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The B-, G- and S-genomic *Chi* genes in family *Triticeae*

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Abstract

As result of a close evolutionary relationship between *Triticeae* B, G, and S genomes, the exchange of genetic material between them is possible and may be beneficial for broadening the genetic diversity of cultivated bread wheat. However, the extent to which regulatory networks are conserved remains poorly researched. Here, the structural organization and transcriptional activity of the B, S, and G genome copies of a gene encoding flavonoid biosynthesis enzyme chalcone-flavanone isomerase (CHI) were explored using introgression lines which differ from the wild type by carrying a non-bread wheat *Chi-1* gene. *Chi-S1*, *Chi-G1*, and *Chi-B1* all mapped to a comparable region of chromosomes 5S, 5G, and 5B, respectively. Nucleotide sequences of *Aegilops speltoides Chi-S1* and *Triticum timopheevii Chi-G1* were determined and compared with *T. aestivum Chi-B1* sequences. The enzymes encoded by these three genes shared the same predicted tertiary structure and active sites. However, the replacement of *Chi-B1* by *Chi-S1* or *Chi-G1* in a wheat background resulted in a significant decrease in the global amount of the *Chi-1* transcript present in the seedling shoot indicating divergence in regulation of expression of the orthologous *Chi-1* genes among *Triticeae* ssp.

Additional key words: *Aegilops speltoides*, chalcone-flavanone isomerase, flavonoid biosynthesis, gene cloning and mapping, *Triticum aestivum*, *Triticum timopheevii*, wheat genome evolution.

Introduction

The *Triticum* genus comprises four distinct sections: *Monococcum* Dum. (diploid einkorn wheat with genomes A^u and A^b), *Dicoccoides* Flaksb. (tetraploid emmer wheat with genome BA^u), the *Triticum* section (hexaploid wheat species with genome BA^uD), and the *Timopheevii* A. Filat. *et* Dorof. section (tetraploid and hexaploid wheat species with genomes GA^u and GA^uA^b, respectively) (Goncharov 2002). Both the B and G genomes are related to the S genome present in *Aegilops speltoides* (Kimber 1974). The close evolutionary relationship between the B, S, and G genomes allows for a relatively straightforward transfer of genetic information from one species to the other.

The S and G genomes harbor a reservoir of genetic diversity available for improvement of various cultivated *Triticum* spp. For example, genes conferring resistance to fungal diseases have been transferred from both *Triticum timopheevii* and *Ae. speltoides* into *T. aestivum* (Schneider *et al.* 2008, McIntosh *et al.* 2013). Overall, the exchange of genetic material between the B, S, and G genomes is beneficial for broadening the genetic diversity of cultivated bread wheat. However, the extent to which regulatory networks of these three genomes are conserved remains poorly researched. The *T. aestivum* lines with single introgressions from *T. timopheevii* and *Ae. speltoides* (Adonina *et al.* 2012, Timonova *et al.* 2013) represent a proper plant model to investigate this issue, and the flavonoid biosynthesis genetic network can provide a convenient model genes. Here, we investigate the structural organization and transcriptional activity of the B, S, and G genome copies of the flavonoid biosynthesis gene encoding the enzyme chalcone-flavanone isomerase (CHI; EC 5.5.1.6.). It catalyzes cyclization of chalcone into (*2S*)-naringenin participating

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Abbreviations: CHI - chalcone-flavanone isomerase; *Chi* - gene encoding chalcone-flavanone isomerase; K_a - number of non-synonymous substitutions; K_s - number of synonymous substitutions; RT-qPCR - reverse transcription quantitative polymerase chain reaction; *Ubc* - gene encoding ubiquitin.
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thereby in synthesis of nine major classes of flavonoids. Due to their antioxidant activity, osmoregulatory function, and metal ion-chelating ability, these compounds are involved in plant response to a broad spectrum of biotic and abiotic stresses (reviewed by Chalker-Scott 1999, Khlestkina 2013). In addition, the flavonoid biosynthesis genetic network is convenient model for studying genetic and epigenetic phenomena (Grotewold 2008), and this system has been also used for investigation of gene function in an alien genetic background (Khlestkina *et al.* 2012).

Materials and methods

Plant materials and PCR primers: The copies of *Chi* present in *Triticum timopheevii* Zhuk. var. *typica* (from the collection of the Institute of Cytology and Genetics, Novosibirsk, Russia), *Aegilops speltoides* Tauch. cv. AE 325 (from the collection of IPK-Gatersleben, Germany), and cv. TS01 (from the collection of the Weizmann Institute of Science, Rehovot, Israel) were PCR amplified using primer pairs Chi_1F/1R (Table 1 Suppl., Fig. 1 Suppl.), and TaChi_LP/RP (Himi *et al.* 2005).

The intrachromosomal positions of *Chi-S1*, *Chi-G1*, and *Chi-B1* were obtained by analysis of mapping populations of *Ae. speltoides* 25/*Ae. speltoides* 37 (Dobrovolskaya *et al.* 2011) and *Triticum aestivum* L. cv. Skala/Line 842 (*T. aestivum* cv. Saratovskaya 29 × *T. timopheevii*, Leonova *et al.* 2002) with primer pairs Chi_3F/3R and 4F/4R (Table 1 Suppl., Fig. 1 Suppl.). The DNA and cDNA of introgression lines *T. aestivum* cv. Saratovskaya 29 × *T. timopheevii* cv. 832-5B-BC3, carrying a single introgression in 5BL from 5GL of *T. timopheevii* (Timonova *et al.* 2013), and *T. aestivum* cv. Rodina × *Ae. speltoides* 5SL, having an introgression in 5BL from *Ae. speltoides* (Adonina *et al.* 2012), were analyzed using primer pairs Chi_3F/3R and 5F/5R, respectively (Table 1 Suppl., Fig. 1 Suppl.). The total *Chi* expression was assessed in roots and shoots of these introgression lines by reverse transcription quantitative PCR (RT-qPCR) using a primer pair Chi_6F/6R (Table 1 Suppl., Fig. 1 Suppl.). Quantification of the cDNA in RT-qPCR was obtained by amplification with ubiquitin (*Ubc*) as internal reference (Himi *et al.* 2005).

Extraction of DNA and RNA, and RT-qPCR: A DNA was extracted from fresh leaves of plants grown in the Institute of Cytology and Genetics greenhouse core facilities (Novosibirsk, Russia) following Plaschke *et al.* (1995). An RNA was extracted from roots and shoots of 4-d-old seedlings germinated in a climatized chamber (*RUMED*, www.rumed.de) at a temperature of 20 ºC and 12-h photoperiod (in three replicates) employing a *Zymo Research* plant RNA MiniPrep™ kit (www.zymoresearch.com). During RNA extraction, the RNA samples were treated with a *Qiagen* RNase-Free DNase set (www.qiagen.com) to remove a genomic DNA contamination. A single-stranded cDNA was then synthesized from 3 μg of the total RNA using a $(dT)_{15}$ primer and a *Fermentas RevertAid*™ first strand cDNA synthesis kit (www.thermoscientificbio.com/ fermentas/) in a 0.02 cm^3 reaction volume. A RT-qPCR was performed applying a *Syntol SYBR Green I* kit (http://www. syntol.ru). The amplifications were performed in an *ABI Prism 7000* sequence detection system (*Applied Biosystems*, www.lifetechnologies.com). Pre-determined amounts of the cloned cDNA were used to generate standard curves. Each sample was run in three replicates. Differences among lines were tested by Mann-Whitney's *U*-test taking $P \leq 0.05$ as significant.

Cloning, sequencing, and data analysis of PCR products: Each 0.05 cm^3 PCR sample contained 50 ng of template (the genomic DNA or cDNA), 67 mM Tris HCl, pH 8.8, 1.5 - 1.8 mM $MgCl_2$, 0.01 % (v/v) *Tween 20*, 18 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 0.25 μM of each primer, and 1 U of *Taq* DNA polymerase (Table 1 Suppl.). Two PCR regimes were used for different primer pairs (Table 1 Suppl.). Amplification program 1 was initiated with a denaturing step of 95 °C / 2 min, 45 cycles of 95 °C / 45 s, 60 °C / 1 min, 72 °C / 2 min, and a final extension of 72 °C / 10 min. Amplification program 2 was initiated with a denaturing step of 94 °C / 2 min followed by 13 cycles of 94 °C / 15 s, 65 °C / 30 s (decreasing by 0.7 °C / cycle), 72 °C / 45 s, 24 cycles of 94 °C / 15 s, 56 °C / 30 s, 72 °C / 45 s, and a final extension of 72 °C / 5 min. The amplicons were separated in a 2 % (m/v) agarose gel (*Low EEO* standard agarose, *Medigen*, www.medigen.ru). The amplicons to be sequenced were recovered from a 1 % (m/v) agarose gel, purified using a *Cytokine* DNA extraction kit (www.cytokine.ru), and sequenced in both directions or cloned with a *Qiagen* PCR cloning kit (www.qiagen.com) and then sequenced. In diploid *Ae. speltoides*, the amplified fragments were sequenced directly whereas in allotetraploid *T. timopheevii*, the PCR products were cloned, the clones carrying putative G-genomic *Chi* were selected using S-genomic *Chi* specific primers and sequenced. Sequencing was performed using an *ABI PRISM Dye* terminator cycle sequencing ready reaction kit (*Perkin Elmer*, www.perkinelmer.com). The sequenced samples were analyzed using resources of the *SB RAS* genomics core, Novosibirsk, Russia, http://www.niboch.nsc.ru/ doku.php/corefacility.

The gene structure was determined with the *FGENESH* + program (http://linux1.softberry.com/berry.phtml, Solovyev 2007).

Sequence clustering was performed using the *MEGA v. 5.1* software (Tamura *et al.* 2011) based on the neighbor-joining algorithm. The numbers of synonymous

(K_s) and non-synonymous (K_a) substitutions were estimated as suggested by Nei and Gojobori (1986). The *MULTALIN* software (http://www-archbac.u-psud.fr/genomics/multalin.html, Corpet 1988) was used to obtain multiple sequence alignments. The 3D structures of CHIs were predicted using the *SWISS-MODEL* program (http://swissmodel.expasy.org, Arnold *et al.* 2006).

Linkage maps were constructed with *MAPMAKER v. 2.0* (Lander *et al.* 1987) using the Kosambi mapping function (Kosambi 1944).

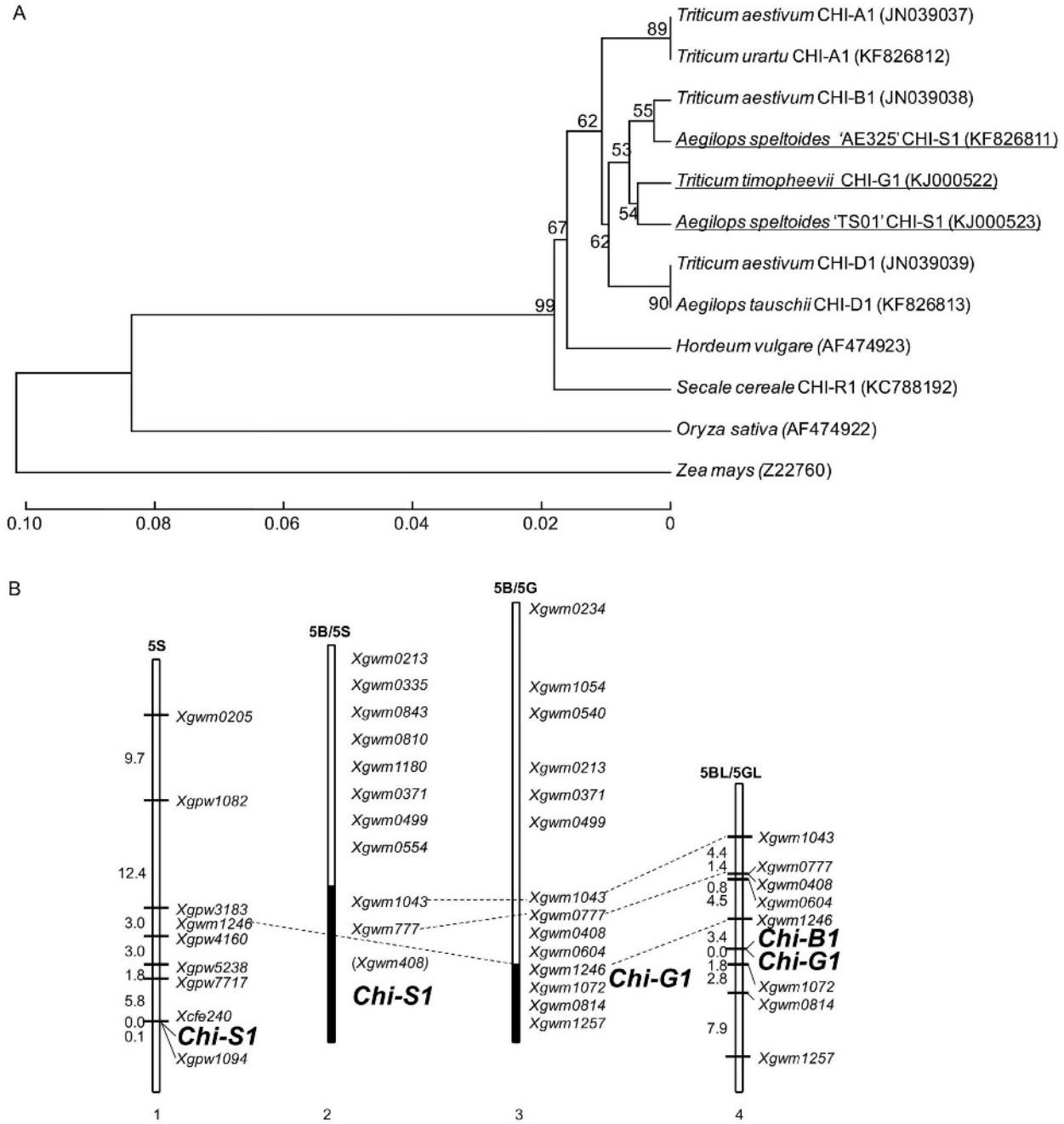

Fig. 1. *A* - Phylogeny of chalcone-flavanone isomerase (CHI) polypeptides. Those determined in the current study are *underlined*. Relevant GenBank accession numbers are given to the right. The numbers at each node represent a bootstrap value as percentage of 1 000 replicates. The genetic distance is displayed under the tree. *B* - Genetic (schemes 1 and 4) and physical (schemes 2 and 3) mapping *Chi-1* genes. Intrachromosomal positions of *Chi-S1*, *Chi-G1*, and *Chi-B1* were obtained from genotypic data acquired in the current study. The microsatellite loci *Xgwm*, *Xgpw*, and *Xcfe* were placed according to Dobrovolskaya *et al.* (2011), Adonina *et al.* (2012), Leonova *et al.* (2002 and unpublished), and Timonova *et al.* (2013). Introgression fragments of alien genetic material in bread wheat chromosome 5B are shown in *black* (schemes 2 and 3). The genetic distances are given in centimorgans to the left of each linkage group.

Results

Full length nucleotide sequences of the *T. timopheevii* and *Ae. speltoides* genomic copies of *Chi* were obtained (deposited in GenBank as accession numbers KF826811, KJ000522, and KJ000523). The level of identity among the coding *Chi-S1*, *Chi-G1*, and *Chi-B1* sequences was 98 %. Comparison of the B, G, and S *Chi* sequences revealed 28 exonic and 43 intronic along with 9 indels with lengths between 1 and 6 bp (Fig. 1 Suppl.). These polymorphisms translated into six polymorphisms at the peptide level (Fig. 2 Suppl.). The ratio of the number of non-synonymous substitutions and the number of synonymous substitutions (K_a/K_s) ranged from 0.07 (*Chi-S1* of cv. TS01 *vs. Chi-S1* of cv. AE325) to 0.24 (*Chi-G1 vs. Chi-S1* of cv. AE325). Divergence among the deduced CHI-S1, CHI-G1 and CHI-B1 protein sequences was in a range of 1 to 2 %. They shared about a 50 % identity with *Medicago sativa* CHI (standard CHI with a known crystal structure; Jez *et al.* 2000). Although some amino acid substitutions among CHI-S1, CHI-G1, and CHI-B1were noted (Fig. 2 Suppl.), each of the predicted proteins included all residues known to be required for proper folding and functional activity (Fig. 2 Suppl.), and their three dimensional conformation, constituted by α-helices and β-strands (Fig. 3 Suppl.), was indistinguishable from that of *Medicago sativa* CHI (Jez *et al.* 2000). These data allow suggesting that all studied CHI enzymes were functional.

The phylogeny of the deduced CHI sequences is shown in Fig. 1*A*. The *Triticum* and *Aegilops* spp. copies formed two sub-clusters, one comprising the A genome, and the other the B/G/S/D genome copies.

To demonstrate the chromosomal location of *Chi-G1* and *Chi-S1* in the introgression lines developed in the bread wheat cvs. Saratovskaya 29 or Rodina, PCR assay based on primer pairs Chi_3F/3R and 5F/5R (Table 1 Suppl.) was performed. The successful amplification of the expected fragment in the introgression lines Saratovskaya 29 × *T. timopheevii* cv. 832-5B-BC3 and cv. Rodina × *Ae. speltoides* 5SL but not in original either cv. Rodina or cv. Saratovskaya 29 (Fig. 2*A*) shows that the donor *Chi* genes were transferred into the two lines along with introgression fragments localized distally

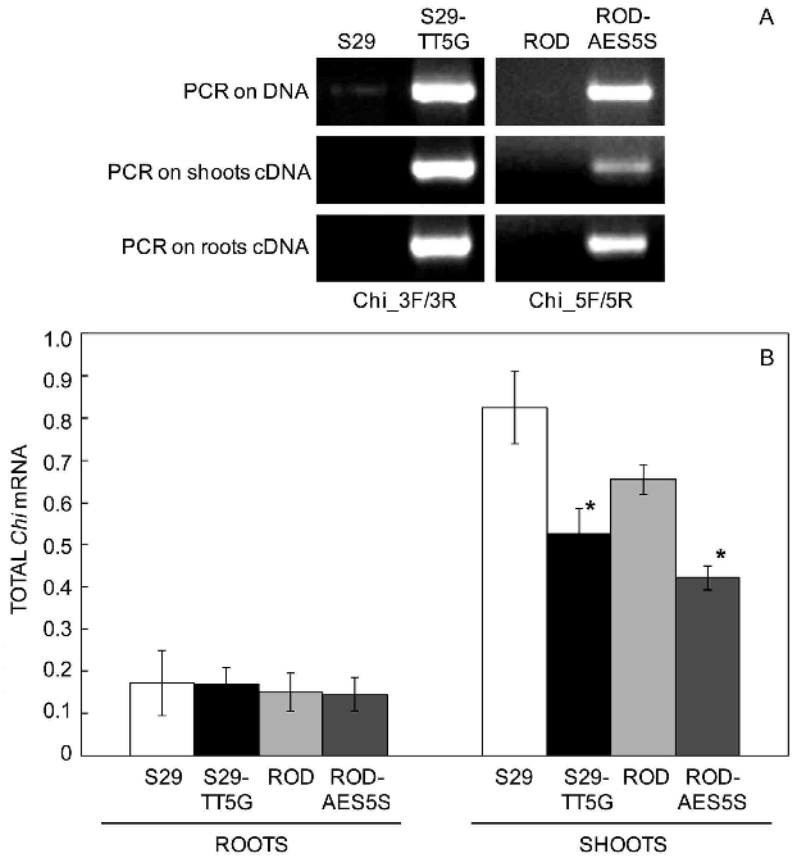

Fig. 2. *A* - Polymerase chain reaction (PCR) and RT-PCR profiles of introgression lines Saratovskaya 29 × *T. timopheevii* cv. 832-5B-BC3 (S29 × TT5G), cv. Rodina × *Ae. speltoides* 5SL (ROD × AES5S), and their parental wheat cvs. Saratovskaya 29 (S29) and Rodina (ROD) obtained using primer pairs Chi_3F/3R and Chi_5F/5R. *B* - *Chi-1* transcript abundance in seedling roots and shoots of S29 × TT5G, ROD × AES5S, and their parental wheat cultivars as assessed by RT-quantitative PCR using a primer pair Chi_6F/6R. Means ± SE ($n = 3$). * indicates statistically significant differences at $P \leq 0.05$.

to microsatellite loci *Xgwm 1246* and *Xgwm 1043* (Fig. 1*B*). The chromosomal location of *Chi-S1* in *Ae. speltoides* was shown by linkage analysis to be close to a microsatellite locus *Xcfe240* (Fig. 1*B*). *Chi-B1* and *Chi-G1* were mapped in an F_2 population bred from crossing cv. Skala × (cv. Saratovskaya 29 × *T. timopheevii* 842). The two genes behaved as alleles and mapped between microsatellite loci *Xgwm1246* and *Xgwm1072* (Fig. 1*B*).

Analysis by RT-PCR based on primer pairs Chi_3F/3R and 5F/5R was used to establish in which plant organs the *Chi-1* homologs had been transcribed (Fig. 2*A*). This experiment shows that *Chi-G1* and *Chi-S1* were both active in the root and shoot of 4-d-old seedlings of the introgression lines cv. Saratovskaya 29 × *T. timopheevii* cv. 832-5B-BC3 and cv. Rodina × *Ae. speltoides* 5SL (Fig. 2*A*). The effect of replacing *Chi-B1* by either *Chi-G1* or *Chi-S1* on *Chi-1* transcript abundance was assessed using RT-qPCR based on a primer pair Chi_6F/6R (Table 1 Suppl; this pair amplifies *Chi-A1*, *-B1*, *-D1*, *-G1*, and *-S1*). In the root, global *Chi* transcript abundance was uniform in the two cultivars and the two introgression lines, but in the shoot, there was a significant decrease in abundance in the introgression lines (Fig. 2*B*).

Discussion

Two introgression lines bread wheat × *T. timopheevii* and bread wheat × *Ae. speltoides* obtained by Adonina *et al.* (2012) and Timonova *et al.* (2013) carried the donor copy of *Chi-1* (Fig. 2*A*). These copies mapped within their respective genomes to a comparable region of the group 5 homoeolog (Fig. 2), as do the three endogenous bread wheat *Chi-1* copies (Shoeva *et al.* 2014), and *Chi-D1* in *Ae. tauschii* (Li *et al.* 1999). A homolog is also present in cereal rye (Khlestkina *et al.* 2009) and maps to an expected chromosome arm (Khlestkina and Shoeva 2014) as does the barley gene *Chi-H1* (Druka *et al.* 2003). Also rice and maize homologs each maps to a region syntenic with barley chromosome 5HL (Druka *et al.* 2003). The phylogeny of the cereal CHI sequences (Fig. 1*A*) is consistent with a standard taxonomic treatment (Dorofeev and Korovina 1979, reviewed by Feldman 2001). The B, G, and S genome CHI polypeptide sequences are closely related to one another. The DNA/DNA hybridization behavior of certain sequences has suggested that the B and G genomes are related to different *Ae. speltoides* accessions, in particular highlighting the similarity of the G genome and the cv. TS01 S genome (Khlestkina and Salina 2001). The same conclusion was drawn in the current study (Fig. 1*A*) and also previously on the basis of microsatellite alleles (Adonina 2007) and *F3h* gene sequences (Khlestkina *et al.* 2008). The relation of the B and G genomes to different *Ae. speltoides* accessions is in line with other evidences that hybridization events producing the BA^u and GA^u tetraploids have occurred independently in both time and space (Mori *et al.* 1995, Kilian *et al.* 2007).

The enzymes encoded by *Chi-S1*, *Chi-G1*, and *Chi-B1* shared the same predicted tertiary structure (Fig. 3 Suppl.) and active sites (Fig. 2 Suppl.). However, the global amount of the *Chi-1* transcript present in the seedling shoot was noticeably lower in the introgression lines than in their respective wheat parent (Fig. 2*B*). The same was not the case in the root although this may simply reflect a smaller contribution made by *Chi-B1* to the overall content of *Chi-1* transcript compared to that made by both *Chi-A1* and *Chi-D1* (Shoeva *et al.* 2014). A decreased expression of the total *Chi-1* in the introgression lines demonstrates that in the wheat background, the expression efficiency of the alien *Chi-G1* or *Chi-S1* was lower than that of the replaced *Chi-B1* gene copies. Thus, some differences in regulation of *Chi-1* gene expression among *Triticeae* ssp. exist, which can be related with divergence between *cis*- or *trans*-regulatory elements of the donor and recipient species. A similar conclusion has been made studying expression of wheat and rye *F3h-1* gene copies (a flavonoid biosynthesis gene encoding flavanone 3-hydroxylase) in a wheat × rye chromosome substitution line. In this case, expression of the alien rye *F3h-R1* gene in the wheat background was reduced significantly in comparison with the wheat orthologs as well as with *F3h-R1* gene expression in the rye donor cultivar (Khlestkina *et al.* 2009). The results obtained on the model genes at the transcriptional level (Fig. 2*B*; Khlestkina *et al.* 2009, 2012) and the observations made at phenotypic, physiological, or biochemical levels (Gustafson and Sears 1993) suggest that a careful parental selection is essential when making wide-crosses because expression of new gene complexes in the wheat background cannot be guaranteed.

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