Primer efficiency determination for flavanone 3-hydroxylase gene in wheat (*Triticum aestivum* L.)

MARIA PRESINSZKA[1], KLARA STIASNA[1], TOMAS VYHNANEK[1], VACLAV TROJAN[1], WOLFGANG SCHWEIGER[2], JAN BARTOS[3], LADISLAV HAVEL[1]
[1]Department of Plant Biology
Faculty of Agronomy
Mendel University in Brno
Zemedelska 1, 613 00 Brno
CZECH REPUBLIC
[2]Institute for Biotechnology in Plant Production
Department for Agrobiotechnology
BOKU/IFA-Tulln
Konrad-Lorenz-Str. 20, 3430 Tulln
AUSTRIA
[3]Centre of Plant Structural and Functional Genomics
Institute of Experimental Botany AS CR
Slechtitelu 31, 783 71 Olomouc-Holice
CZECH REPUBLIC

maria.presinszka@mendelu.cz

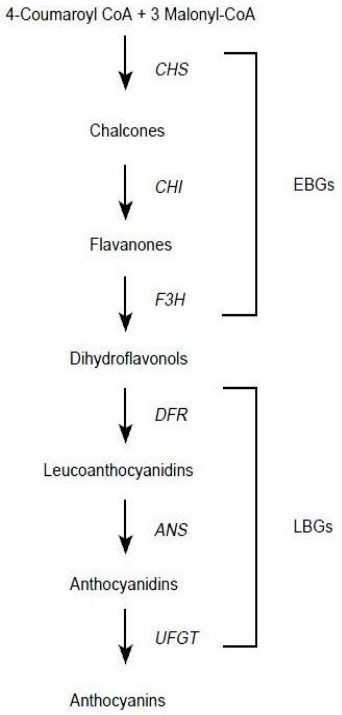
Abstract:
Anthocyanins are water-soluble pigments that may be red, purple, or blue. They belong to a parent class of molecules called flavonoids. Anthocyanins occur in all tissues of higher plants, including leaves, stems, roots, blossoms, and fruits. Flavanon 3-hydroxylase (F3H) is one of the key enzymes of the flavanoid biosynthesis pathway. F3H converts flavanones to dihydroflavonols. The main aim was to study the *F3H* gene in wheat (*Triticum aestivum* L.). In our experiment 5 genotypes with nonstandard coloured caryopses were used (2 genotypes with blue aleurone, 2 genotypes with purple pericarp and 1 genotype with white caryopses- without anthocyanins). Total RNA was isolated by phenol-chloroform method and complementary DNA was obtained by reverse transcription. Specific primers for each of the *F3H* genes (*F3H_A*, *F3H_B* and *F3H_D*) that were found in the National Center for Biotechnology Information (NCBI) database were designed. The first results of quantitative PCR (qPCR) contained two or more products. New primers for *F3H-D* gene were found and qPCR was repeated. The only one PCR product of *F3H_D* amplification from all five genotypes was sent for sequence analysis. Obtained sequences of *F3H_D* have 99-100% conformity with the database NCBI.

Key Words: wheat, *Triticum aestivum* L., flavanone 3-hydroxylase, anthocyanins

Introduction

One of the major cereal crops, *Triticum aestivum* L. (genome 2n = 6x= 42, BBAADD), can also have anthocyanin pigmentation of different organs. High anthocyanin content in cereal plants has its role in plant defense, but it has also positive effects on human health [1]. Anthocyanins are phenolic phytochemicals classified within flavonoids together with flavonols, flavones, flavanols, flavanones, and isoflavonoids [2]. Flavonoids are a class of plants secondary metabolites which have an important role in pigmentation [3]. Biosynthesis of anthocyanins start with the formation of malonyl-CoA and p-coumaroyl-CoA derived from the phenylpropanoid biosynthetic pathway under the action of seven enzymes: chalconsynthase, chalconisomerase, flavanon 3-hydroxylase, flavonoid 3-hydroxylase, dihydroflavonol-4-reductase, anthocyanin synthase, and flavonoid-3-O-glucosyltransferase [4]. Anthocyanins are as pigments responsible for blue, purple, red, or orange coloration of plant tissues and organs [4]. There are known more than six hundreds natural anthocyanins. Six of the most frequented are cyanidins, delphinidins, malvidins, pelargonidins, petunidins, and peonidins [6].

Fig. 1 Scheme of the flavonoid biosynthetic pathway [5]



Legend: CHS- chalcone synthase, CHI- chalcone isomerase, F3H- flavanone 3-hydroxylase, DFR- dihydro-flavanone reductase, ANS- anthocyanidin synthase, UFGT- UDP glucose: flavonoid-3-O-glucosyltransferase, EBGs- early genes of biosynthesis pathway, LBGs- late genes of biosynthesis pathway

The purple color of wheat is caused by anthocyanins accumulated in the pericarp, while the blue color is in the aleurone layer of wheat grains [7]. The purple color of pericarp originated from purple Ethiopian tetraploid and hexaploid wheat (*Triticum aethiopicum Jakubz.*). The main anthocyanin of pericarp in purple wheat is cyanidine-3-glucoside and it is a predominant anthocyanin in these seeds [8]. The most frequent anthocyanins in blue wheat seeds are delphinidine-3-glucoside and delphinidine-3-rutinoside, cyanidine-3-glucoside and peonidine-3-glucoside [9].

F3H (*flavanone 3-hydroxylase*) is a member of a gene family involved in the biosynthesis of flavonoid compounds [10]. F3H catalyzes an early step in flavonoid metabolism - the formation of flavonols from dihydroflavonols, and therefore provides precursors for many classes of flavonoid compounds [11]. *F3H* belongs to the early genes of the biosynthesis pathway. The protein is classified as a soluble 2-oxoglutarate-dependent dioxygenase based on its requirements for 2-oxoglutarate, molecular oxygen, ferrous iron (Fe^{2+}) and ascorbate [12].

Material and Methods

The common wheat (*Triticum aestivum* L.) genotypes with anthocyanin pigmentation of grain were used: two genotypes with purple pericarp (ANK- ANK28B and AA- Abyssinskaya Arraseita), two genotypes with blue aleurone (UC- UC66049 and TBS- Tschermaks Blaukörniger Sommer-weizen) and one genotype with white caryopsis (N67- Novosibirskaya 67). Genotype Novosibirskaya 67 was used as a standard, because it does not contain anthocyanins.

All seed samples were sown in Botanical Garden and Arboretum of the Mendel University in Brno. The caryopses were collected 10th, 15th, 20th, 25th, 30th, 35th and 40th days after anthesis (days *post anthesis* - dpa) during maturation that is 7 samples for each genotype. For isolation of total RNA, RNA blue (Top Bio) was used. Reverse transcription from RNA to cDNA was performed using Enhanced Avian HS RT PCR kit (Sigma Aldrich). The allohexaploid genome of bread wheat carries four copies of the *F3H* gene, i.e. three homoeologs (orthologs) in A, B, and D genomes (labeled in NBCI: *F3H-A1* - AB223024.1, *F3H-B1* - AB223025.1, and *F3H-D1* - AB223026.1) and one paralog (labeled in NCBI *F3H-B2* - JN384122.1) in the B genome [13]. Specific primers for all *F3H* genes were designed using program Primer3. Sequence for *F3H* genes was found in the National Center for Biotechnology Information (NCBI) database. Quantitative polymerase chain reaction (qPCR, CFX96 Real Time Systems: Bio-Rad) was performed. One reaction generally run for 40 cycles and in Table 1 are the used temperatures.

Table 1 qPCR reaction conditions

Step	Temperature	Time
Initialization	94°C	5 min
Denaturation	94°C	30 sec
Annealing	60/62°C	30 sec
Extension	72°C	30 sec

For sequence analysis of *F3H_D* the PCR products were purified, ligated and cloned using p GEM®-T vector (Promega). After ligation recombinant p GEM®-T plasmid was transferred into cells of bacteria (electrocompetent cells - *E. coli*) using electroporation. SOC medium (Super-optimal broth with catabolite repression) was added to the samples and shaken 30 minutes. The samples were transferred under sterile conditions to Petri dishes with ampicillin (100 mg/ml) and components for blue/white selection (Xgal: 5-bromo-4-chloro-3-indolyl-β-D-galactosid and IPTG: Isopropyl-1-thio-

β-D-galactosid) to LB medium (Lysogeny broth). After blue-white screening, clones with recombinant plasmids were transferred into 1.5 ml of liquid LB medium and cultivated overnight at 37 °C [14]. PCR with these amplified plasmids was performed and PCR products were sent to Macrogen (Netherlands) for sequence analysis. After the analysis the sequences were evaluated using programme BLAST and Clustal W.

Results and Discussion

In the last years, studies of flavonoids attracted attention of many scientists [15]. Seeds of cereals are not typical sources of them; therefore relevant knowledge has been acquired also in maize, wheat, barley, oat, and rice as the most important world food sources. Colored-grain wheat varieties with good genetic stability, excellent stress resistance and high yield are still required [16].

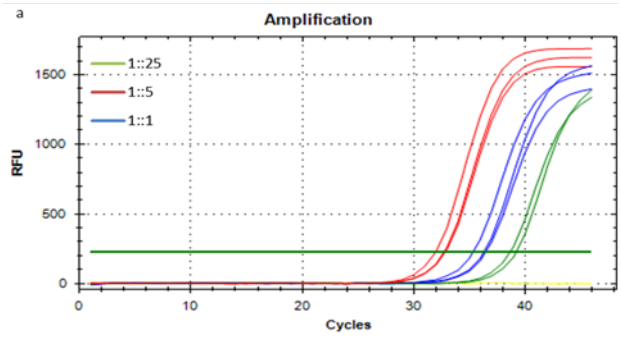
F3H genes were genetically mapped in wheat, barley and rye. Wheat contains three homoeologous copies of *F3H*: *F3H-A1*, *F3H-B1* and *F3H-D1* that are expressed in colored coleoptiles of different wheat genotypes [17]. Specific primers for sequences of the *F3H* genes (*F3H_A*, *F3H_B* and *F3H_D*) that were found in the National Center for Biotechnology Information (NCBI) database were designed. Primer efficiency test with primers for *F3H* genes and *GAPDH* (housekeeping gene: *glyceraldehyd-3-phosphate-dehydrogenase*) was performed. The cDNA samples were used in dilutions 1:1, 1:5 and 1:25. On the agarose gel we detected two or more products, because our *F3H* primers were not specific for studied genotypes. The best result was obtained for *F3H_D* gene with a PCR product approx. 240 bp and one longer. If for the qPCR reactions cDNA and genomic DNA were used, the same curves for all samples and the same size of the main PCR product (240 bp) were obtained. This is due to fact that designed primer for *F3H_D* gene was in the coding region that did not contain introns. For further work the *F3H_D* gene was chosen.

Himi et al. [3] designed *F3H-D* primers in their experiments that we tested on our genotypes (Table 2). The primer efficiency test with *F3H_D* and housekeeping gene (*GAPDH*) was repeated with the same cDNA dilutions (1:1, 1:5, 1:25). We obtained positive results. For all dilutions were done three repeats. In figure 2 the qPCR curves are highlighted. We detected for undiluted samples (1:1) and for dilution samples (1:5) three very similar curves. The size of PCR product was 229 bp. Only one run from three repeats was not successful with dilutions 1:25 with the same size of PCR product 229 bp. The flavonoids biosynthesis pathway was investigated and the most enzymes involved in the pathways of major flavonoids classes were determined, e.g. CHS, CHI, F3H, DFR, FLS, FNS and ANS [15].

Table 2 Sequences of F3H [3]

Name	sequences 5→3
F3H_F	CAA GAA GCA GGC CAA GGA C
F3HD_R	CTG CTA CAC ACG TAC GGA TAC C

Fig. 2 Primer efficiency test with F3H_D primer using cDNA mix



For the sequence analysis of the *F3H_D* gene suitable *F3H_D* sequences were obtained from PCR products by cloning. We cloned 5 genotypes with nonstandard colored caryopses (Abissinskaya arrasaita, ANK-28B, Novosibirskaya 67, UC66049 and Tschermaks Blaukörniger Sommerweizen). The sequences were 155 - 156 bp long and the variation in the number of bp was caused by insertions/deletions. The sequence analysis has shown that TBS has one, two, three and four nucleotide polymorphisms at the start of the sequence and ANK-28B has single nucleotide indel in the end of the sequence (Figure 3).

Comparing the sequences of putative proteins there were no differences among the genotypes AA, ANK-28B, N67 and UC66049 only the single nucleotide indel in sample ANK-28B caused protein shortening. One, two, three and four nucleotide polymorphisms in genotype TBS had different putative protein compared to the other genotypes. The similarity among all sequences of analyzed five genotypes and sequences obtained from NCBI was between 99 to 100%. The sequences of *F3H_D* gene were localized on the long arm of homoeologous chromosomes group 2 [18, 3]. *F3H* genes are critical for anthocyanin production in wheat coleoptiles. Within the *Triticeae* tribe it will be interesting to further investigate the extents to which are these

wheat regulatory genes specific in correlation with the resulting color [18].

Fig. 3 Comparison of the sequences of *F3H* genes with NBCI database using Clustal W

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CLUSTAL 2.1 multiple sequence alignment


NCBI            GCGCCCATGCCCACCGCCACCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
ANK             GCGCCCATGCCCACCGCCACCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
AA              GCGCCCATGCCCACCGCCACCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
N67             GCGCCCATGCCCACCGCCACCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
UC              GCGCCCATGCCCACCGCCACCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
TBS             ATAGTAC GGTGATCGCCGTCAAGTCTCTCAACGAAATTCTTGCCTAGATCATTCCGGCG 60
                **    **   ****** ****************************************


NCBI            GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
ANK             GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
AA              GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
N67             GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
UC              GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
TBS             GGGCGCGATTCAATATTTCAATTGATTAATGCGTGGGATTTGATTCTCCTAAGTACGAGA 120
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NCBI            TAAATTATGCATATGGTATCCGTACGTGTGTAGCAG 156
ANK             TAAATTATGCATATGGTATCCGTACGTGTGTAGC-- 155
AA              TAAATTATGCATATGGTATCCGTACGTGTGTAGCAG 156
N67             TAAATTATGCATATGGTATCCGTACGTGTGTAGCAG 156
UC              TAAATTATGCATATGGTATCCGTACGTGTGTAGCAG 156
TBS             TAAATTATGCATATGGTATCCGTACGTGTGTAGCAG 156
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Legend: NBCI- National Center for Biotechnology Information database, samples: ANK- ANK-28B, AA- Abissinskaya arrasaita, N67- Novosibirskaya 67, UC- UC66049, TBS- Tschermaks Blaukörniger Sommerweizen

Conclusion

The aim of the work was to test the primers for qPCR of *F3H* genes that will be used for studies of differently coloured wheat varieties and to obtaining the sequence of gene *F3H_D*. The first designed primer tests showed two or more products which was caused by non-specificity individual homoeologs of *F3H*. We obtained positive results from second qPCR with new *F3H_D* primers, where we obtained a single PCR product with the size 229 bp. We have used 5 genotypes of wheat with nonstandard coloured caryopses for qPCR and subsequently for sequence analysis the amplicons were cloned. The comparison of the *F3H_D* sequences with sequence from NCBI showed high degree of similarity (99-100%). The sequences have shown nucleotide polymorphisms in Tschermaks Blaukörniger Sommerweizen, which caused changes in the putative protein and one indel, which resulted in shortening of the protein.

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