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Allelic state of the genes conferring purple pigmentation in different wheat organs predetermines transcriptional activity of the anthocyanin biosynthesis structural genes

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ABSTRACT

In wheat, dominant alleles of the *Pc*, *Plb*, *Pls*, and *Pp* genes determine purple (red) colour of culm, leaf blades, leaf sheaths, and grain pericarp, respectively. These genes have been mapped in the wheat genome. However, their structural and functional organization remained unknown. We used near-isogenic lines differing by allelic state of the *Pc*, *Plb*, *Pls*, and *Pp* genes and compared transcriptional activity of the anthocyanin biosynthesis structural genes (*Chs*, *Chi*, *F3h*, *Dfr*, and *Ans*) in the studied organs. It was shown that alleles conferring strong pigmentation induced more intensive transcription of the structural genes, suggesting the genes *Pc*, *Plb*, *Pls*, and *Pp* to be transcriptional regulators in the anthocyanin biosynthesis network. Some species-specific peculiarities of anthocyanin biosynthesis structural gene regulation in wheat *Triticum aestivum* L. are discussed.

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1. Introduction

The anthocyanin biosynthesis pathway (ABP) is considered to be one of the best characterized plant secondary metabolite pathways at the biochemical and molecular-genetic levels in plant species (Winkel-Shirley, 2001). In the model plant species (maize, arabidopsis, snapdragon, and petunia), the identification and genetic studies of mutants impaired in anthocyanin accumulation in different organs allowed identification and then cloning of genes participating in the ABP (Mol et al., 1998). It has been shown that these genes encode enzymes participating in the biosynthesis (structural genes) and transcription factors that determine the temporal and spatial pattern of the structural gene expression (regulatory genes).

One of the major cereal crops, *Triticum aestivum* L. (genome BBAADD, $2n = 6x = 42$), can also have anthocyanin pigmentation on different organs. There is a renewed interest among breeders for high anthocyanin content in cereals, not only because of its role in plant defense (in all tissues; Gould, 2004), but also because of its beneficial effects on human health (in grain; Lila, 2004). By genetic analysis, it has been revealed that the loci *Rc*, *Pc*, *Plb*, *Pls*, *Pan*, and *Pp1*, determining anthocyanin pigmentation in the coleoptile, culm, leaf blades, leaf sheaths, anthers, and grain pericarp of wheat, respectively, clustered in homoeologous group 7 chromosomes (Khlestkina et al., 2008a) and the loci *Pg* and *Pp3*, determining anthocyanin pigmentation in glumes and pericarp, respectively, were closely linked in chromosome 2A (Khlestkina et al., 2010a). However, because of the large size and complex wheat genome organization (Wu et al., 2010), the nucleotide sequences of these genes have not been isolated yet and their function in ABP is not clear thus far. Comparative mapping data in grasses indicate that these loci may encode Myb- (genes on chromosome 7) and Myc-(genes on chromosome 2A) -like transcription activators, respectively (Khlestkina, 2012). But these assumptions are needed to be confirmed by comparative transcriptional analysis of the ABP genes in the genotypes differing by alleles at the *Rc*, *Pc*, *Plb*, *Pls*, *Pan*, *Pg*, and *Pp* loci. Earlier, such comparative analysis was performed using substitution, recombinant, or introgression lines, but for the *Rc* gene only (Himi et al., 2005; Khlestkina et al., 2008b, 2010b).

The most suitable genetic model to study gene functions in wheat is near-isogenic lines (NILs) differing by the allelic state of the genes of interest. In the current investigation, comparative transcriptional analysis of the five anthocyanin biosynthesis structural genes: *Chs* (chalcone synthase), *Chi* (chalcone-flavanone isomerase), *F3h* (flavanone 3-hydroxylase), *Dfr* (dihydroflavonol 4-reductase), and *Ans* (anthocyanidin synthase) was performed in contrastingly colored coleoptile, culm, leaf blades, leaf sheaths, and grain pericarp of NILs differing by the *Rc*, *Pc*, *Plb*, *Pls*, *Pp* alleles.

Abbreviations: ABP, anthocyanin biosynthesis pathway; *Ans*, anthocyanidin synthase; *Chi*, chalcone-flavanone isomerase; *Chs*, chalcone synthase; *Dfr*, dihydroflavonol 4-reductase; *F3h*, flavanone 3-hydroxylase; NIL, near-isogenic line.

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2. Materials and methods

2.1. Plant material

The wheat genotypes with contrasting anthocyanin pigmentation of grain pericarp (colored/uncolored) and varying in intensity in the vegetative organ coloration (weak/strong) were used: cultivar Saratovskaya 29 (S29) and NILs, i:S29Pp1Pp3^P (NIL(P)) and i:S29Pp1Pp2^{PF} (NIL(PF)), developed on S29 background (Arbuzova et al., 1998). S29 has a pericarp without anthocyanin pigment and weakly colored coleoptile, culm, leaf blades, and leaf sheaths, whereas NIL(P) and NIL(PF) have strong purple pigment on the organs listed, inherited from the donor cultivars Purple and Purple Feed, respectively. The lines have been characterized earlier using microsatellite markers (Tereshchenko et al., 2012), and genes determining differences in colour intensity have been identified (Table 1).

2.2. Anthocyanin extraction

Anthocyanins were extracted from different organs using 1%-HCl/methanol according to Khlestkina et al. (2011). The relative anthocyanin content was evaluated by a spectrophotometer SmartSpec™Plus (BioRad) at 530 nm as described previously (Christie et al., 1994). Anthocyanin extractions and measurements were performed in triplicate. The significance of differences between S29 and the NILs was assessed using non-parametric Mann–Whitney *U*-test (Mann and Whitney, 1947).

2.3. DNA/RNA extraction

DNA of S29, NIL(P), and NIL(PF) was available from a previous investigation (Tereshchenko et al., 2012) and used in the current study to optimize conditions of PCR. RNA from coleoptiles and roots (uncolored control) was extracted at the 5th day after seed germination from four seedlings grown in Rubarth Apparate (RUMED GmbH) at 20 °C under a 12 h day/12 h night regime. RNA from grain pericarp, culm, leaf blades, and leaf sheaths was extracted from plants grown in the greenhouse, upon appearance of anthocyanin pigment on the corresponding organ. For S29, RNA was extracted in 2 replicates. For RNA extraction, the QIAGEN Plant Rneasy Kit was used. Extracted RNA was treated by DNAse (QIAGEN RNase-Free DNase Set).

2.4. RT-PCR/PCR

Single-stranded cDNA was synthesized from 0.5 to 1 μg of total RNA using $(dT)_{15}$ and the QIAGEN Omniscript Reverse Transcription Kit in a 20 μl reaction mixture. RT-PCR was performed in two replicates with primers amplifying coding sequences of the genes *Ubc* (endogenous control), *Chs*, *Chi*, *F3h* (primer sequences published in Himi et al., 2005), *Dfr* (forward primer 5′atcgagg agcggcagcgg3′, reverse primer 5′cgtcgtgggaggagcaga3′), and *Ans* (forward primer 5′aagagggagtgggaggacta3′, reverse primer 5′cgagggagaggatggcga3′). Primers were designed by using OLIGO software (Offerman and Rychlik, 2003). PCR reaction mixtures (20 μl) contained 50 ng of DNA/cDNA template, 67 mM Tris–HCl, pH 8.8, 1.8 mM $MgCl_2$, 0.01% Tween 20, 18 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 0.25 μM of each primer and one unit of *Taq* DNA polymerase (Medigen, Novosibirsk). PCR amplifications with primer pairs designed in the current study were performed on an Eppendorf Mastercycler with the following profile: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2 min; 1 cycle at 72 °C for 10 min. PCR amplification with the other primer pairs was conducted using protocols offered by developers (Himi et al., 2005). PCR-fragments were separated by 1% agarose gel electrophoresis and photographed with Molecular Imager Gel Doc™ XR+ System (Bio-Rad).

3. Results

In addition to the dominant *Rc*, *Pc*, *Plb*, *Pls*, and recessive *Pp* alleles, inherited from S29, the NIL(P) and NIL(PF) carry dominant alleles of these genes, inherited from Purple and Purple Feed, respectively (Table 1), resulting in more intense anthocyanin pigmentation in the corresponding tissues of these lines in comparison with S29 (Fig. 1). Anthocyanin relative content (OD_{530} values of the extracts) measured in the wheat organs was found to be significantly lower in the coleoptile, culm, leaf blades, leaf sheaths, and pericarp of S29 than those in the organs of NIL(P) and NIL(PF) (Table 2, Fig. 1). It can be concluded that additional dominant alleles of the *Rc*, *Pc*, *Plb*, *Pls*, and *Pp* genes affect anthocyanin content in coleoptile, culm, leaf blades, leaf sheaths, and pericarp of the NILs, respectively (Table 2). Although anthocyanins are not synthesized in roots and in S29 pericarp, the very low OD_{530} values (about 0.1) are still detectable in these tissues, that can be explained by the small overlap in absorbance by anthocyanins and some uncolored substances at the 530 nm wavelength.

Comparative transcriptional analysis of the structural genes *Chs*, *Chi*, *F3h*, *Dfr*, and *Ans* was carried out in differently colored organs of S29, NIL(P), NIL(PF), and in uncolored roots as a control. Total RNA from roots, coleoptile, culm, leaf blades, leaf sheaths, and grain pericarp were analyzed by RT-PCR with the specific primers. The results obtained for the pair of lines S29/NIL(P) are presented in Fig. 1. The five structural ABP genes transcribed more intensively in the coleoptile, leaf blades, leaf sheaths, culm, and grain pericarp of NIL(P) in comparison with S29. Similar observations were made for the pair of lines S29/NIL(PF) (data are not presented). Thus, the differences in transcriptional level of the ABP structural genes were tightly related with the presence of the additional dominant alleles *Rc-D1*, *Plb-D1*, *Pls-D1*, *Pc-D1*, and *Pp* in the lines NIL(P) and NIL(PF) in comparison with S29 (Table 1). The obtained results suggest the allelic state of the genes conferring purple pigmentation in different wheat organs to predetermine level of transcriptional activity of the anthocyanin biosynthesis structural genes *Chs*, *Chi*, *F3h*, *Dfr*, and *Ans*.

It is worth noting that in uncolored tissues (roots in all lines and pericarp of S29), low transcriptional level of the genes *Chs*, *Chi*, *Dfr*, and *Ans* is still detectable, whereas *F3h* is transcribed neither in

Table 1
The dominant alleles of the genes determining purple coleoptile, culm, leaf blades, leaf sheaths, and grain pericarp in NILs and its parents (S29, Purple, Purple Feed), Tereshchenko et al., 2012.

Cultivar/line	Coleoptile	Culm	Leaf blade	Leaf sheath	Grain pericarp
S29	*Rc-A1*^{S29}	*Pc-A1*^{S29}	*Plb-A1*^{S29}	*Pls-A1*^{S29}	*pp1*, *pp3*
Purple	*Rc-D1*^{P}	*Pc-D1*^{P}	*Plb-D1*^{P}	*Pls-D1*^{P}	*Pp-D1*^{P}, *Pp3*^{P}
Purple Feed	*Rc-D1*^{PF}	*Pc-D1*^{PF}	*Plb-D1*^{PF}	*Pls-D1*^{PF}	*Pp-D1*^{PF}, *Pp3*^{PF}
NIL(P)	*Rc-A1*^{S29}, *Rc-D1*^{P}	*Pc-A1*^{S29}, *Pc-D1*^{P}	*Plb-A1*^{S29}, *Plb-D1*^{P}	*Pls-A1*^{S29}, *Pls-D1*^{P}	*Pp-D1*^{P}, *Pp3*^{P}
NIL(PF)	*Rc-A1*^{S29}, *Rc-D1*^{PF}	*Pc-A1*^{S29}, *Pc-D1*^{PF}	*Plb-A1*^{S29}, *Plb-D1*^{PF}	*Pls-A1*^{S29}, *Pls-D1*^{PF}	*Pp-D1*^{PF}, *Pp3*^{PF}

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