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Nonsense-mediated decay of sucrose synthase 1 mRNA with induced premature chain termination codon during cold acclimation in winter wheat

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Abstract: Cold acclimation induces the expression of cold-regulated genes needed to protect plants against freezing damage. Fourteen candidate genes were identified as being differentially expressed (presence/absence) between cold acclimated and nonacclimated crown and leaf tissues of 2 winter wheat lines using the cDNA-AFLP method. Two TILLING populations originating from the 2 winter wheat lines were further developed in order to create mutant forms of the candidate genes to verify their role in freezing-tolerance formation. Exon 8 of the identified differentially expressed *sucrose synthase* 1 (*Ss1*) gene was chosen for mutation detection by high-resolution melting analysis. A total of 75.68 kb of DNA was screened and 2 novel alleles of the Ss1 gene were identified, of which 1 was silent and 1 was a nonsense (premature stop codon) mutation. qRT-PCR analysis showed that premature stop codon mutation has a strong negative effect on Ss1 gene expression in nonacclimated leaves as well as in crowns and leaves collected at 2, 4, and 6 weeks of cold acclimation compared with the wild-type winter wheat line. Further work will reveal the effect of the mutation on cold tolerance of winter wheat and will enable the assessment of the role of Ss1 in the cold acclimation process.

Key words: High-resolution melting analysis, premature stop codon, *Triticum aestivum* L., TILLING

1. Introduction

Bread wheat (*Triticum aestivum* L.), being one of the most important agricultural crops, is a staple food crop for a large portion of the world's population (Rahaie et al., 2013). Cold is one of the main abiotic stresses causing winterkill in winter wheat. Freezing tolerance of winter wheat is one of the main factors governing winter survival (Armonienė et al., 2013b). Freezing tolerance can be defined as the ability of plants to survive freezing temperatures, prevent damage to the vegetative tissues, and minimize other negative effects of freezing temperatures on yield potential (Reinheimer et al., 2004). Freezing tolerance is associated with the occurrence of a cold acclimation that is triggered by the induction of cold responsive (*Cor*) genes after exposure of plants to low nonfreezing temperatures for certain periods of time (Winfield et al., 2010). In particular, *Cor* genes are transcriptionally activated during cold acclimation, and the accumulated COR proteins lead to protection of the integrity and functionality of cell structures from freezing damage (Kosová et al., 2010). Cold acclimation can also lead to changes in lipid composition, protein, and nucleic acid conformation, as well as the accumulation of carbohydrates (Thomashow, 1999; Kosová et al., 2010).

Carbohydrates, in particular, are found to play an important role in freezing tolerance (Livingston et al., 2006; Nägele et al., 2012), and the accumulation of simple sugars such as trehalose, raffinose, and sucrose has been shown to be correlated with enhanced freezing tolerance (Kaplan et al., 2006; Nägele et al., 2012). Such accumulation of sucrose and other simple sugars during cold acclimation contributes to the stabilization of membranes (Thomashow, 1999; Kosová et al., 2010) and may play a key role in protecting the cells from freezing and dehydration (Steponkus, 1984). Sugars are also recognized as important regulatory molecules with both signaling and putative ROS scavenging functions in plants (Bolouri-Moghaddam et al., 2010). Significantly higher capacity for sucrose synthesis in cold-tolerant versus cold-sensitive accessions of *Arabidopsis* was observed. Sucrose synthesis was found to be a metabolic bottleneck under challenging environmental conditions (Nägele et al., 2012). Sucrose synthase (Ss) (E.C. 2.4.1.13) catalyzes the reversible conversion of sucrose and a nucleoside diphosphate into the corresponding nucleoside diphosphate-glucose and fructose, being one of the main enzymes of carbohydrate metabolism. Two genes corresponding to the Ss1 and Ss2 proteins were characterized in wheat. Both genes

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were located on the short arms of homoeologous group 7 chromosomes in wheat (Maraña et al., 1988); however, they showed different expression patterns. *Ss2* was expressed in the endosperm only, whereas *Ss1* mRNA was expressed in both roots and leaves (Martinez de Ilarduya et al., 1993). Sucrose synthase activity increases during cold acclimation in leaves (Crespi et al., 1991; Sasaki et al., 2001; Janská et al., 2011). In wheat exposed to low temperature, Ss1 activity was found to be higher in the winter wheat cultivar Monopol than in the spring wheat cultivar Katepwa (Savitch et al., 2000). Zhang et al. (2011) demonstrated that accumulation of greater amounts of soluble sugars coupled with higher CBF3 and cold acclimation-specific transcript levels in *Medicago falcate* may play a role in conferring greater tolerance of *M. falcate* to freezing than that of *M. truncatula*. However, the exact mechanism of sucrose and Ss role in freezing tolerance remains unclear.

Creation and testing of *Ss1* gene mutant forms would be one of the ways to determine the role of Ss in the cold acclimation process. During the last decade, the use of chemically induced mutagenesis has had a renaissance with the development of TILLING (Targeting Induced Local Lesions in Genomes) technology. In TILLING, mutagenesis is complemented by the isolation of chromosomal DNA from every mutated line and screening of the population at the DNA level using advanced molecular techniques (Sikora et al., 2011). High-resolution melting (HRM) analysis is one of many mutation detection techniques available that measures the disassociation of double-stranded DNA at high-temperature resolution (Martino et al., 2010). HRM analysis is able to detect all single base changes, but shows greater sensitivity for G/A and C/T changes than for A/T and G/C changes (Liew et al., 2004). HRM is therefore suitable for TILLING, especially EMS-TILLING. HRM has been successfully applied in identification of point mutations in wheat (Dong et al., 2009; Botticella et al., 2011), tomato (Gady et al., 2009), *Brassica rapa* (Lochlainn et al., 2011), tobacco (Reddy et al., 2012) and peach (Chen and Wilde, 2011).

The objectives of this study were (1) to create a TILLING population of winter wheat; (2) to detect new alleles of *Ss1* gene identified as being differentially expressed in cold acclimated and non-acclimated wheat leaf and crown tissues; (3) to compare relative expression of *Ss1* gene in leaves and crowns of mutant and wild type plants during cold acclimation.

2. Materials and methods

2.1. Generation of wheat TILLING library

Two winter wheat breeding lines, '5450-1' and '5899-16', developed at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, were used in this study. Seeds of both lines were treated with ethyl methanesulfonate (EMS) according to Slade et al. (2005) with minor modifications. Batches of 100 seeds were transferred to 50-mL Falcon tubes and surface sterilized in 30 mL of 50% ACE solution (commercial ACE containing 5%–15% sodium hypochlorite) for 15 min. The seeds were then washed 3 times with deionized water (ddH_2O) and soaked with agitation (18 h at 125 rpm on shaker) in 45 mL of EMS solution with concentrations ranging from 0.5 to 1.0%. Treated seeds were washed extensively under running tap water for at least 4 h and then sown in plastic pots filled with a peat and sand (1:1) substrate. Seeds were allowed to germinate in the greenhouse and the number of germinating seeds was scored. The number of surviving seedlings was scored 2 months after sowing. The mutagenized seeds were grown into M_1 plants and allowed to self-pollinate. The M_1 population was advanced to M_2. M_3 seeds from individual M_2 plants were collected and cataloged.

2.2. Plant material for cDNA-AFLP and qRT-PCR

Seeds of winter wheat breeding lines '5450-1' and '5899-16' were sown in plastic pots (125 cm^3) filled with universal peat substrate GP0428 (Durpeta, Lithuania). Plants were grown at 20 °C in a plant growth chamber (CLF Plant Climatics, Wertingen, Germany) with a 12-h photoperiod at a light intensity of 600 μmol m^{-2} s^{-1} at 70% relative air humidity until the seedlings reached a 3-leaf stage. The plants were then cold-acclimated by transferring them to a growth chamber set at 5 °C with an 8-h photoperiod at a light intensity of 200 μmol m^{-2} s^{-1} and 80% relative air humidity for 6 weeks. Leaves and crowns were collected in 3 biological replications at 0, 2, 4, and 6 weeks of cold acclimation, flash frozen in liquid nitrogen, and stored at –80 °C until RNA extractions.

For qRT-PCR analysis, seeds of wheat line '5899-16' and 2 mutant lines, M631 and M692, of the M_3 generation were sown and samples were collected under the same conditions. All M_3 generation seedlings were tested by HRM analysis for the presence of the mutation.

2.3. cDNA-AFLP analysis

The cDNA-AFLP procedure was conducted as described earlier (Armonienė et al., 2013a) with minor modifications. Poly(A)+RNA was isolated from the total RNA with a Dynabeads mRNA Purification Kit (Invitrogen Dynal AS, Oslo, Norway). Total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Poly(A)+RNA was reverse-transcribed with an anchored oligo-dT18 primer using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. Twenty microliters of reverse transcription reaction product were used for second-strand cDNA synthesis by adding 30 U of DNA Polymerase I (Thermo Scientific) and incubating for 2 h at 15 °C. The

double-stranded cDNA was purified by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), washed with ethanol, resuspended in a final volume of 25 µL of nuclease-free ddH2O, and quantified by running 8 µL of cDNA on 1% agarose gel. Selective amplification PCR mix contained 10 µL of 20X diluted preamplification product, 0.25 µM of *Ase*I selective primer with 2 selective nucleotides, 1.5 µM of *Taq*I selective primer with 2 selective nucleotides, 0.2 mM dNTP mix, and 0.5 U DreamTaq DNA polymerase (Thermo Scientific) in a 20-µL total reaction volume. The sequences of primers that showed differential gene expression between cold-acclimated and nonacclimated wheat are indicated in Table 1. Products of selective PCR were fractionated in 2% agarose gel by electrophoresis at 100 W for 2 h. The fragments were visualized using ethidium bromide and UV light. The reproducibility of transcript-derived fragments (TDFs) was tested by repeating PCR amplifications 2 to 3 times for each primer combination used.

2.4. Isolation, cloning, and sequence analysis of TDFs

TDFs based on the presence/absence in the samples under comparison were excised from the gel with a surgical blade while avoiding any contaminating fragments, purified with a GeneJET Gel Extraction Kit (Thermo Scientific), and eluted with 20 µL of sterile water. An aliquot of 1 µL was used for reamplification with Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) in a total volume of 20 µL using the combination of corresponding AFLP amplification primers. All PCR amplifications were conducted in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation of 10 s at 98 °C; followed by 30 cycles of 1 s at 98 °C, 5 s at 60 °C, and the 36-s elongation step at 72 °C; then followed by 1 min at 72 °C for a final extension. Reamplified DNA fragments were cloned into the pJET1.2/blunt cloning vector using the GeneJet PCR Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. Insert sizes were checked by colony PCR and restriction enzyme digestion. Plasmid DNA was isolated with the GeneJet Plasmid Miniprep Kit (Thermo Scientific) and sequenced at GATC Biotech (Köln, Germany). At least 2 clones for each reamplified TDF were DNA-sequenced to ascertain integrity of the excised TDF. Sequence homology analysis of the fragments was carried out against the publicly available nonredundant genes/transcripts (http://www.ncbi.nlm.nih.gov/BLAST) using the BLASTX algorithm.

Table 1. cDNA-AFLP primer sequences that showed differential gene expression between cold-acclimated and nonacclimated wheat.

No.	Primer pairs	Sequences (5'→3')
1	OLIGO47/A14	GACTGCGTACCTAATAT
	OLIGO67/T14	GATGAGTCCTGACCGAAT
2	OLIGO44/A11	GACTGCGTACCTAATAA
	OLIGO75/T22	GATGAGTCCTGACCGAGT
3	OLIGO56/A23	GACTGCGTACCTAATTA
	OLIGO70/T17	GATGAGTCCTGACCGACG
4	OLIGO54/A21	GACTGCGTACCTAATGG
	OLIGO69/T16	GATGAGTCCTGACCGACC
5	OLIGO48/A15	GACTGCGTACCTAATCA
	OLIGO72 /T19	GATGAGTCCTGACCGAGA
6	OLIGO44/A11	GACTGCGTACCTAATAA
	OLIGO67/T14	GATGAGTCCTGACCGAAT
7	OLIGO48/A15	GACTGCGTACCTAATCA
	OLIGO67/T14	GATGAGTCCTGACCGAAT
8	OLIGO53/A20	GACTGCGTACCTAATGC
	OLIGO67/T14	GATGAGTCCTGACCGAAT

2.5. Genomic DNA isolation

Genomic DNA was extracted from 0.2-g samples of wild-type '5450-1' and '5899-16' and M_2 generation leaf material according to Lassner et al. (1989) with several modifications. These included an additional wash step with chloroform and RNA digestion with ribonuclease A. The DNA was quantified, diluted to 20 ng/µL in deionized water, and 2-fold pooled according to Botticella et al. (2011).

2.6. HRM analysis

Oligonucleotide primers (forward and reverse) for the target *sucrose synthase 1* (*Ss1*) gene were designed according to the known DNA sequences from TDFs (Table 1) using PrimerBlast (http://www.ncbi.nlm.nih.gov). Primers were selected to specifically amplify a 120-bp fragment of *sucrose synthase type 1* gene. The sequences of primers were 5'-TCCTCGGTGTATGTCTCCAG-3' and 5'-GGTTATTGGAACCGAGCACA-3' for the forward and reverse primer, respectively. HRM analysis was carried out using MeltDoctor HRM Master Mix (Life Technologies, Foster City, CA, USA) following the manufacturer's instructions. PCR reactions contained 20 ng of genomic DNA, 0.3 µM of forward and reverse HRM primers, and 5 µL of MeltDoctor HRM Master Mix in a total volume of 10 µL. The PCR was carried out on a 7500 Fast Real-Time PCR System (Life Technologies). The reaction conditions were as follows. Enzyme activation was at 95 °C for 10 min, with 2-step (denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min) cycling of 40 cycles. Melt curve/dissociation analysis was done by denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, and HRM at 95 °C, followed by annealing at 60 °C for 15 s. Individuals were genotyped using HRM V.2.0.2

software (Life Technologies). Positive pools were selected by analyzing the melting temperature profiles; the pool containing a mutation shows lower melting temperature compared to the wild type. Samples identified as putative mutants were selected, reamplified for each individual in the pool, and sequenced at GATC Biotech for the mutation identification.

2.7. qRT-PCR and data analysis

Total RNA from leaf and crown tissue was extracted with the RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's protocol. The on-column DNase digestion was performed using RNase-free DNase (QIAGEN). An aliquot of RNA samples (1 μg) was reverse-transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qRT-PCR was performed in triplicate with the primers used in HRM on a 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with a SYBR Green PCR master mix kit (Applied Biosystems). The PCR thermal cycling parameters were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The wheat ADP-ribosylation factor gene (*Ta2991*) was used as an endogenous control using primers developed by Paolacci et al. (2009). Relative expression of the genes of interest was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using a control wheat line, '5899-16', at 0 weeks of cold acclimation as the calibrator. Dissociation curves were generated for each reaction to ensure a single specific product was amplified for each gene. For both crown and leaf tissue, 3 biological replicates × 3 technical replicates were used in qRT-PCR experiments.

2.8. Statistical analysis

Statistical analysis was performed using Statistica 7 software (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was used to determine significance ($P < 0.01$ and $P < 0.05$) of the differences between wheat genotypes, EMS concentrations, and relative gene expression. The differences between means of wheat genotypes were inspected using Fisher's protected significant differences post hoc analysis.

3. Results

3.1. Development of TILLING population

Concentration of the mutagen EMS had an effect on both germination and development of wheat seedlings. A significant negative relationship ($R = -0.59$, $P < 0.01$) between EMS concentration and percentage germination average at the 15th day after sowing (when the number of germination reached a plateau) was observed in both genotypes of winter wheat. Analysis of the results of wheat percentage germination average revealed significant differences ($P < 0.05$) between different EMS concentrations and wheat genotypes. Percentage germination average ranged from 79.9% to 63.3% and from 80.7% to 72.3% in '5450-1' and '5899-16', respectively (Figure 1A). The '5899-16' genotype showed significantly ($P < 0.01$) higher germination rate at the highest (1.0%) EMS concentration treatment compared to '5450-1'.

Analysis of wheat survival after 2 months revealed significant differences ($P < 0.01$) of percentage survival between different EMS concentrations and wheat genotypes. The '5899-16' genotype showed a higher survival rate at 0.6% and at higher (0.8%, 0.9%, and 1.0%) concentrations of EMS treatment in comparison to '5450-1' (Figure 1B). A significant negative relationship ($R = -0.7$, $P < 0.01$) was identified between different EMS concentrations and percentage survival rate of winter wheat. Percentage survival average declined from 77.4% to 0% and from 79.3% to 31.7% for genotypes '5450-1' and '5899-16', respectively.

A total of 2147 M_2 lines were produced (1386 and 761 for '5899-16' and for '5450-1', respectively). M_2 seeds were harvested from each M_1 line and one seed per line was sown for the generation of M_2 populations. A total of 946 seeds (94.6%) germinated out of 1000 seeds sown (500 per genotype). Genomic DNA was extracted from the leaf material of the 4-week-old plants from 486 of the '5450-1' line and from 460 of the '5899-16' line in the M_2 population. Seeds were harvested from each of the 756 fertile lines. These seeds made up the M_3 TILLING population.

3.2. cDNA-AFLP analysis of differentially expressed genes

cDNA-AFLP was performed using 48 primer pair combinations, which generated 522 TDFs with an average of 11 fragments per primer pair. The lengths of the TDFs were in the range of 200–2000 bp. Twenty TDFs (3.8%) were identified as being differentially expressed (presence/absence) between cold-acclimated and nonacclimated wheat (Table 2). Thirteen TDFs (65%) were upregulated and 7 TDFs (35%) were downregulated within 2 weeks after the start of the cold acclimation. About 85% of TDFs exhibited upregulation in leaf tissue compared to 15% in crown tissue. All of the downregulated TDFs were detected in leaf tissue profiles. Twenty TDFs were extracted from agarose gels, cloned, and sequenced. A total of 14 TDFs revealed significant (E value < 1e-10) sequence similarities in a BLASTX search against the nonredundant (nr) protein database of GenBank (Table 2).

3.3. Mutant discovery by HRM analysis

Exon 8 of differentially expressed *Ss1* gene was chosen for mutation screening by HRM analysis in wheat TILLING M_2 population. A total of 75.68 kb of DNA was tested and 2 putative mutants were identified. These 2-fold pooled samples of putative mutants had peaks shifted towards a lower temperature (reduced by 0.3 °C) compared to the wild-type genotype (Figure 2). HRM analysis was repeated

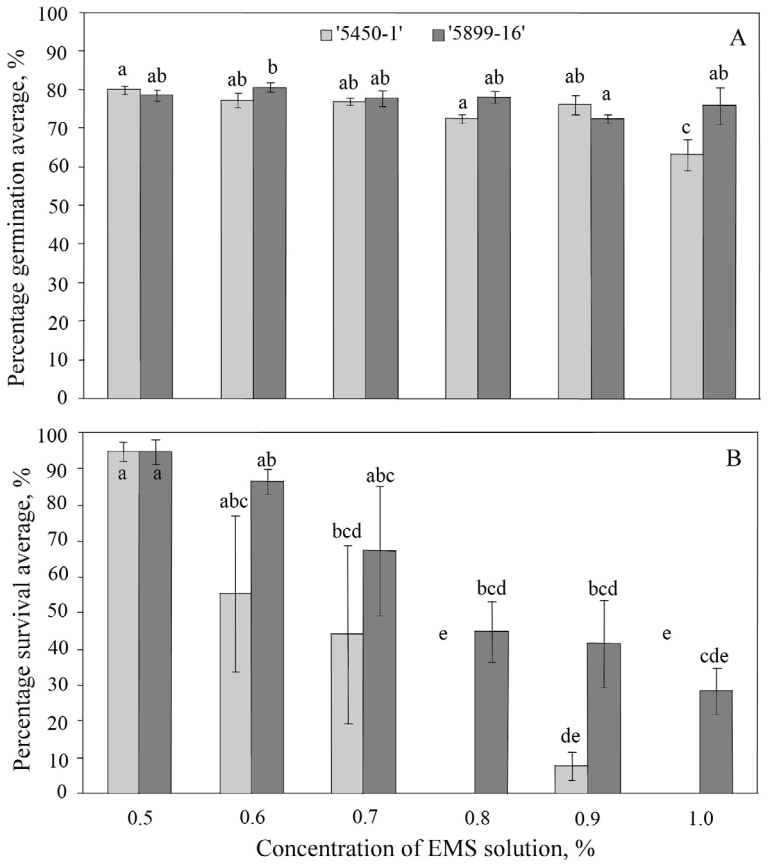

Figure 1. Dose optimization of a mutagen EMS. **A)** Percentage germination average at 15th day after sowing; **B)** percentage survival average at 2 months after sowing of wheat genotypes '5450-1' and '5899-16' exposed to various concentrations of EMS solution. Vertical bars represent standard deviation of the mean. The letters above the boxes indicate statistically significant ($P < 0.05$) differences between treatments.

in separate wells for each sample with predicted mutation. As expected, only single samples of the 2-fold pooled samples showed lower melting temperature. Sequencing results revealed 2 novel alleles of the *Ss1* gene. Both mutations were transitions of the G > A type as expected for the EMS mutagenesis, which acts via alkylation of G residues. Mutant no. 692 was found to have a silent mutation (G1119A, K373K) and mutant no. 631 had a nonsense (premature stop codon) mutation (G1122A, W374*). Both mutations were in the homozygous state.

3.4. qRT-PCR analysis of *Ss1* gene

qRT-PCR was performed to evaluate the effect of the identified mutations on the expression level of the *Ss1* gene in crown and leaf tissues during cold acclimation. qRT-PCR analysis showed that the premature stop codon mutation has a strong effect on *Ss1* gene expression (Figure 3). Putative knock-out mutant M631 had significantly lower relative expression of the *Ss1* gene in nonacclimated leaves as well as in crowns and leaves collected at 2, 4, and 6 weeks of cold acclimation compared with the wild-type winter wheat line '5899-16' (control) and M692 mutant (silent mutation).

4. Discussion

High-throughput TILLING permits a rapid and low-cost discovery of new alleles induced in plants. The main advantage of TILLING is its applicability to any species, regardless of its genome size and ploidy level (Kurowska et al., 2011). In this work, TILLING was employed as a tool to create novel allelic forms for identified genes to verify their role in cold acclimation. Firstly, we optimized the dose of a mutagen, EMS, for 2 winter wheat breeding lines to achieve a substantial mutation rate while avoiding serious defects in germination and plant development. The results showed that higher concentrations of EMS (0.8%–1.0%) have a significant impact on the development of wheat seedlings. Although germination rate was in the range of 70% to 80%, less than 50% of the germinated seedlings

Table 2. Functional annotation of the 14 TDFs revealing significant (E < 1e-10) sequence similarities in BLASTX search against the nonredundant (nr) protein database of GenBank along with the TDF size and accession numbers in GenBank protein database.

TDF identifier	Plant tissue	Status after2 weeks of cold acclimation	Size (bp)	Homologous protein	E value[a]	Accession no.	Species
TDF01	Leaf	Upregulated	1130	Sucrose synthase 1	0.0	EMT33394.1	*A. tauschii*
TDF02	Leaf	Upregulated	1072	Heat-shock protein	0.0	CAA82945.1	*S. cereale*
TDF03	Leaf	Upregulated	887	Serine carboxypeptidase-like	4E-99	EMT33269.1	*A. tauschii*
TDF04	Leaf	Upregulated	997	Vacuolar-sorting receptor 1	0.0	EMT18407.1	*A. tauschii*
TDF05	Leaf	Upregulated	1103	Zinc finger AN1 domain	4E-57	EMS61459.1	*T. urartu*
TDF06	Crown	Upregulated	813	Beta-3-tubulin	4E-169	Q9ZRB0.1	*T.aestivum*
TDF07	Crown	Upregulated	901	Phototropin-2-like	0.0	XP_003581132.1	*B. distachyon*
TDF08	Crown	Upregulated	1111	Digalactosyldiacylglycerol synthase 2, chloroplastic	0.0	EMS58077.1	*T. urartu*
TDF09	Leaf	Downregulated	704	Phosphoglycerate kinase, chloroplastic	3E-122	EMT12348.1	*A. tauschii*
TDF10	Leaf	Downregulated	1139	Signal recognition particle 54 kDa protein, chloroplastic	0.0	XP_003577835.1	*B. distachyon*
TDF11	Leaf	Downregulated	936	Predicted protein	3E-145	BAJ89837.1	*H. vulgare*
TDF12	Leaf	Downregulated	614	ATP-dependent Clp protease ATP-binding subunit ClpC homolog 1	9E-130	Q7F9I1.2	*O. sativa*
TDF13	Leaf	Downregulated	676	Clp-P protease subunit	2E-135	ACN54193.1	*T. aestivum*
TDF14	Leaf	Downregulated	469	RuBisCo activase A	7E-110	EMS57012.1	*T. urartu*

a - Expect (E) value describes the probability of the alignment occurring by chance.

had survived. No seedlings of the '5450-1' genotype had survived after treatment with 0.8% or 1.0% EMS. Polyploid species such as wheat have higher tolerance to mutations due to the complementation of essential genes by homologous copies; however, high mutation frequency may increase the number of deleterious mutations, which, if too numerous, will kill the plant (Chawade et al., 2010). The 2 winter wheat genotypes had different optimal doses of EMS. The most appropriate EMS concentration for the '5899-16' genotype was in a range of 0.7% to 1.0%, while it was lower for the '5450-1' genotype, ranging from 0.6% to 0.7% (Figure 1), in accordance with the general rule that the optimum concentration of a mutagen should have a survival rate from 30% to 80% (Wang et al., 2010). Our defined EMS concentrations are in the range of what has been used for polyploids such as bread wheat (0.6%–0.9%) (Rakszegi et al., 2010), durum wheat (0.7%, 1.0%, or 1.2%, Slade et al., 2005; 0.7%–1.0%, Uauy et al., 2009), and oat (0.9%, Chawade et al., 2010), whereas optimal concentrations used for diploid species are much lower. Barley has an optimum EMS concentration of 0.2% to 0.63% (Gottwald et al., 2009) and *Brassica napus* tolerates 0.25%, 0.3%, or 0.4% (Gilchrist et al., 2013), while in *Arabidopsis* it was determined to range from 20 mM to 50 mM (Martín et al., 2009).

The *Ss1* gene, identified as differentially expressed between nonacclimated and cold-acclimated wheat by cDNA-AFLP, was chosen as a candidate to induce allelic mutations and determine its impact on the cold acclimation process. We demonstrate a significant increase of *Ss1* gene expression during cold acclimation in leaves of both wild-type and M692. Zhang et al. (2011) showed *Ss1* gene expression to be upregulated during cold acclimation in leaves of freeze-tolerant *M. falcate*, whereas the expression of *Ss1* in freeze-susceptible *M. truncatula* was much lower in both cold-acclimated plants and nonacclimated plants. *Ss1* expression had even decreased after 72 h of cold acclimation in *M. truncatula*. *M. falcate* showed significantly higher sucrose and total sugar content, as well as SS activity, after 21 days of cold acclimation (Zhang et

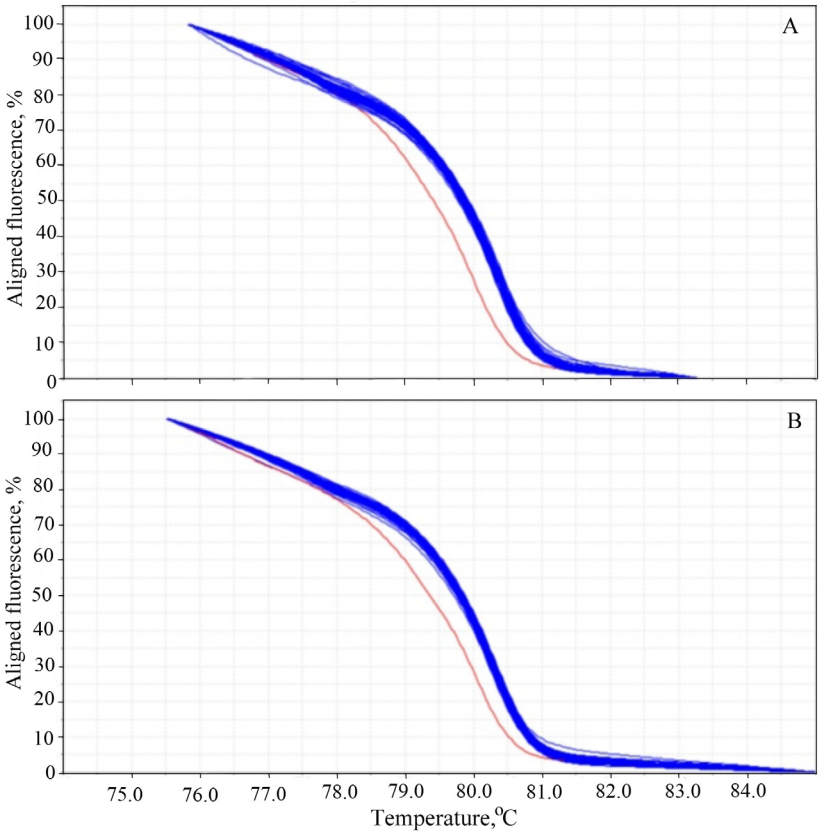

Figure 2. HRM analysis of 96 two-fold pooled samples. Aligned curves of fluorescence vs. temperature showing wild-type (blue) and mutant (red) samples: **A)** M692, **B)** M631.

al., 2011). In wheat, sucrose accumulation and Ss activity increases during the first 14 days of cold acclimation (Crespi et al., 1991). Zhang et al. (2011) also reported that the expression of *M. falcate* CBF3 was greater than that of *M. truncatula* during cold acclimation. The CBF cold-responsive pathway plays an important role in cold acclimation and induces freezing tolerance in many plants (Thomashow, 2010; Winfield et al., 2010). *Ss1* is a CBF target as the promoter possesses CRT/DRE elements (Reulland et al., 2009). CBF3 causes both the acquisition of freezing tolerance and the accumulation of soluble carbohydrates (Gilmour et al., 2000). This implies that sucrose synthase is regulated by CBF during the cold acclimation process and is involved in freezing tolerance formation in plants.

Ss1 gene expression increase in crown tissue was not as large as in leaves during cold acclimation, but there were significant ($P < 0.01$) differences in its expression between genotypes at various cold acclimation time points. Janská et al. (2011) also observed only a marginal effect of low temperature on the expression of genes involved in sucrose/starch metabolism in the crown of winter barley. They hypothesized that sucrose can be exported rather than cleaved into fructose and glucose, as the few genes that were significantly upregulated in the crown were indeed sucrose transporters.

Two novel alleles of the *Ss1* gene were identified by HRM analysis. HRM is especially useful when the gene of interest contains many short exons. An overall mutation density of 1 mutation per 37.84 kb of the DNA was observed in our TILLING population, thus appearing to be in the same range as previously determined for hexaploid wheat (1 mutation per 40 kb) by HRM (Botticella et al., 2011). This is in agreement with mutation densities of 1/40 kb and 1/38 kb identified in tetraploid (Slade et al., 2005) and hexaploid (Uauy et al., 2009) wheat, respectively, by traditional TILLING screening based on Cel 1 nuclease digestion.

qRT-PCR revealed that premature stop codon mutation in the *Ss1* gene sequence of mutant M631 is associated with a significant reduction in the gene expression. The *Ss1* gene was strongly repressed during the whole cold acclimation period in leaf and crown tissues of M631. It was approximately 7-fold and 18-fold lower in the leaves and in the crowns of M631, respectively, in comparison to the wild-type and M692. Hence, the premature stop codon in the *Ss1* gene probably resulted

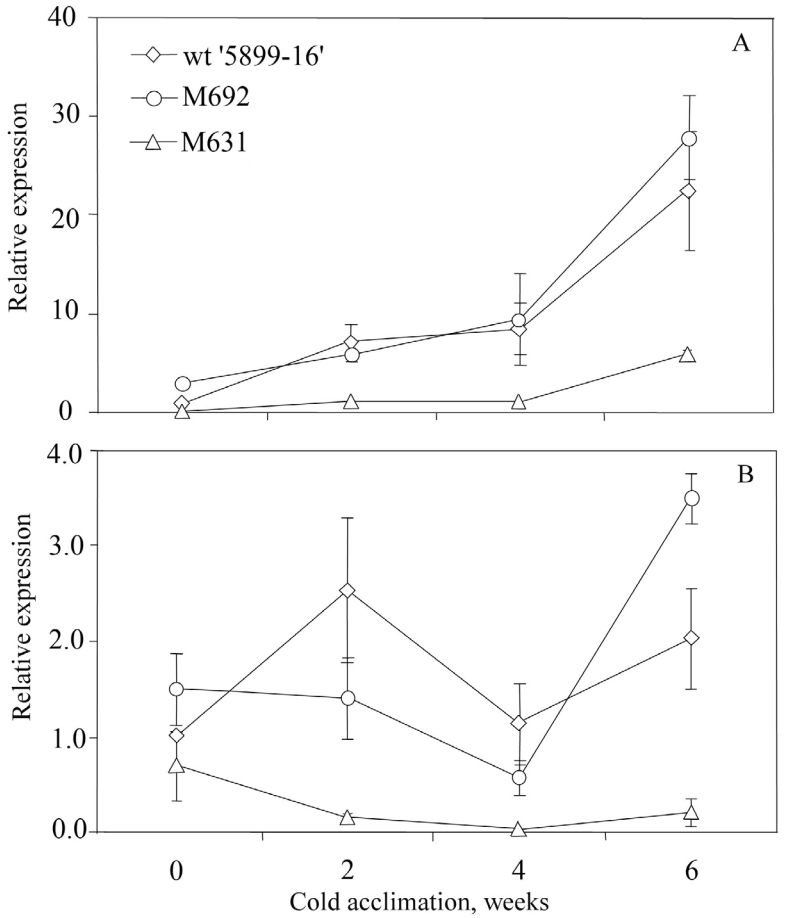

Figure 3. Relative expression of *Ss1* gene during cold acclimation in **A)** leaves and **B)** crowns of wild-type genotype '5899-16' and 2 mutant genotypes, M692 and M631, as revealed by qRT-PCR. Total RNA was extracted from seedling leaves acclimated for 0–6 weeks at 5 °C. *ADP-ribosylation factor* gene (*Ta2991*) was used as the endogenous control. Vertical bars represent standard deviation of the mean.

in the nonsense-mediated mRNA decay (NMD) of the aberrant mRNA. NMD is usually induced if the distance between the premature termination codon and the 3'-end of the transcript exceeds 350 nt (Kalyna et al., 2012). The location of the premature stop codon in our mutant complied with this condition. In wheat, reduced RNA levels have been reported for multiple genes containing premature stop codon mutations including a waxy gene (Saito and Nakamura, 2005), a *SbeIIa* (Botticella et al., 2011; Slade et al., 2012), and a *Vrn1* gene (Chen and Dubcovsky, 2012). Horst et al. (2007) detected premature stop codons in the sucrose synthase *LjSus1* and *LjSus3* genes of *Lotus japonicus*. Interestingly, the mutations were in the same exon as in our study. Both mutants (*sus1-1* and *sus3-1*) showed reduced Ss activity. *sus3-1* plants had decreased Ss content in the nodules and exhibited defective growth in comparison to the wild type plants (Horst et al., 2007). Unfortunately, extensive backcrossing is needed to assess the impact of our identified mutation on freezing tolerance of winter wheat, as highly mutagenized lines of polyploid species require significant backcrossing to remove extraneous mutations before they can be assessed for their phenotypes.

TILLING is a powerful tool for the development of new alleles for cold-responsive genes in bread wheat. We have found a new allele of the *Ss1* gene showing decreased expression during cold acclimation in both leaves and crowns. Further work will reveal the effect of the mutation on the cold tolerance in winter wheat and will enable the assessment of the role of *Ss1* in the cold acclimation process.

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