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SEQUENCE ANALYSIS OF FLAVANONE 3-HYDROXYLASE AND DIHYDROFLAVONOL 4-REDUCTASE GENES IN WHEAT WITH NONSTANDARD COLOURED CARYOPSES

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The DNA sequences of chosen structural genes, *flavanone 3-hydroxylase* (*F3H*) and *dihydroflavonol 4-reductase* (*DFR*), encoding key enzymes from the flavonoid biosynthetic pathway, were studied in this paper. Sequences were gained using different approaches, i.e. direct sequencing from the PCR product for *F3H* and a cloning strategy for *DFR*. Five bread wheat (*Triticum aestivum* L.) genotypes with nonstandard coloured caryopses, purple, blue, and white, were used as plant material. The sequence variability was observed among tested genotypes.

Keywords: anthocyanins, DFR, F3H, flavonoids, *Triticum aestivum* L.

INTRODUCTION

Flavonoid biosynthesis is one of the most studied pathways in plant secondary metabolism. The function of these compounds lies in the pigmentation of tissues such as flowers, fruits, seeds and leaves not only to make them more attractive to pollinators and seed dispersers but also to protect tissues against UV damage and play a role in seed dormancy and stress management (HOLTON and CORNISH, 1995; CHALKER-SCOTT, 1999; HIMI *et al.*, 2002; WINKEL-SHIRLEY, 2002). The regular inclusion of flavonoids in the human diet has a positive effect on the

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consumer's health. Mostly fruits are rich in natural pigments, but also some types of cereals as wheat, barley, rice or maize can be considered as a source of flavonoids (ABDEL-AAL *et al*., 2006; LIN and WENG, 2006; ABDEL-AAL *et al*., 2008).

This diverse group of compounds with aromatic molecules in their structure is derived from phenylalanine and malonyl-coenzyme A and is divided into six major groups present in most higher plants: chalcones, flavones, flavonols, flavandiols, anthocyanins and proanthocyanidins. Another group, aurones, is also common (WINKEL-SHIRLEY, 2001). The unusual colouration of wheat (*Triticum aestivum* L.) caryopses is caused by anthocyanins, particularly cyanidin 3-glucoside and delphinidin-3-glucoside, while in purple wheat cyanidin 3-glucoside is the most abundant anthocyanin and in blue delphinidin-3-glucoside is more prevalent (ABDEL-AAL *et al*., 2003; HOSSEINIAN *et al*., 2008; TROJAN *et al*., 2014). The enzymes involved in the biosynthetic pathways leading to anthocyanin group formation are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) (MOL *et al*., 1998; DAVIES and SCHWINN, 2006; MA *et al*., 2014). Genes were studied in the experiment described in this article encode two important enzymes of mentioned biosynthetic pathway – F3H and DFR. F3H hydroxylates naringenin to dihydroflavonol, while DFR usually reduces dihydroflavonol to leucoanthocyanidin, but in some cases can also convert flavanone to flavan-4-o1 which when polymerized create phlobaphens (WINKEL-SHIRLEY, 2001).

While the genome of most plants contains only a single copy of these genes, three homoeologous copies (orthologs) of the genes encoding the F3H enzyme (EC 1.14.11.9) in common wheat ($2n = 6x= 42$, BBAADD) were located on the telomeric regions of the long arm of the chromosomes of homoeologous group 2 and one paralog *F3H_B2* in the genome B (KHLESTKINA *et al*., 2008; HIMI *et al*., 2011; KHLESTKINA *et al*., 2013), and DFR enzyme (EC 1.1.1.219) genes were located on the long arms of the chromosomes 3A, 3B, and 3D (HIMI and NODA, 2004).

These structural genes of the plant flavonoid biosynthesis are regulated by the MYB-bHLH-WD40 complex. Transcription factors of a MYC gene family which refers to one of the largest families of the plant transcriptional factors – bHLH – are integral part of this regulator complex and the *TaMYC1* gene is known in bread wheat to control the synthesis of flavonoid pigments in the wheat pericarp and recent evidences suggest that *TaMYC1* to be a synonym of *Pp3* (WINKEL-SHIRLEY, 2002; JAAKOLA, 2013; LI, 2014; XU *et al*., 2015; LI *et al*., 2017; STRYGINA and KHLESTKINA, 2017; ZONG *et al*., 2017). The purple coloration of the caryopsis is phenotypic expression of the process of interaction of the products of functionally active alleles of the *TaMyc1* and *Pp1* (MYB encoding gene) (KHLESTKINA *et al*., 2014; SHOEVA *et al*., 2014), while LI *et al*. (2017) suggested the *ThMYC4E* to be the *Ba-1* candidate gene for blue aleurone and associated traits in bread wheat.

This paper describes three partial *F3H* structural gene sequences (*F3H_A*, *F3H_B* and *F3H_D*) and one partial *DFR* sequence obtained from wheat genotypes with blue, purple and white caryopses.

MATERIAL AND METHODS

Plant material

Five genotypes of the spring varieties of bread wheat (*Triticum aestivum* L.) were used in this experiment - two genotypes with purple pericarp, i.e. Abyssinskaya Arraseita (AA) and ANK-28B (ANK), two with blue aleurone, i.e. Tschermaks Blaukörniger Sommerweizen (TBS)

and UC66049 (UC) and one with white caryopses, i.e. Novosibirskaya 67 (N67) (Tab. 1). The seeds were provided by Research Institute Kroměříž, Ltd., Czech Republic, and were sown and harvested in Brno, Czech Republic (N 49° 12' 55.48"; E 16° 36' 53.58"). The surface of the harvested caryopses used for further germination and DNA isolation was sterilized by an aqueous solution of sodium hypochlorite in a concentration 5 gL^{-1}. Genomic DNA was isolated (DNA separation by silica adsorption, DNeasy Plant Mini Kit, Qiagen) from 5–7 day old wheat seedlings planted under controlled laboratory conditions. The concentration and purity of extracted DNA was measured by spectrophotometer Picopet 1.0 (Picodrop, Cambridge, UK).

Table 1. EVIGEZ numbers of used plant material*

Genotype	EVIGEZ number
Abyssinskaya Arraseita (RU 687-12)	01C0200501
ANK-28B	NA
Novosibirskaya 67	01C0204324
Tschermaks Blaukörniger Sommerweizen	01C0205098
UC66049	01C0205100

**Plant Genetic Resources Documentation in the Czech Republic*

Flavanone 3-hydroxylase gene sequencing directly from PCR product

Nine mutually combinable genome specific primer pairs for three homoeologous copies in genome A, B and D, were designed and tested for the purposes of direct sequencing from PCR product. *F3H* gene sequences originating from genomes A, B and D of the cultivar Chinese Spring(https://www.ncbi.nlm.nih.gov/nuccore/AB223024.1,https://www.ncbi.nlm.nih.gov/nuccore/AB223025.1, https://www.ncbi.nlm.nih.gov/nuccore/AB223026.1) were used as a matrix for primer design. The Primer3 online primer design tool (http://primer3.ut.ee/) was used (ROZEN and SKALETSKY, 2000). After difficulties with specificity of the designed primers, finally primers by HIMI *et al.* (2011) were used for F3H sequence data acquisition. The forward primer F3H_F with 5'–3' sequence CAA GAA GCA GGC CAA GGA C was universal for all three reverse primers (primer by HIMI *et al.*, 2011). The universal forward primer with reverse primer for genome A F3HA_R (5'–3' sequence CCA AAC TCA CGA TAA CTC CTT ATT TAC) provided a product of size 303 bp (primer by HIMI *et al.*, 2011). F3H_F with reverse primer for genome B F3HB_R (5'–3' sequence TGG TGG GAT TGA TTA TTC TCC) gave a PCR product 189 bp long (primer by HIMI *et al.*, 2011). F3H_F in combination with reverse primer for genome D F3HD_R (5'–3' sequence GGT ATC CGT ACG TGT GTA GCA G) provided a 229 bp long PCR product (primer by HIMI *et al.*, 2011). The PCR conditions are summarized in table 2. The forward primer lies in the coding region (3rd exon) of the gene, while reverse primers are located in different loci of the 5'–3' untranslated region. The PCR products were purified using the PCR purification kit (QIAquick PCR Purification Kit, Qiagen). Sequence analyses were performed by the Macrogen company (Netherlands). The sequences obtained were processed and evaluated using the Basic Local Alignment Search Tool (BLAST) and the multiple sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). F3H coding sequences were translated to amino acid sequence using ExPASy Translate tool (https://web.expasy.org/translate/) and aligned by Clustal Omega.

Table 2. Flavanone 3-hydroxylase and dihydroflavonol 4-reductase PCR conditions

	Initial denaturation	Denaturation	Annealing	Elongation	
F3H_A	95°C/5 min	95°C/30 s	57°C/30 s	72°C/30 s	40 cycles
F3H_B	95°C/5 min	95°C/30 s	60°C/30 s	72°C/30 s	40 cycles
F3H_D	95°C/5 min	95°C/30 s	61°C/30 s	72°C/30 s	40 cycles
DFR	94°C/5 min	94°C/60 s	60°C/120 s	72°C/120 s	35 cycles

Dihydroflavonol 4-reductase gene sequencing by cloning strategy

The sequence with accession number AB162138.1 (https://www.ncbi.nlm.nih.gov/nuccore/AB162138.1) was used for designing primers specific for the *DFR* gene copy in genome A from a Chinese Spring cultivar. As the primers were derived only from an A genome of mentioned cultivar, cloning method was used to ensure sufficient quality of obtained sequences in case the designed primers were genome non-specific (unlike primers for *F3H* gene designed by HIMI *et al*., 2011, which were genome specific). This approach is recommended by KELLER *et al*. (2005). After picking and testing several primer pairs, the forward primer DFR_F (5'–3' sequence GAA AAA GTC CGC ATG TGG TT) and the reverse primer DFR_R (5'–3' sequence GGG CAG ATA CTT GGT TCA GA) producing 243 bp long PCR fragments situated in the non-coding region were used. The PCR conditions are shown in table 2. Obtained PCR products were cloned with the pGEM-T Vector System (Promega). Purified PCR products were ligated with pGEM-T plasmids (Promega) according to the user manual and transferred into electrocompetent *E. coli* using electroporation. A solid LB medium (lysogeny broth) with the addition of the antibiotic carbenicillin (100 mg L^{-1}) and blue/white selection marker (X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and IPTG-isopropyl β-D-1-thiogalactopyranoside) was used for the cultivation of electroporated *E. coli*. Petri dishes with inoculated LB medium were stored overnight at 37°C. White recombinant colonies (four colonies of each genotype in three repetitions, twelve different colonies in total for every genotype, according to MA *et al*., 2013) were transferred directly to PCR mastermix with a primer pair specific for regions T7 and SP6 of used plasmid (T7 5′-TAA TAC GAC TCA CTA TAG GG-3′ and SP6 5′-ATT TAG GTG ACA CTA TAG-3′). PCR was performed under the following conditions: 5 min at 95°C, then 30 cycles of 30 s at 95°C, 50 s at 55°C and 2 min at 72°C, followed by a final extension step at 72°C for 10 min. PCR products were purified using the PCR purification kit Invisorb Fragment Clean Up (Stratec Molecular, Berlin, Germany). Sequence analyses were performed by the Macrogen company (Netherlands). Sequence data gained were processed in the same way as *F3H* sequences.

RESULTS AND DISCUSSION

F3H sequence data

After the elimination of unreadable and uncertain data at the beginning of each read, sequences of all three copies of the *F3H* wheat gene were obtained. Due to this necessary reduction, full length sequences were not gained. The length of the sequences is as follow: genome A 243–245 bp, genome B 120 bp, genome D 208–209 bp. Unfortunately, the sequence for the genome A copy from the genotype Abyssinskaya Arraseita could not be obtained. Several single nucleotide polymorphisms among tested genotypes were observed in the coding region, especially in the case of white genotype Novosibiskaya 67 compared to reference *F3H_B* and

blue genotype Tschermaks Blaukörniger Sommerweizen compared with the *F3H_D* reference sequence (HIMI *et al.*, 2011) (Figure 1). The similarity between the reference *F3H_A* sequence and the nucleotide sequences obtained from our tested plant material was 98–99%. An analogous situation was observed in the two remaining gene copies. The similarity between the *F3H_B* sequences of the analyzed genotypes and the sequence obtained from Genbank ranged between 95 and 100%. From 94 to 100% was the similarity within reference *F3H_D* and the tested genotypes. In all cases this can be considered a high degree of similarity. Very similar results gave alignment with 3rd exon sequences by KHLESTKINA *et al.* (2008), low structural divergence was observed between *F3H_A*, *F3H_B* and *F3H_D* homoeologues. The paralogous *F3H_B2* sequence (https://www.ncbi.nlm.nih.gov/nuccore/JN384122, KHLESTKINA *et al.*, 2013) departs significantly from the sequences used in this study as a reference (HIMI *et al.*, 2011), so we can assume that partial DNA sequences obtained in our experiment would show similar range of divergence with *F3H_B2*. Sequences longer than 200 bp, i.e. *F3H_A* and *F3H_D*, were submitted to the Genbank. The next phase is to determine the genome specific expression of *F3H* genes and to obtain sequences of paralog *F3H_B2*.

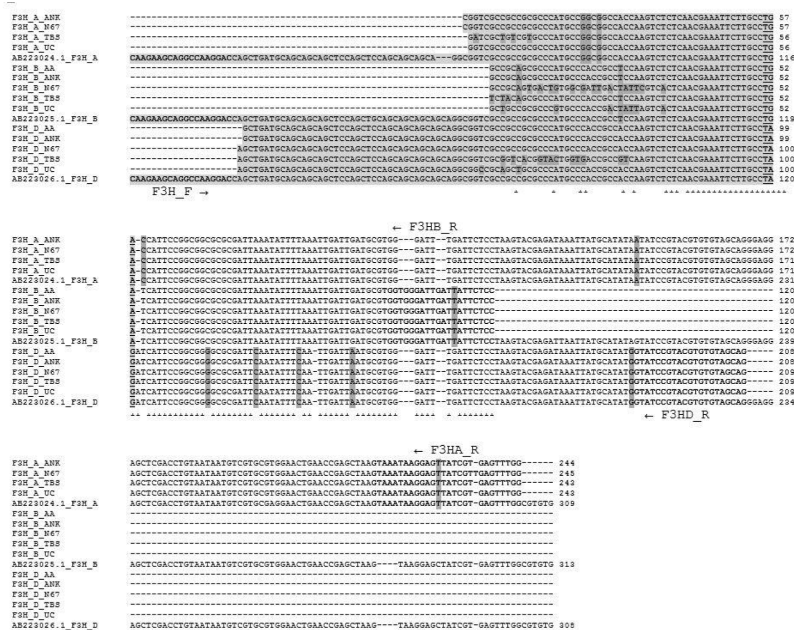

Figure 1. Nucleotide sequences alignment of *F3H* wheat genes in five tested genotypes and original sequences from Genbank. Letters in light grey boxes show exon, while white space indicates 5’–3’ untranslated region. Dark grey boxes demonstrate single nucleotide polymorphisms and differences between sequences. The stop codons TGA and TAG are underlined. Primers are written in bold font with the name and arrow showing their direction, i.e. right arrow – forward primer; left arrow – reverse primer. Abbreviations: AA – Abyssinskaya Arraseita, ANK – ANK-28B, N67 – Novosibirskaya 67, TBS – Tschermaks Blaukörniger Sommerweizen, UC – UC66049.

Figure 2 shows multiple sequence alignment of obtained coding sequence translated to amino acid sequence. Previously mentioned single nucleotide polymorphisms in coding regions resulted in several missense mutations. The most significant change in coding region was observed in case of genotype UC66049 genome B, where transversion from A to T on the 28th position caused formation of stop codon instead of lysine what could potentially result in functional changes of given protein.

Full sequence data of *F3H* gene obtained in this study can be seen in Genbank database under accession numbers given in Table 3.

```
F3H_A_ANK          --------------------------VAAAPMPAATKSLNEILA
F3H_A_N67          --------------------------VAAAPMPAATKSLNEILA
F3H_A_TBS          --------------------------IAVVPMPAATKSLNEILA
F3H_A_UC           --------------------------VAAAPMPAATKSLNEILA
AB223024.1_F3H_A   DLAKRKKQAKDQLMQQQLQLQQ-QQAVAAAPMPAATKSLNEILA
F3H_B_AA           ---------------------------AAAPMPTASKSLNEILA
F3H_B_ANK          ---------------------------AAAPMPTASKSLNEILA
F3H_B_N67          ---------------------------AAVTVAIDYSSLNEILA
F3H_B_TBS          ---------------------------STAPMPTASKSLNEILA
F3H_B_UC           ---------------------------AAAPVPTDY*    <- stop codon
AB223025.1_F3H_B   DLAKRKKQAKDQLMQQQLQLQQQQQAVAAAPMPTASKSLNEILA
F3H_D_AA           ------------LMQQQLQLQQQQQAVAAAPMPTATKSLNEILA
F3H_D_ANK          ------------LMQQQLQLQQQQQAVAAAPMPTATKSLNEILA
F3H_D_N67          ------------LMQQQLQLQQQQQAVAAAPMPTATKSLNEILA
F3H_D_TBS          ------------LMQQQLQLQQQQQAVAVTVLVTAVKSLNEILA
F3H_D_UC           ------------LMQQQLQLQQQQQAAAAAPMPTATKSLNEILA
AB223026.1_F3H_D   DLAKRKKQAKDQLMQQQLQLQQQQQAVAAAPMPTATKSLNEILA
```

Figure 2. Amino acid sequence alignment of *F3H* polypeptide. Abbreviations: AA – Abyssinskaya Arraseita, ANK – ANK-28B, N67 – Novosibirskaya 67, TBS – Tschermaks Blaukörniger Sommerweizen, UC – UC66049

Table 3. Genbank accession numbers of the F3H sequence data

GenBank accession number	F3H sequence designation
KP420721	F3H_A_ANK-28B
KP420722	F3H_A_Novosibirskaya 67
KP420723	F3H_A_Tschermaks Blaukörniger Sommerweizen
KP420724	F3H_A_UC66049
KP420725	F3H_D_Abyssinskaya Arraseita
KP420726	F3H_D_ANK-28B
KP420727	F3H_D_Novosibirskaya 67
KP420728	F3H_D_Tschermaks Blaukörniger Sommerweizen
KP420729	F3H_D_UC66049

DFR sequence data

Using cloning strategy, difficulties with poor data quality in the beginning of the read are no longer a problem compared to the direct sequencing method, i.e. full length sequences in several versions were gained. A disadvantage is the rather protracted work flow and more

sophisticated equipment needed. Unfortunately, no data for genotype Novosibirskaya 67 were obtained. After multiple alignment of the acquired sequences and reference sequence from Genbank (HIMI *et al.*, 2004), low variability represented by few single nucleotide polymorphisms was observed, and these may be considered as allelic variations of the *DFR* gene. BI *et al.* (2013) noted allelic variants in genome A and B among Chinese wheat cultivars. In some locations, all the tested genotypes from our collection had the same nucleotide in the same position, but the reference *DFR* sequence in the same position differed, for example in the 98th and 100th positions (Figure 3). In these cases, the nucleotide sequence corresponded with homoeologous copies in genomes B and D rather than genome A (https://www.ncbi.nlm.nih.gov/nuccore/AB162139 and https://www.ncbi.nlm.nih.gov/nuccore/AB162140, HIMI *et al.*, 2004) and from the present partial sequence we were not able to distinguish them from each other. Otherwise no significant differences were present. Also, very poor inter-genotype variability was observed. The similarity between the reference *DFR* and the tested genotypes was very high, at 98–99%. The next step in our research is to design genome specific primers for *DFR* genes for further sequence variability analyses and gene expression experiments.

Full sequence data of *DFR* gene obtained in this study can be seen in GenBank database under accession numbers given in Table 4.

Figure 3. Nucleotide sequences alignment of the wheat gene *DFR* in tested genotypes and their variants, and original sequences from GenBank. Dark grey boxes demonstrate single nucleotide polymorphisms and differences between sequences. Primers are written in bold font with the name and arrow showing their direction, i.e. right arrow – forward primer; left arrow – reverse primer. Abbreviations: AA – Abyssinskaya Arraseita, ANK – ANK-28B, TBS – Tschermaks Blaukörniger Sommerweizen, UC – UC66049

Table 4. Genbank accession numbers of the DFR sequence data

GenBank accession number	DFR sequence designation
KP420730	DFR_Abyssinskaya Arraseita 1
KP420731	DFR_Abyssinskaya Arraseita 2
KP420732	DFR_Abyssinskaya Arraseita 3
KP420733	DFR_ANK-28B 1
KP420734	DFR_ANK-28B 2
KP420735	DFR_ANK-28B 3
KP420736	DFR_Tschermaks Blaukörniger Sommerweizen 1
KP420737	DFR_Tschermaks Blaukörniger Sommerweizen 2
KP420740	DFR_UC66049 1
KP420741	DFR_UC66049 2

CONCLUSIONS

Anthocyanins contribute to the quality characteristics of many crops in a significant way and are therefore targets of many breeding programmes. We believe that obtained sequence data of genes involved in their biosynthetic pathway - *F3H* and *DFR* - will be a valuable source of information for on-going research and breeding. Knowing the sequences of transcription factors and structural genes from the biosynthetic pathway could possibly be a tool of complex studies focusing on expression profiles during maturation. The on-going release of transcriptome and genome information will increase our understanding of this topic.

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ANALIZA SEKVENCI GENA FLAVANONE 3-HIDROKSILAZE I DIHIDROFLAVONOL 4-REDUKTAZE PŠENICE SA NESTANDARDNOM BOJOM KARIOPSISA

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Izvod

DNK sekvence odabranih strukturalnih gena, *flavanone 3-hidroksilaze* (*F3H)* i *dihidroflavonol 4-reduktaze* (*DFR*), ključnih kodirajućih enzima iz biosintetskog puta flavonoida, proučavano je u ovom radu. Sekvence su dobijene korišćenjem različitih pristupa, tj. direktnog sekvenciranja iz PCR proizvoda za F3H i strategije kloniranja za DFR. Kao biljni materijal korišćeno je pet genotipova hlebne pšenice (*Triticum aestivum* L.) sa nestandardnim bojama kariposisa, ljubičastom, plavom i belom. Varijabilnost sekvenci je uočena među testiranim genotipovima.

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