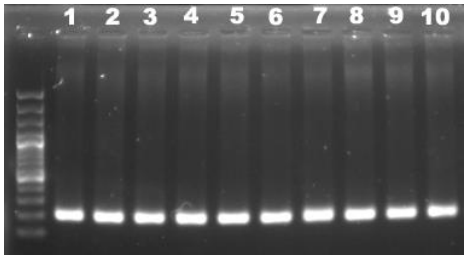

Figure 1. 2% Agarose gel electrophoresis

Legend – 1. Ladder 100bp, 2. Predel,3. Severina, 4. Superdur, 5. M-287, 6. M-431, 7. M-376, 8. D-8367, 9. D-8326, 10. D-8370, 11. TD-97.

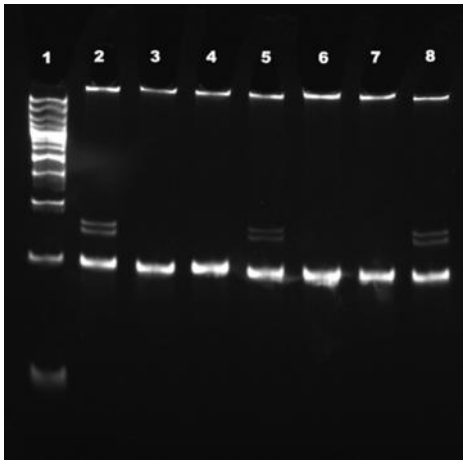

Figure 2. 6% Polyacrylamide gel electrophoresis

Legend – 1. Ladder 100bp, 2. M-431, 3. TD-97, 4. Predel, 5. Severina, 6. M-287, 7. M-376, 8. Superdur.

For correct determination of the length of the amplified fragments, electrophoresis of the PCR products of several genotypes with different carotene content was performed: M-431(10.71), TD 97 (9.83), Predel (9.25), Superdur (9.09), Severina (6.25), M–287 (6.27), M-376 (7.95) on 6% PAGE.

Figure 2 shows the identified allelic variants in the studied gene. The *Psy-A1* codominant marker, based on a 37 bp insertion at the 5' end of intron 2 of the *PsyA1* gene amplifies two fragments. The first fragment is 194 bp in length and corresponds to the *Psy-A1a* allele that binds to a higher carotene content. The second fragment is 231 bp in length and corresponds to the

Psy-A1b allele in 6 x wheat associated with a lower carotene content (HE *et al*., 2008). The *Psy-A1a* allele with a 194 bp fragment in length was observed in all 7 studied cultivars and lines of durum wheat. The 231-bp *Psy-A1b* allele, although of much lower intensity, is found in 3 genotypes: the M-431 line, the Bulgarian Severina cultivar and the foreign Superdur cultivar. The M-431 line has the highest content of yellow pigments, the Superdur cultivar - relatively high, and the Severina cultivar - medium high. The presence of this allele in the 3 genotypes of different yellow pigment content is most likely due to a known impurity or residual segregation of this gene in the population of the studied genotypes.

The first results obtained from the analysis of polymorphism in one of the phytoene synthase genes on chromosome 7A cannot be associated with the phenotypic differences in the yellow pigment content of the studied genotypes.

There are other publications reporting a lack of association between the *PsyA1* gene and the concentration of yellow pigments. QUINN (2012) reported that the *PsyA1* gene in a large set of Australian durum wheat genotypes could not be associated with the observed phenotypic variation by this trait. STEPANENKO *et al*. (2017) found that 97.8% of all 162 studied Ukrainian durum wheat genotypes had the *Psy-A1a* allele and only 0.8% had *Psy-A1b*.

Different percentages have been reported to explain the variation in the yellow color associated with the *PSY1A* gene - low, medium, high and very high in common and durum wheat (HE *et al*., 2008; ZHANG *et al*., 2009; HOWITT *et al*., 2009; BLANCO *et al*., 2011; RAVEL *et al*., 2013). There are also studies that illustrate that the *PSY1* locus accounts for a very small part of the variation in the yellow pigment content (ZHANG *et al*., 2008; RONCALLO *et al*., 2012). Some authors have reported large phenotypic differences between genotypes that share the same allelic variants at the *PSY1* locus (HE *et al*., 2009), most likely reflecting the polygenic nature of this trait. According to HE *et al*. (2009) the highest content of carotenoids was observed in the presence of the *Psy-A1a* and *Psy-B1c* alleles. ZHANG and DUBCOVSKY (2008) allow the possibility of a second gene or other regulatory element to be involved in the control of the yellow color of the endosperm localized very close to *PSY1* on the long arm of chromosome 7. SCHULTHESS and SCHWEMBER (2013), summarizing previous studies on this topic, conclude that the marker phenotypic associations of the *PSY1* loci with the yellow endosperm are not perfect, and sometimes even lead to unexpected values of the trait.

Our research in this regard should continue by analyzing polymorphism in relation to other loci such as *Psy-B1* (7B), *Psy-A2* (2A) as well, about which there is information that they are also responsible for carotene synthesis in durum wheat grain.

CONCLUSION

The studied genotype sample revealed a significant variety in yellow pigment content and pigment losses along the grain-semolina-pasta chain. Genotypes high in yellow pigments in the grain, semolina and pasta and with relatively low pigment losses have been identified, which can be used in the hybridization program to improve the color potential of Bulgarian durum wheat varieties. For the three years of study on average the highest is the yellow pigment content in the grain of lines M-431 (10.71 ppm), TD-97 (9.83 ppm) against 9.25 ppm for the standard Predel. High levels of yellow pigments (8-9 ppm) have other 4 breeding lines (D-8370, D-8326, D-8367, M-376) and the foreign variety Superdur (9.09 ppm). The TD-97 line was also characterized by

the lowest total pigment losses (13.6%), followed by the Superdur variety (15.1%) and the M-431 line (16.9%). The M-431 breeding line with the highest yellow pigment content (10.71 ppm) has medium high coefficient of variation of 9.73%, i.i. acceptable stability by this trait.

The first results obtained from the analysis of polymorphism in one of the phytoene synthase genes on chromosome 7A cannot be associated with the phenotypic differences in the yellow pigment content of the studied genotypes. Research along these lines should continue by analyzing polymorphism in relation to other loci such as *Psy-B1*(7B), *Psy-A2*(2A), for which there is information that they are also responsible for carotene synthesis in durum wheat grain.

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ISPITIVANJE SADRŽAJA KAROTENOIDA U DURUM PŠENICI

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Izvod

Cilj istrživanja je da se odredi varijabilnost i stabilnost osobina povezanih sa količinom žutog pigmenta u zrnu, grizu i testenini. Osim toga, otkriven je polimorfizam u jednom od gena fitoen sintaze na hromozomu 7A povezan sa sintezom karotenoida. U istraživanje je uključeno 10 genotipova tvrde pšenice - sorte i linije različitog porekla - Bugarska - Institut za ratarstvo - Poljoprivredni institut Čirpan i Dobroudja - General Toševo, Evropa, CIMIT -Meksiko. Svi genotipovi su uzgajani u poljskim uslovima u konkurentnim ogledima sorti u tri ponavljanja u godini berbe 2014/2016. Dobijeni rezultati su statistički obrađeni pomoću osnovnih statistika (programski paket TIBCO statistica 13.3.0). Utvrđena je velika varijabilnost među genotipovima za ispitivane osobine. Linije: M-431 (10,71 ppm u zrnu, 10,42 ppm u grizu i 8,96 ppm u testeninama) i TD-97 (9,83 ppm u zrnu, 9,58 ppm u grizu i 8,52 ppm u testeninama) i bugarska standardna sorta Predel (9,25 ppm u zrnu, 8,87 ppm u grizu i 7,27 ppm u testeninama) karakteriše najveći sadržaj žutog pigmenta u zrnu, grizu i testenini. Obe linije se odlikuju i najmanjim ukupnim gubicima pigmenta (2,6%) pri mlevenju zrna do griza i pri preradi do testenine (11-11,4%), dok linija TD-97 ima najmanji ukupni gubitak pigmenta (13,6%). Nadalje, genotipovi koje karakteriše najveći sadržaj žutog pigmenta (M-431 i TD-97) imaju prihvatljivu stabilnost i mogu se koristiti u programu hibridizacije za poboljšanje potencijala boje za bugarske sorte durum pšenice. Kodominantni marker Psi-A1, zasnovan na umetanju od 37 bp na 5 'kraju introna 2 gena PsiA1, pojačava dva fragmenta. Alel Psi-A1a sa fragmentom od 194 bp je primećen u svih 7 proučavanih sorti i linija durum pšenice. Alel Psi-A1b od 231 bp nalazi se u 3 genotipa. Dobijeni rezultati se ne mogu dovesti u vezu sa fenotipskim razlikama u sadržaju žutog pigmenta u ispitivanim genotipovima durum pšenice.

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