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The homoeologous genes encoding C24-sterol methyltransferase 1 in *Triticum aestivum*: structural characteristics and effects of cold stress

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Abstract

A unique structural feature of plant sterols is the presence of a 24-alkyl group in the sterol side chain, which is synthesized by C24-sterol methyltransferase (SMT). Here we report for the first time that the bread wheat genome (AABBDD) contains at least three homoeologous genes encoding C24-sterol methyltransferase 1. While these copies have similar coding regions, they differ markedly in the nucleotide sequences of their non-coding regions. Sequencing *de novo* of the promoter regions of the *TaSMT1* homoeologs demonstrated the occurrence of common and specific stress-sensitive *cis*-elements such as LTR, the *cis*-element involved in low temperature response. These *cis*-elements, along with other factors, determine the differences in the effects of stress on the expression of homoeologous *TaSMT1* genes. For example, *TaSMT1-5A* is constitutively expressed in the roots and leaves, while *TaSMT1-4D* gene is highly stress-responsive. Another important enzyme involved in sterol biosynthesis is C22-sterol desaturase, which converts β -sitosterol into stigmasterol. This enzyme is encoded by homoeologous *TaCYP710A8* genes, which, in contrast to *TaSMT1*, are all up-regulated in response to stress. Cold-induced expression of *TaCYP710A8* is greater in roots than in leaves. This may be due to the higher cold sensitivity of the roots and the necessity to increase the amount of stigmasterol known as a “stress sterol”. Our findings suggest that the existence of homoeologous genes of sterol biosynthesis in polyploid plants supports the diversity of genetic mechanisms of sterol-mediated response of plants to stresses.

Additional key words: gene expression, C22-sterol desaturase, *cis*-elements.

Introduction

Sterols are essential structural elements of membranes and also components of signaling pathways, which are involved in the regulation of plant growth and development, and the responses of plants to stresses (Benveniste 2004). Plant membranes contain a complex mixture of sterols with β -sitosterol, stigmasterol, and campesterol as predominant species. Manipulation of sterols by binding and depletion greatly affects not only membrane permeability but also other biochemical and physiological characteristics in plants (Roche *et al.* 2008, Bonneau *et al.* 2010, Valitova *et al.* 2011, 2014). Sterols are isoprenoid derived lipids with a fused four-ring core structure and an aliphatic side chain (Benveniste 2004). A

unique structural feature of plant sterols is the presence of a 24-alkyl group in this side chain, the synthesis of which is catalyzed by S-adenosyl-L-Met-dependent C24-sterol methyltransferases (SMT). Alkylation happens in two steps where primary methylation is catalyzed by SMT1 (EC 2.1.1.142) resulting in the formation of methyl sterols (campesterol), and secondary methylation is catalyzed by SMT2 (EC 2.1.1.143) resulting in the formation of ethyl sterols (β -sitosterol, stigmasterol).

The SMT is an integral protein localized in endoplasmic reticulum (Boutté and Grebe 2009). SMTs contain several conserved domains such as three sterol binding sites (Bouvier-Navé *et al.* 1997, Nes 2000) and

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Abbreviations: CDS - coding DNA sequences; LTR - low-temperature responsiveness; SMT - C24-sterol methyltransferase; qPCR - quantitative polymerase chain reaction; UTR - untranslated region.

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S-adenosylmethionine binding site (Kagan and Clarke 1994) required for proper folding and functional activity (Nes *et al.* 2002). SMTs are present in plants, fungi, and protozoa but is absent in mammals (Nes 2000). Various plant species possess a diversity of *SMT* genes. For example, SMTs are encoded by three genes in *Arabidopsis thaliana*, three genes in *Glycine max*, four genes in *Nicotina tabacum*, and three genes in *Oryza sativa* (Bouvier-Navé *et al.* 1997, Schaller *et al.* 1998, Neelakandan *et al.* 2009, 2010, Carland *et al.* 2010). Some of these *SMT* genes have been cloned and their role in the regulation of 24-alkylsterol biosynthesis in plants has been characterized (Schaeffer *et al.* 2001, Neelakandan *et al.* 2009, Carland *et al.* 2010, Nakamoto *et al.* 2015).

Higher plants synthesize 24-methyl sterols and 24-ethyl sterols in defined proportions. Interestingly, in the *Arabidopsis smt2* null mutant, the enzyme SMT1 can perform both primary and secondary methylation reactions to appropriate sterol substrates, although with different kinetics (Diener *et al.* 2000). Studies using mutants revealed that SMT plays roles in cell division, expansion, polarity and proliferation, embryogenesis, vein, shoot and root patterning, gravitropism, fertility, and hormone signaling (Diener *et al.* 2000, Schaeffer *et al.* 2001, Carland *et al.* 2002, Willemsen *et al.* 2003, Souter *et al.* 2004, Men *et al.* 2008, Nakamoto *et al.* 2015). Several studies have demonstrated that plant sterols, particularly β -sitosterol, possess antioxidant activity (Vivancos and Moreno 2005, Li *et al.* 2007, Paniagua-Pérez *et al.* 2008, Posé *et al.* 2009). *Arabidopsis* mutants with an up-regulated expression of *SMT* genes and an increased content of β -sitosterol showed higher resistance to oxidative stress compared to the wild type (Wang *et al.* 2012). Thus, SMT is a key enzyme in plant sterol biosynthesis, whose functioning provides the optimal balance of molecular species of sterols during plant ontogenesis and stress response.

In *Triticum aestivum*, a single gene encoding SMT1 has been annotated so far (GenBank: U60755.1; Subramaniam *et al.* 1999). Information about sequences and structure of other *SMT* genes in *T. aestivum* is still

unknown. Wheat is probably the most important crop in the world, yet it has one of the most challenging genomes. Bread wheat is an allohexaploid consisting of seven groups of chromosomes, each group containing a set of three homoeologous chromosomes belonging to the A, B, and D genomes, derived from a common ancestor (Marcussen *et al.* 2014). Thus, three representatives of each single copy of a gene are present in the wheat genome. They are referred to as homoeologous genes. The large size and intricate organization of the wheat genome hamper both the genome-wide sequencing and gene annotation.

Another enzyme involved in sterol biosynthesis is C22-sterol desaturase, or CYP710A8 from the plant cytochrome P₄₅₀ subfamily, which converts β -sitosterol into stigmasterol (Morikawa *et al.* 2006, Arnqvist *et al.* 2008). Stigmasterol in most higher plants and ergosterol in most fungi and some algae have a double bond at C22. This double bond is formed by a desaturation reaction, which is the final stage of plant sterol biosynthesis (Ohta and Mizutani 2013). Earlier, full-length cDNAs of wheat CYP710A8 genes were isolated and mapped on chromosome 3A, 3B, and 3D (Tang *et al.* 2011).

We hypothesize the existence of homoeologous *SMT1* genes in polyploid wheat and suggest that they are involved in a variety of sterol-mediated responses to stresses. Therefore, the aim of the present study was to sequence and analyze the structure and expression of homoeologous *TaSMT1* genes. For this purpose, we firstly determined the DNA sequence of the three homoeologous *TaSMT1* genes in wheat. The structures of the gene regulatory and coding regions were analyzed. Secondly, we studied the effect of cold stress on the expression of *TaSMT1* and *TaCYP710A8* genes in the roots and leaves of wheat seedlings. Earlier it was shown that cold stress can induce the overexpression of a number of enzymes of sterol biosynthesis in cereals, which increases the amount of membrane sterols (Uemura and Steponkus 1994). Thirdly, the upstream regions of the homoeologous *TaSMT1* genes were sequenced and potential stress regulatory motifs were analyzed *in silico*.

Materials and methods

Plants and cultivation: Wheat (*Triticum aestivum* L. cv. Kazanskaya Jubileinaya; *Niva Tatarstana*, Kazan, Russia) seedlings were grown hydroponically in 0.25 mM CaCl₂ solution in a growth chamber at a temperature of 25 °C., a 12-h photoperiod, and an irradiance of 460 μ mol m⁻² s⁻¹ for 5 d. To induce cold stress, 5-d-old seedlings were transferred to 4 °C for 1 and 12 h.

Isolation of plant DNA and molecular cloning of target gene fragments: Genomic DNA from *T. aestivum* seedlings was isolated using cetyltrimethyl ammonium bromide (CTAB) buffer. DNA concentration and quality

was evaluated spectrophotometrically using *NanoDrop ND-1000* spectrophotometer (*Thermo Scientific*, Waltham, USA) and 1 % (m/v) agarose gel electrophoresis. For the amplification of the fragments of homoeologous *TaSMT1* genes, primers were constructed on the conserved regions of *TaSMT1*. To amplify promoter regions of homoeologous *TaSMT1*, gene-specific primers were designed based on their sequencing data from *IWGSC* database. Forward and reverse primers are listed in Table 1 Suppl. PCR was performed by means of *C1000 Touch™* thermal cycler (*Bio-Rad*, Hercules, USA) using genomic DNA, primers and polymerases *Maxima Hot Start Taq*

DNA (*Thermo Scientific*) and GoTaq Flexi DNA (*Promega*, Madison, USA).

For promoter sequencing, the PCR was conducted with a *Genome Walker* universal kit (*Clontech*, Mountain View, USA) using the genomic DNA as template following the manufacturer instructions. Genomic DNA from *T. aestivum* was digested with four blunt end cutting enzymes (DraI, EcoRV, PvuII, StuI) to construct the DNA libraries. DNA fragments were further purified and ligated to the *Genome Walker* adaptor provided in the kit. A primary PCR was performed with the outer adaptor primer (AP1) and the corresponding outer *TaSMT1*-gene specific primer to walk in the 5' end direction (GSP1). The product of this primary PCR was then diluted (1:50) and used as a template for a second walk using the nested adaptor primer (AP2) and one of the *TaSMT1*-gene-specific primers (GSP2). Using the GSP2 primer, the purified PCR products were then sequenced. The gene-specific oligo sequences used in PCR amplification are as follows: GSP1: 5'-CGGTGTAGTTGGATTTCCTCGATTCC TCC-3'; GSP2: 5'-GCAACAGTACAGGGAATGAAG ACACTCGGC-3'.

PCR products were purified using *AxyPrep*TM PCR cleanup kit (*Axygen Biosciences*, Union City, USA) and cloned into the *pGEM*[®]-*T Easy* vector system (*Promega*) according to the manufacturer protocol. Nucleotide sequences were determined using an automatic *ABI 3130* genetic analyzer (*Applied Biosystems*, Foster City, USA) using *Big Dye Terminator v.3.1* cycle sequencing kit (*Applied Biosystems*).

Analysis of gene expression by real-time quantitative PCR: Total RNA was isolated from wheat using *RNeasy Plant Mini* kit (*Qiagen*, Hilden, Germany) according to the manufacturer protocols. RNA concentration and purity were assessed using *NanoDrop*[®] ND-1000 spectrophotometer and the integrity was verified by 1 % agarose gel electrophoresis. The reverse transcription (RT) reaction was performed using *C1000 Touch*TM thermal cycler with reverse transcriptase *RevertAid RT* (*Thermo Scientific*) and Oligo(dT)₁₆ primers (*Sintol*, Moscow, Russia) according to the manufacturer protocol.

Real-time qPCR was performed using an *ICycler IQ 2 Multicolor* real-time PCR detection system (*Bio-Rad*). The templates were amplified three times at 95 °C for 3 min

followed by 40 cycles of amplification (94 °C for 10 s and 55/60 °C for 40 s). ADP-ribosylation factor (*TaARF*) and RNase L inhibitor-like protein (*TaRLI*) genes were used as reference genes (Paolacci *et al.* 2009). The gene-specific primers and *TaqMan* probes used for real-time qPCR are listed in Table 1 Suppl. For *TaCYP710A8* genes, previously published primers were used (Tang *et al.* 2011). Quantification was performed according to Pfaffl (2001).

Bioinformatic analysis: Nucleotide sequences and chromosomal localization of the *TaSMT1* genes were determined using the *BLAST* facility of the international wheat genome sequencing consortium database (*IWGSC*) (<http://www.wheatgenome.org/>) hosted by *URGI* server (<http://urgi.versailles.inra.fr>). The complete sequences of A, B and D *TaSMT1* genes of hexaploid wheat were obtained following the initial query during the survey of the coding DNA sequences (CDS) of *T. aestivum* Δ-24-sterol methyltransferase mRNA (*NCBI* accession U60754; *GenBank*: U60754.1). Search and comparison of homologous sequences was performed using the *BLAST* program at the *NCBI* server. Design of gene-specific primers, translation of CDS, sequence alignment, and estimation of molecular mass of predicted protein was performed using the program *Vector NTI Suite 9* (*Invitrogen*, Carlsbad, USA). Exon-intron structures of *TaSMT1*-5A, *TaSMT1*-4B, and *TaSMT1*-4D genes were predicted by using online mRNA-to-genomic alignment program *Spidey* (<http://www.ncbi.nlm.nih.gov/spidey/>). The phylogenetic tree of selected *SMTs* was constructed by the bootstrap test (1 000 replicates) using *MEGA7* software.

The promoter sequences were analyzed *in silico* for the presence of potential *cis*-acting elements using *PLANTCARE* (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot *et al.* 2002) and *PLACE* databases (Higo *et al.* 1999). The database of CpG islands and analytical tool (*DBCAT*) (<http://dbcat.cgm.ntu.edu.tw/>) were applied to recognize comprehensive methylation profiles of DNA alteration in the promoter sequences.

Statistics: All experiments were performed with at least three biological and four analytical replicates. Data presented are means and standard errors. Statistical analysis was performed using the two-tailed Student's *t*-test in *Microsoft Excel 2013*.

Results

The full-length coding regions of three homoeologous genes of wheat *SMT1* were found using *URGI* database. These genes are located on the long arms of 5AL, 4BL, and 4DL chromosomes and were named as *TaSMT1*-5A, *TaSMT1*-4B and *TaSMT1*-4D, respectively. The nucleotide sequences of *TaSMT1* homoeologous genes were confirmed experimentally by several rounds of amplifications and cloning with specific pair of primers.

Multiple alignments of *TaSMT1* clones suggested the presence of several products in the PCR amplification mix. The sequenced fragments corresponded to the putative sequences from the *URGI* database from different wheat genomes (A, B, and D). A comparison analysis of the genomic sequences of the three *TaSMT1* genes with their corresponding cDNA sequences showed that these genes are characterized by structural similarities and consist of

twelve (*TaSMT1-5A*), ten (*TaSMT1-4B*), and eleven (*TaSMT1-4D*) exons, with all the splicing sites complying with the GT–AG rule (Fig. 1A). Analysis allowed us to identify four conserved exons with identical sequences and sizes among three homoeologous *SMT1* genes from wheat and *SMT1* genes from other plants (Fig. 1A). Comparative analysis of the CDS of the three *TaSMT1* genes confirmed that they had a high degree of similarity: 96.5 %

(*TaSMT1-5A* vs *TaSMT1-4B*), 97.7 % (*TaSMT1-5A* vs *TaSMT1-4D*), and 97.6 % (*TaSMT1-4B* vs *TaSMT1-4D*) (Table 1). Despite these similarities in CDS, there are small differences due to insertions/deletions and single nucleotide polymorphisms (Fig. 1B). Differences in the sizes of the intron sequences among three homoeologous *SMT1* genes in wheat were also observed (Fig. 1A).

Fig. 1. A - Schematic representation of the structure of three homeologous *TaSMT1* genes of wheat. The rectangles show exons, the lines show introns, and the arrows show the conserved exons (<http://www.ncbi.nlm.nih.gov/spidey/>). At the 5'- and 3' ends of the splicing site, the introns have conserved base pairs (GT ... AG). B - Alignments of the coding DNA sequence (CDS) of three homeologous *TaSMT1* genes using *Vector NTI* program.

Table 1. Identity of *TaSMT1* sequences.

Alignment	Identity [%] <i>TaSMT1-5A/4B</i>	<i>TaSMT1-5A/4D</i>	<i>TaSMT1-4B/4D</i>
Genomic DNA sequence	54.7	74.3	70.5
CDS	96.5	97.7	97.6
Promoter sequence	32.6	37.3	81.1

Fig. 2. Phylogenetic tree of the CDS of *SMT* gene family from various plant species constructed by the bootstrap test using *MEGA7* software. The numbers indicate confidence values.

A phylogenetic tree was constructed on the basis of the analysis of CDS of *SMT1* genes in wheat and other plant species. The *TaSMT1* sequences of wheat, as expected, were most similar to *SMT1* of cereals such as *Aegilops* and barley (Fig. 2). Furthermore, comparison of the full-length deduced amino acid sequences of mature TaSMT1 protein from wheat with those of protein sequences of other SMT1 demonstrated that these sequences were highly conserved among the other plant species and yeasts (Table 2 Suppl.). TaSMT1 shared the highest similarity with SMT1 from barley, *Brachypodium*, and *Aegilops* with similarities of 98, 96, and 94 %, respectively, and the least similarity with SMT1 from *Saccharomyces cerevisiae*.

SMTs contained several conserved domains such as three sterol binding sites and S-adenosyl methionine binding site required for proper folding and functional enzyme activity (Fig. 3). Important amino acid residues Glu₈₂, His₉₀, Asp₁₂₅, Glu/Asp₁₅₂, Glu₁₉₅, Glu₂₂₄, Asp₂₇₆, which are directly involved in the binding sterols and S-adenosyl methionine to the enzyme *via* hydrogen bonds (Nes *et al.* 2004) were found in TaSMT1.

In order to characterize the expression of different copies of the *TaSMT1* gene, we estimated the relative transcriptions of *TaSMT1*-5A, *TaSMT1*-4B, and *TaSMT1*-4D genes in the roots and leaves of control wheat seedlings and seedlings stressed with a low temperature (4 °C). PCR analysis followed by electrophoretic DNA

separation in agarose gel revealed the presence of *TaSMT1*-5A and *TaSMT1*-4D transcripts in control plants both in the roots and leaves. Unfortunately, we were unable to quantify the *TaSMT1*-4B transcript in the roots or the leaves, probably because *TaSMT1*-4B was silent or inactive. Expression of *TaSMT1*-5A was higher in the leaves (Fig. 4A), whereas the *TaSMT1*-4D gene was more strongly expressed in the roots (Fig. 4B). In addition, the expression of the *TaSMT1*-5A gene in roots and leaves at 4 °C remained practically unchanged (Fig. 4A). Conversely, cold increased the expression of the *TaSMT1*-4D gene in both the roots and leaves, with the greatest (4-fold) changes observed in roots exposed to cold for 12 h.

The effect of cold stress on the expression of homoeologous genes encoding C22-sterol desaturase, or CYP710A8, was analyzed in the roots and leaves of wheat seedlings. Using specific primers for *TaCYP710A8*-3A and common primers for *TaCYP710A8*-3B and *TaCYP710A8*-3D homoeologous genes, which share 97.8 % identity, we estimated the expression of *TaCYP710A8* in control plants and also after short-term (1 h) and long-term (12 h) exposure to 4 °C (Fig. 5). Analysis of gene expression demonstrated co-expression of *CYP710A8* homoeologous genes in the roots and leaves. In the roots of cold-treated wheat seedlings, the expression of all genes steadily increased (Fig. 5). Conversely, in the leaves of cold-treated seedlings, the expression profile of

TaCYP710A8 genes was characterized by initial up-regulation followed by down-regulation after longer

exposure to cold (Fig. 5).

Fig. 3. Alignments of the deduced amino acid sequences of the SMT1 using *Vector NTI* program. The rectangles show conserved domains of SMT1: I, III, and IV are sterol binding sites; II – AdoMet binding site. The conserved amino acid residues, which are directly involved in the binding sterols and adenosyl methionine to the enzyme *via* hydrogen bonds: Glu₈₂, His₉₀, Asp₁₂₅, Glu/Asp₁₅₂, Glu₁₉₅, Glu₂₂₄, and Asp₂₇₆ are indicated with arrows.

Fig. 4. Relative expressions of *TaSMT1-5A* (A) and *TaSMT1-4D* (B) genes assessed with real-time qPCR in wheat roots and leaves treated with cold stress. One, two, or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively (two-tailed Student's *t*-test; $n = 3 - 6$).

Differential expression of wheat homoeologous genes *TaSMT1-5A* and *TaSMT1-4D* in response to cold treatment may depend on the presence of specific sequences in the promoter structure. Therefore, the 1 - 1.5 kb upstream regions to the ATG initiation codon of *TaSMT1-5A*, *TaSMT1-4B*, and *TaSMT1-4D* were sequenced. The alignment of promoter sequences of *TaSMT1* homoeologues revealed differences in the

upstream regions of these genes. An alignment using *Vector* software showed that the homology in upstream regions were as following: 32.6 % (*TaSMT1-5A* vs *TaSMT1-4B*), 37.3 % (*TaSMT1-5A* vs *TaSMT1-4D*) and 81.1 % (*TaSMT1-4B* vs *TaSMT1-4D*) (Table 1). Potential *cis*-acting elements were identified based on their homology to previously characterized regulatory motifs in plants, as documented in *PLACE* and *PLANTCARE*

databases (Higo *et al.* 1999, Lescot *et al.* 2002) and listed in Table 3 Suppl. Analysis of the sequenced promoter regions of *TaSMT1* genes showed that they contain conserved sites (TATA-box and CAAT-box) and the following stress-sensitive motifs: *cis*-elements induced by

light (ACE, AE, and G-box), drought (MBS), low and high temperatures (LTR and HSE), and hormones such as ABA (ABRE), gibberellin (GARE and P-box), ethylene (ERE), salicylic acid (TCA element), auxin (TGA element), and methyl jasmonate (TGACG motif) (Table 3 Suppl.).

Fig. 5. Relative expression of *TaCYP710A8-3A* (A) and *TaCYP710A8-3B/-3D* (B) genes assessed with real-time qPCR in wheat roots and leaves treated with cold stress. One, two, or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively (two-tailed Student's *t*-test; $n = 3 - 6$).

Fig. 6. Venn diagram illustrating the distribution of common (in intersections) and specific (in brackets) potential *cis*-elements in the promoter regions of three homoeologous *TaSMT1* genes.

Discussion

The ability to synthesize C24 alkyl sterols and more specifically C24 ethyl sterols is unique to the plant kingdom (Neelakandan *et al.* 2009, Nakamoto *et al.* 2015). The *SMT1* gene catalyzes the first step towards the synthesis of C24 alkyl sterols (Carland *et al.* 2002, Willemsen *et al.* 2003). In work reported here we showed that the bread wheat genome contains at least three homoeologous genes encoding C24-sterol methyltransferase 1. We also demonstrated different patterns of expression of *TaSMT1* and *TaCYP710A8* genes in roots and leaves in control conditions as well during cold stress.

Furthermore, we suggested that LTR, the *cis*-element involved in low temperature responsiveness, which we found in the *TaSMT1-4D* gene, together with other stress-responsive motifs in the promoter was involved in the regulation of *TaSMT1* expression in response to cold treatment. These findings suggest that the existence of homoeologous genes of sterol biosynthesis in polyploid plants supports the diversity of genetic mechanisms of sterol-mediated response of plants to stresses.

It was suggested that *SMT* lineages are likely concurrent with the origin of the eukaryote domain, and

then lost variably in some lineages because no SMT homologs from advanced animals (above the sponges) were identified (Haubrich *et al.* 2015). The phylogenetic tree of the *SMT1* family demonstrated that three wheat *SMT1*s showed the highest similarity (more than 98 %) with *Aegilops tauschii*, other cereals species also showed high identity with wheat (Fig. 2). Therefore, the sequence similarity exists between wheat *SMT* and typical *SMT* gene family from other plant species. Furthermore, analysis of the full-length deduced amino acid sequences of mature TaSMT1 protein demonstrated that highly conserved regions correspond to substrate binding segments for sterol and S-adenosyl methionine binding site (Fig. 3) designated as regions I, III, and IV and region II according to Nes *et al.* 2004.

Three homoeologous *TaSMT1* genes were located on the 5A, 4B, and 4D chromosomes of the hexaploid wheat genome. Interestingly, two genes (*TaSMT1-4B* and *TaSMT1-4D*) were located on homoeologous group 4 chromosomes and one gene (*TaSMT1-5A*) was located on homoeologous group 5 chromosomes. Homoeologous genes are usually located on the same group of chromosomes. The pattern of chromosome arm locations showed that genes located on 5AL chromosome could originally have been derived from the 4AL chromosome. Possibly during the evolution of modern-day chromosome 4A a translocation event occurred involving the distal regions of the long arm of chromosomes 4A and 5A. The non-homoeologous 4AL/5AL translocation occurred at the diploid level, *e.g.*, in *T. monococcum* (Devos *et al.* 1995, Ma *et al.* 2013). Various chromosome translocations have also been reported for other wheat genes, such as *TaEPSPS* (*TaEPSPS-7A1*, *TaEPSPS-4A1*, and *TaEPSPS-7D1*) (Aramrak *et al.* 2015) and *Waxy* genes (*Wx-7A*, *Wx-4A* and *Wx-7D*) (Huang *et al.* 2010).

Comparison of *SMT* genomic structures from different taxa indicates that the *SMT* genes consist of three major classes based on their intron pattern. Class 1 or algal *SMT1*s have five introns, class 2 or vascular plant *SMT1*s have five to twelve introns and class 3, comprising both *SMT2*s of plants and *SMT1*s of non-photosynthetic organisms, have no introns (Neelakandan *et al.* 2009). The *SMT* genes in wheat all have structural similarities and consist of twelve (*TaSMT1-5A*), ten (*TaSMT1-4B*), and eleven (*TaSMT1-4D*) exons, and are similar to homologous *SMT1* genes in other plants. An analysis of gene structure revealed that these genes include four conserved exons with identical sequences and sizes (Fig. 1A). It is likely that that these exons encode functionally important domains of the SMT1 protein in plants. Translation of the nucleotide sequence of these exons into the amino acid sequence confirmed that these exons encode sterol-binding sites, which are required for the functional activity of the protein. Thus, based on their introns and exons, *TaSMT1* genes correspond to the structure of the homologous genes of the *SMT1* family of higher plants (Neelakandan *et al.* 2009).

The alignment of the CDS revealed the presence of polymorphic sites where nucleotide substitutions and deletions occurred (Fig. 1B). These differences in nucleotide sequences might allow specificity of gene functioning. There may be different evolutionary consequences of the structural and functional divergence of homoeologous genes in polyploids. Possible outcomes include first subfunctionalization, *i.e.*, separation of functions between copies (co-expression of genes, inhibition/stimulation of transcription of certain homoeologs, as well as tissue-, organ-, and stage-specific expression). Second, neofunctionalization can occur, where one gene may acquire new functions. Finally, in pseudogenization there is complete loss of function by one of the homoeologs, and it subsequently becomes a pseudogene (Wendel 2000). Polyploid organisms have the advantage that they can display structural and functional divergence of homoeologous genes, which can increase the adaptive potential of the organism in a changing environment.

In wheat, the three related *TaSMT1* homoeologs possess conserved exon/intron structure and CDS but contain significant sequence variations within non-coding regions. These variations may result in the differences in gene expression. Earlier, we demonstrated that cold stress greatly changes the sterol content of wheat roots (Sulkarnayeva *et al.* 2014). In the present work, we found that cold stress upregulated the *TaSMT1-4D* gene expression in both the roots and the leaves (Fig. 4B), which might facilitate the acclimation of membranes to cold. The role of *TaSMT1* genes in the response of plants to stress was further supported by the presence of potentially stress responsive *cis*-elements in the promoter region of homoeologous *TaSMT1* genes. The promoter regions contained several *cis*-regulatory elements related to the response to biotic and abiotic stresses and hormonal stimuli (Table 3 Suppl.). It is known that the regulatory elements ABRE, LTR, MBS, G-box, and CBF/DREB transcription factors are also present in the promoters of other genes whose expression is induced by cold stress (Fowler and Thomashow 2002, Ruelland *et al.* 2009). Interestingly, the cold-sensitive *cis*-element LTR was present in the promoter regions of *TaSMT1-4B* and *TaSMT1-4D* but not in *TaSMT1-5A*. We suggested that LTR *cis*-element can influence the expression of *TaSMT1-4D* in response to cold stress, but the expression of *TaSMT1-4B* was not cold stimulated. This indicated complex regulation of the activity of these homoeologous genes, and thus more work is needed to unravel the fine mechanisms governing the activity of *TaSMT1* in a polyploid organism. Furthermore, the presence of CpG island, which was found in the promoter sequences of *TaSMT1-4D* (Fig. 1 Suppl.) might contribute to the stable expression of this gene, as CpG islands usually remain unmethylated (Ashikawa 2001, Papadakis *et al.* 2004). Despite the fact that CpG islands were not found in the promoter of *TaSMT1-5A*, it cannot be ruled out that an

increase in the length of sequenced region will give us more information about the presence of CpG islands.

Analysis of the expression of homoeologous genes encoding C22-sterol desaturase (CYP710A8), another enzyme involved in sterol biosynthesis, showed different patterns of co-expression of these genes in the roots and leaves. In the roots of cold-treated wheat seedlings, the expression of all genes steadily increased over the time-course of cold exposure, while leaves displayed a bell-shaped response, with gene up-regulation during early stress response and down-regulation after longer exposure to cold (Fig. 5). Thus, our data demonstrated that the expression of homoeologous *TaCYP710A8* genes differed in the roots and leaves. Considering that C22-sterol desaturase converts β -sitosterol into stigmasterol, continuous up-regulation of *TaCYP710A8* genes in roots supports the higher cold sensitivity of roots than leaves as demonstrated by Zhang *et al.* (2017) and the necessity to raise the content of stigmasterol known as a “stress sterol”.

In conclusion, this is the first report that shows that allohexaploid wheat possesses three homoeologous *TaSMT1* genes located on chromosomes A, B, and D. Despite significant similarities in their coding regions, the nucleotide sequences of the non-coding regions markedly differed. The presence of specific stress-sensitive *cis*-elements in their promoter regions, along with other factors, determined the differences in the patterns of expression of the *TaSMT1* genes: only the *TaSMT1*-4D gene was highly responsive to cold stress. Conversely, expression of the homoeologous *TaCYP710A8* genes changed in the same way in response to cold stress. These findings suggest that the existence of homoeologous genes of sterol biosynthesis in polyploid plants supports the diversity of epigenetic mechanisms of sterol-mediated response of plants to stresses. New data obtained will allow us in future to develop novel experimental tools of increasing stress tolerance and sustainability of crops by targeted regulation of sterol metabolism.

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