TaMYB13-1, a R2R3 MYB transcription factor, regulates the fructan synthetic pathway and contributes to enhanced fructan accumulation in bread wheat

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Abstract

Fructans are the major component of temporary carbon reserve in the stem of temperate cereals, which is used for grain filling. Three families of fructosyltransferases are directly involved in fructan synthesis in the vacuole of Triticum aestivum. The regulatory network of the fructan synthetic pathway is largely unknown. Recently, a sucrose-upregulated wheat MYB transcription factor (TaMYB13-1) was shown to be capable of activating the promoter activities of sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose:fructan 6-fructosyltransferase (6-SFT) in transient transactivation assays. This work investigated TaMYB13-1 target genes and their influence on fructan synthesis in transgenic wheat. TaMYB13-1 overexpression resulted in upregulation of all three families of fructosyltransferases including fructan:fructan 1-fructosyltransferase (1-FFT). A γ-vacuolar processing enzyme (γ-VPE1), potentially involved in processing the maturation of fructosyltransferases in the vacuole, was also upregulated by TaMYB13-1 overexpression. Multiple TaMYB13 DNA-binding motifs were identified in the Ta1-FFT1 and Taγ-VPE1 promoters and were bound strongly by TaMYB13-1. The expression profiles of these target genes and TaMYB13-1 were highly correlated in recombinant inbred lines and during stem development as well as the transgenic and non-transgenic wheat dataset, further supporting a direct regulation of these genes by TaMYB13-1. TaMYB13-1 overexpression in wheat led to enhanced fructan accumulation in the leaves and stems and also increased spike weight and grain weight per spike in transgenic plants under water-limited conditions. These data suggest that TaMYB13-1 plays an important role in coordinated upregulation of genes necessary for fructan synthesis and can be used as a molecular tool to improve the high fructan trait.

Key words: Fructans, fructosyltransferases, gene regulation, MYB transcription factor, γ-vacuolar processing enzyme, wheat.

Introduction

Fructans are oligo- and polysaccharides that are produced by many plant species, including temperate monocots and dicots, as well as by some bacteria and fungi (Pollock, 1986). In angiosperms, fructans can be found in about 15% of all species (Hendry, 1987), including many temperate cereals like wheat and barley. Fructans are mainly synthesized and stored in plant vacuoles by a group of fructosyltransferases belonging to plant glycose hydrolyase family 32 enzymes (Pollock, 1986; Darwen and John, 1989; Vijn et al., 1997; Ritsema and Smeekens, 2003a; Chalmers et al., 2005; Altenbach et al., 2009; Van den Ende et al., 2009). Plant fructosyltransferases have been extensively researched, including characterization of the 3D structure of Pachysandra terminalis fructosyltransferase (Lammens et al., 2012). In wheat and barley, three enzyme families that synthesize graminan-type fructans consisting of β-2,6 linked fructosyl units with β-2,1 branches are sucrose:sucrose 1-fructosyltransferase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT), and fructan:fructan 1-fructosyltransferase (1-FFT) (Lücher et al., 1996; Müller et al.,...
Despite many studies on fructans and genes involved in fructan synthesis and regulation, regulatory pathways involving transcription factors, protein kinases, and phosphatases are largely unknown. Recently, one transcription factor has been identified to be potentially involved in the regulation of Ta1-SST and Ta6-SFT genes. Xue et al. (2011a) have shown three highly homologous R2R3 MYB transcription factor genes (TaMYB13-1, TaMYB13-2, and TaMYB13-3) that are closely co-regulated with Ta1-SST and Ta6-SFT genes in wheat, and TaMYB13-1 is a predominantly expressed gene among these three. The expression of TaMYB13-1 is upregulated by sucrose and during stem development. The activation of the expression of Ta1-SST and Ta6-SFT promoter-driven reporter genes by TaMYB13-1 has been demonstrated in transient transactivation assays.

To gain further insight into the role of TaMYB13-1 in the fructan synthetic pathway in wheat, this work characterized transgenic wheat overexpressing TaMYB13-1. Expression analysis of TaMYB13-1-overexpressing lines using Affymetrix wheat genome array and quantitative reverse-transcription PCR revealed that TaMYB13-1 upregulated the expression of not only Ta1-SST and Ta6-SFT but also Ta1-FFT family genes and other genes associated with fructan accumulation (e.g. fructokinase 1 (FK1) and γ-vacuolar processing enzyme 1 (γ-VPE1)). TaMYB13 DNA-binding motifs were also found in the promoter regions of the Ta1-FFT1 and Taγ-VPE1 genes and verified with in vitro DNA-binding assays. These two new target genes identified in this study showed positive correlation with TaMYB13-1 expression in all datasets analysed. The transgenic plants had higher fructan and WSC levels as well as higher spike weight and grain weight per spike in comparison with wild-type control plants, demonstrating that a TaMYB13-1-mediated regulon plays an important role in modulating fructan accumulation and conferring the high fructan trait.

Materials and methods

Plant material and growth conditions

Spring wheat (Triticum aestivum cv. Bobwhite SH 98 26) was grown in a controlled environment room in 1.5-l pots containing a mixture of sand:soil:peat (3:1:1). The room had the following day/night settings: 16/8 light/dark (500 μmol m⁻² s⁻¹), 20/16 °C and 60/80% relative humidity (Kam et al., 2008). Two organs selected for gene expression and WSC analyses are flag leaf (an important organ of photosynthesis for contributing to wheat grain yield) and stem (a major organ for fructan storage). As the top internode (peduncle) of the stem contains a much more abundant amount of RNA than the lower internodes, only top internode samples were used for main analyses in this study. To minimize diurnal fluctuations of gene expression samples were harvested 6–7 h after the lights turned on. Harvested samples were frozen in liquid nitrogen immediately and stored at –80 °C.

For evaluation of yield-related phenotypes under mild drought conditions, plants were grown in 14.3-l pots with a 29-cm top diameter. Each pot grew six plants, which mimics plants grown in a small plot. The plants were watered only when they showed mild water-deficit stress.

RNA extraction and cDNA synthesis

Samples were ground in liquid nitrogen using mortar and pestle. RNA was isolated using Plant RNA Reagent from Invitrogen.
according to the manufacturer’s instructions. DNA was removed as described by Xue and Loveridge (2004) and the isolated RNA was purified using the RNeasy Plant mini-kit column (Qiagen), following the directions of the kit. For quantitative reverse-transcription PCR, 5 μg total RNA was retro-transcribed using Superscript III first strand synthesis kit from Invitrogen, according to the manufacturer’s instructions.

Real-time PCR

Relative transcript levels of genes were determined from cDNA with an ABI Prism 7900 sequence detection system using SYBR Green PCR Master mix (Applied Biosystems) according to the manufacturer’s instructions. PCR amplification efficiencies of gene-specific primers were determined by serial cDNA dilutions. Relative gene expression levels were calculated as described by Shaw et al. (2009), where the relative mRNA value of each gene in a given sample is estimated using the mean of two normalized values against that of two internal control genes, TaRPII36, RNA-polymerase II 36 kD subunit (Xue et al., 2006) and TaRPI5, RNA polymerases I, II, and III 15 kD subunit (Xue et al., 2008b). Primers used for the amplification of Tal-SST1, Tal-SST2, Tal-SST5, Tal-FFT1, Tal-FFT2, TaRPI15, and TaRPIII60 were as published by Xue et al. (2008a, b, 2011a). The following real-time PCR primer pairs were used for the quantification of Tey-VEP1 and TaFK1 mRNA levels: 5′-CGAGCTGATTTGGAAACCTTCT-3′ (forward) and 5′-AGCGACCATCTGGTGTATCCAA-3′ (reverse) for Tey-VEP1; 5′-TCTTTGAGATCAAGGTCAAGT-3′ (forward) and 5′-CACCAGCACCCTGTTGATCAA-3′ (reverse) for TaFK1.

Transformation of wheat with TaMYB13-1 construct

TaMYB13-1 expression construct (Hv6-SFT:TaMYB13-1:ricercbS3′) was made by inserting the coding region of TaMYB13-1 after the barley Hv6-SFT promoter (Nagaraj et al., 2001), using expression construct plasmids in the cloning vector pSP72 as described by Xiao and Xue (2001) and Xue et al. (2003), followed by nucleotide sequencing. The selectable marker cassette containing rice actI:bar:nos 3′ was used to co-transform Bobwhite wheat plants. Both cassettes were PCR amplified and used for transformation of immature Bobwhite SH 98 26 embryos using the particle bombardment method as described by Pellegrineschi et al. (2002). Transgenic plants were selected with the herbicide phosphinothricin and grown in a controlled environmental growth room as described above. The presence of the Hv6-SFT:TaMYB13-1:ricercbS3′ cassette was verified by real-time PCR using genomic DNA as described previously (Xue et al., 2011b).

WSC extraction and analysis

The flag leaf and top internode samples were harvested from plants at anthesis grown in controlled environment as described above. The WSC levels were measured using the modified anthrone procedure (Xue et al., 2009). The levels of sugars (sucrose, glucose, and fructose) were determined using HPLC (Waters, Massachusetts, USA) and separated on an analytical column (CarboPac PA-100; Dionex, California, USA) using 50–150 mM NaOH as a mobile phase. The fructan fraction of the WSC extracts was analysed by HPLC measurement of the glucose and fructose levels of the WSC extracts before and after mild acid hydrolysis of fructans. The mild acid hydrolysis of fructans was performed according to the method of Van den Ende et al. (2003). For TLC analysis, approximately 80 μg WSCs per lane were loaded on 20 cm × 20 cm silica-gel-coated plates (0.2 mm thick, TLC Silica gel 60 F254, Merck). The TLC was run as described by Incoll et al. (1989) with 1-propanol:ethyl-ethanoate:water (5:3:2, v/v/v). Sugars and fructans separated on TLC plates were visualized by spraying the plates with urea-phosphoric acid and heating at 110 °C (Wise et al., 1955). WSCs extracted from Helianthus tuberosus were used as markers for fructans.

Expression analysis using Affymetrix Wheat GeneChip Array

RNA from the flag leaves of plants at anthesis grown in controlled environment was extracted and processed as described above. RNA quality check, cRNA preparation, labelling, hybridization, and data acquisition of Affymetrix Wheat GeneChips were performed by the microarray service at the Australian Genome Research Facility (Melbourne, Australia). The Affymetrix GeneChip data were normalized using GeneChip robust multiarray average, developed by Wu et al. (2004), using the Affymetrix package within Bioconductor, running within the R statistical programming environment (www.r-project.org). The dataset (the accession number GSE42000) was deposited at the NCBI (www.ncbi.nlm.nih.gov/geo). Probesets with expression levels below 100 in both the control and transgenic groups were discarded, so were probesets that had a differential expression value with a P-value >0.05.

Determination of the genomic DNA sequences of TaMYB13-1 target genes

The sequences of the Affymetrix probes for the target genes were used as query sequences to blast the genomic sequence of wheat from CerealsDB (Wilkinson et al., 2012). The retrieved sequence was subsequently used in a new blast search to retrieve additional sequences. The final retrieved genomic sequence contigs were cross checked with EST data from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the DFCI Triticum aestivum Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=wheat). The blast search was continued until at least 1000bp upstream of the ATG was obtained if it was possible.

In vitro DNA-binding assays

In vitro DNA-binding assays using cellulase D (CELD) as a reporter were performed as described by Xue (2002), using streptavidin-coated 96-well plate and binding/washing buffer (25 mM HEPES/KOH pH 7.0, 50 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT) containing 0.15 μg ml−1 shared herring sperm DNA, 0.3 mg ml−1 bovine serum albumin, 10% glycerol, and 0.025% Nonidet P-40. For each assay, 40000 fluorescent units h−1 of the CELD activity of TaMYB13-1-CELD protein and 2 pmol of biotinylated probes were used. The cellulase activity of TaMYB13-1-CELD proteins bound to immobilized biotinylated probes was assayed by incubation in 100 μl of the CELD substrate solution (1 mM methylumbelliferyl β-D-cellubioside in 50 mM Na-citrate buffer, pH 6.0) at 40 °C for 3 h. A biotin-labelled double-stranded oligonucleotide without a TaMYB13-1-binding site was used as a control of background activity. For the synthesis of the biotin-labelled probes, oligonucleotides were designed around the predicted TaMYB13-1 DNA-binding sites present in the genomic sequence as described above, including 10 bp upstream and downstream these sites. The oligonucleotides were manufactured by Geneworks (Adelaide, Australia). The biotinylated double-stranded oligonucleotides were synthesized as described by Xue (2005).

Results

Overexpression of TaMYB13-1

To gain a better insight into the target genes of TaMYB13-1, this work expressed the coding sequence of the TaMYB13-1 cDNA derived from correctly spliced mRNA under the control of the 6-SFT promoter from barley (Hv6-SFT) and rice rbcs 3′ region (Fig. 1A) in wheat, as Hv6-SFT is a well-studied fructosyltransferase gene and is expected to be expressed in organs where fructan accumulation normally occurs. The presence of TaMYB13-1 in T0 transgenic wheat

According to the author, TaMYB13-1 regulates fructan synthesis in wheat. The expression analysis using Affymetrix Wheat GeneChip Array showed significant changes in the expression levels of various genes involved in fructan synthesis. The determination of the genomic DNA sequences of TaMYB13-1 target genes revealed several potential binding sites. In vitro DNA-binding assays confirmed the interaction of TaMYB13-1 with cellulase D (CELD) as a reporter. Overall, the study provided insights into the target genes regulated by TaMYB13-1, contributing to a better understanding of fructan synthesis in wheat.
plants was verified by genomic quantitative PCR (data not shown), using primers that amplify the 3′-untranslated region of rice rbcS (Xue et al., 2011b). In the T₁ generation, expression of TaMYB13-1 transgene was confirmed in the mature leaves of transgenic plants using quantitative reverse-transcription PCR and rice rbcS 3′ region primers (data not shown). The T₂ plants of five independent TaMYB13-1 transgene expressing lines (a20, a21, b2, b13, and b36) were analysed for the expression level of TaMYB13-1 in the flag leaves at anthesis. The primers used amplified both the correctly spliced endogenous TaMYB13-1 as well as the transgene TaMYB13-1. The expression levels of TaMYB13-1 in the flag leaves of transgenic plants at anthesis ranged from 3- (a21) to 9-times (b36) higher than that of wild-type control plants (Bobwhite). Subsequently, the flag leaf and top internode samples of plants in the fructan accumulation phase (at anthesis) were selected for comparative analysis of expression levels. The average increases in the expression levels of TaMYB13-1 in the flag leaf and top internode (peduncle) of transgenic plants at anthesis, in comparison with wild-type control plants, are shown in Fig. 1B and C.

**Upregulation of TaMYB13-1 target genes in transgenic wheat**

Transgenic lines were analysed to examine changes in the expression levels of TaMYB13-1 target genes. Since the expression levels of many genes might be affected by the upregulation of a transcription factor, this work used the Affymetrix Wheat GeneChip to study the expression of the wheat transcriptome, comparing the three transgenic lines (a20, b2, and b36) containing the highest TaMYB13-1 expression with three wild-type control plants. The flag leaf samples were used for Affymetrix array analysis because it was thought that overexpression of TaMYB13-1 might also affect the expression levels of genes related to the sucrose synthetic pathway in the leaf. From the generated dataset, genes were selected with expression levels notably increased or decreased (factor 2; P < 0.05). Thirty-two probesets (27 genes) had an increased expression level in the transgenic lines compared to the control plants (Table 1), whereas 18 probesets (18 genes) had a lower expression level (Supplementary Table S1, available at JXB online). This study decided to focus on the upregulated genes, since this would more likely result in the identification of direct targets of TaMYB13-1.

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**Fig. 1.** Expression levels of TaMYB13-1 and its target genes in TaMYB13-1 transgenic lines and Bobwhite (BW) control plants. (A) TaMYB13-1 expression cassette. (B, C) Relative expression levels of TaMYB13-1 and its target genes in the flag leaf (B) and top internode (peduncle) (C) of the plants at anthesis; values are means ± SD of 4–5 independent T₂ transgenic lines or three biological replicates of Bobwhite; the mean expression value of each gene in the Bobwhite control group was set at 1. (D, E) Expression correlation between TaMYB13-1 and its putative target genes in the flag leaf (D) and top internode (E); the expression level in each sample is relative to the mean expression value in the Bobwhite control group, which was set at 1. * P < 0.05; ** P < 0.01.
As expected, the hybridization signal of the Ta.12834.1.S1_s_at probeset, which can hybridize three highly homologous TaMYB13 genes (TaMYB13-1, TaMYB13-2, and TaMYB13-3) in both correctly and wrongly spliced forms (Xue et al., 2011a) as well as the H6-SFT promoter-driven TaMYB13-1 transgene, was higher in the transgenic lines than control plants (Table 1). Strikingly, the Ta.24195.1.A1_at probeset (an unknown-function gene) had a 17-fold increase in expression in the transgenic lines compared to the control plants. Among the upregulated genes with known function, the only pathway that is overrepresented was the one related to fructan synthesis. Of 32 upregulated probesets, 10 are related to the sucrose (Ta.10107.2.S1_a_at, Ta.10107.2.S1_x_at, and Ta.10107.1.S1_at) and fructan (Ta.3475.2.S1_at, Ta.3475.1.A1_at, Ta.2788.1.A1_at, Ta.2789.1.S1_a_at, Ta.2789.2.S1_x_at, Ta.2789.2.S1_at, and Ta.2789.1.S1_at) synthetic pathways. Among these 10 probesets, five belong to Ta1-SST and Ta6-SFT, which can be transactivated by TaMYB13-1, as shown in the previous study (Xue et al., 2011a).

Interestingly, Ta1-FFT1 and Ta1-FFT2, represented by the probeset Ta.3475.2.S1_at, had an increased hybridization signal in transgenic lines, which was >5-times higher than non-transformed Bobwhite control plants (Table 1). A similar increase in the signal of the Ta1-FFT2-specific probeset was observed. These data indicate that TaMYB13-1 has an impact on the expression of all three families of fructosyltransferases in wheat. Besides fructosyltransferases, other upregulated genes relevant to the fructan synthesis were a fructokinase gene (TaFK1) represented by three probesets and a γ-vacuolar processing enzyme gene (Taγ-VPE1).

To validate the upregulation of these genes, their expression levels were measured by quantitative reverse-transcription PCR in both the flag leaf and top internode. As shown in Fig. 1B, the upregulation of all genes was observed in the flag leaf, although the marked increases in the Ta6-SFT2 and Ta1-FFT2 mRNA levels were not statistically significant due to the large variation in expression among individual transgenic lines (a20, b2, b13, and b36). Therefore, this work tested the correlation in expression between TaMYB13-1 and its upregulated genes. The expression correlations between TaMYB13-1 and these tested genes in the flag leaf were all statistically significant (Fig. 1D). Similar results were obtained in the top internode, where the expression levels of all these tested genes were significantly increased in the five transgenic lines and were significantly correlated with the TaMYB13-1 mRNA level, except for Ta1-FFT2 and TaFK1 (Fig. 1C and E).

Because fructosyltransferase proteins are located in the vacuole and because plant mature fructosyltransferases generally consist of two subunits derived from proteolytic cleavage of their pre-proteins except the 1-FFT from Helianthus tuberosus (Koops and Jonker, 1994, 1996; Sprenger et al., 1995; Van den Ende et al., 1996, 2000), it was suspected that the TaMYB13-1-upregulated vacuolar processing Taγ-VPE1 might be a candidate for processing the maturation of fructosyltransferase proteins. Therefore, this work examined whether Taγ-VPE1 and fructosyltransferase genes were closely co-regulated in the expression datasets. Correlation analysis showed that the Taγ-VPE1 mRNA levels were highly correlated with these fructosyltransferase mRNA levels in the stem of recombinant inbred lines with contrasting fructan levels, during stem development and among samples of transgenic and control plants (Supplementary Fig. S1).

Table 1. Genes increased in expression levels by at least 2-fold in TaMYB13-1-overexpressing transgenic lines compared to Bobwhite control plants in Affymetrix wheat genome array data

<table>
<thead>
<tr>
<th>Probeset</th>
<th>Description</th>
<th>EXPR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td>0.02</td>
</tr>
<tr>
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<td>Putative retro-element</td>
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<td>0.00</td>
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<td>Ta1-FFT2 and Ta1-FFT1</td>
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<td>0.04</td>
</tr>
<tr>
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<td>Ta1-FFT2</td>
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<td>0.02</td>
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Enhanced fructan accumulation in TaMYB13-1-overexpressing transgenic wheat

The expression data clearly points to the fructan synthesis pathway as a target for TaMYB13-1 regulation. It is known that a good correlation exists between Ta1-SST mRNA levels and its enzyme activity levels in wheat (Xue et al., 2011a),
as well as $Hv6-SFT$ mRNA levels and its enzyme activity levels in barley (Sprenger et al., 1995). Since genes from $Ta1-SST$ and $Ta6-SFT$ families, together with $Ta1-FFT$ family genes, were highly upregulated in the transgenic lines, the fructan and WSC concentrations in these transgenic lines were compared with wild-type control plants. As can be seen in Fig. 2A, the fructan levels in the top internode as well as the flag leaf were higher in all of the four transgenic lines than in the control plants. The increase in fructan levels in the flag leaf and top internode of the transgenic group was statistically significant in comparison with the wild-type control group (Fig. 2B). There was also a slight increase in the sucrose level in the top internode of transgenic lines, but not in the leaf (see TLC analysis in Fig. 3). Correlation analysis showed highly significant positive relationships between $TaMYB13-1$ mRNA levels and fructan levels in the flag leaf and top internode in the dataset of these transgenic and control plants (Fig. 2C and D). Also, the WSC concentrations in the flag leaf and top internode were increased by 36 and 17%, respectively ($P < 0.05$; Table 2). The increase of WSC concentration in the flag leaf and top internode in the transgenic plants compared to control plants corresponds almost exclusively to the increased portion of fructan accumulation (about 6 mg (g freshweight)$^{-1}$, Fig. 2B). TLC analysis clearly illustrates an increase in the levels of fructans with various polymerization degrees in the transgenic lines compared to control plants (Fig. 3). The difference is especially clear in the top internode samples at anthesis, where the fructans in the control plants were barely visible, whereas the transgenic plants accumulated a significant amount of fructans.

**Presence of $TaMYB13$-binding sites in regulatory regions of target genes**

As the $TaMYB13$-1-upregulated genes in its overexpressing transgenic plants can be direct or indirect target genes, this work investigated whether $TaMYB13$-1-binding sites

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**Fig. 2.** Fructan concentrations in the flag leaf and top internode of transgenic plants overexpressing $TaMYB13$-1 and relationship between $TaMYB13$-1 mRNA levels and fructan levels. (A) Fructan concentration changes in individual transgenic lines in comparison with Bobwhite control plants (BW43/44/45); values are means ± SD of three replicates from the same plant. (B) Group comparison of fructan concentrations; values are means ± SD of four independent transgenic lines or three biological replicates of Bobwhite (BW). (C, D) Relationship between $TaMYB13$-1 mRNA levels and fructan levels in the flag leaf (C) and the top internode (D) in the datasets of the transgenic and Bobwhite control plants; the $TaMYB13$-1 expression level in each sample is relative to the mean expression value in the Bobwhite control group, which was set at 1. All samples were collected at anthesis. *$P > 0.05$; **$P > 0.01$.**
were present in their promoter sequences as a supporting line of evidence for directly targeted genes. In the case of Ta1-SST and Ta6-SFT, Xue et al. (2011a) have shown that TaMYB13-binding sites are present in the regulatory regions of these genes. The wheat genome sequence database in the CerealsDB website (Wilkinson et al., 2012) was used to identify the regulatory regions of the genes that were upregulated in the transgenic lines. The obtained sequences and probable gene structures were assembled from accessions, as displayed in Supplementary Table S2.

This work was able to identify the regulatory regions of 11 genes (Table 3), in addition to the regulatory regions of Ta1-SST, Ta1-FFT1, Ta6-SFT1, and Ta6-SFT2, which were published previously (Nagaraj et al., 2001; Gao et al., 2010; Xue et al., 2011a). The length of the upstream regulatory regions identified ranged from 0.6 to 2 kb (Table 3). Most of these newly identified genes contained at least one core TaMYB13 DNA-binding sequence motif (DTTHGGT; Xue et al., 2011a) in their upstream regulatory regions. In addition to the presence of TaMYB13-binding motifs in the promoter regions of Ta1-SST, Ta6-SFT1, and Ta6-SFT2 (Xue et al., 2011a), Ta1-FFT1 contained three predicted TaMYB13-binding motifs in its upstream regulatory region and Taγ-VPE1 had two predicted motifs.

Several genes are known to be regulated by sequences that are present in their introns, for example AGAMOUS and SEEDSTICK (Deyholos and Sieburth, 2000; Kooiker et al., 2005). Therefore, this work searched for TaMYB13 DNA-binding motifs in the introns of the identified genes as well. As shown in Table 3, at least one binding site was present in the introns of nine of the analysed genes and three of them (TaFK1, Taγ-VPE1, and amino acid permease) even had four or more of these motifs in their introns.

TaMYB13 binding to target genes

Xue et al. (2011a) have shown that flanking regions play an important role in the determination of the binding affinity of TaMYB13-1. Therefore, CELD reporter-based in vitro DNA-binding assays were performed to determine the affinity of TaMYB13-1 to the identified motifs in the upstream regulatory regions of the following upregulated genes: Ta1-FFT1 (one site), TaMYB13-1 (one site), TaMYB13-2 (one site), TaMYB13-3 (one site), TaH2B (one site), and Taγ-VPE1 (two sites) (Fig. 4) and oligonucleotides 10 bp upstream and downstream of the core sequence of TaMYB13 DNA-binding motif were designed. As shown in Fig. 4, TaMYB13-1 bound strongly to the motifs present in the upstream regulatory regions of Ta1-FFT1 and Taγ-VPE1. The strongest interaction was the motif present in the promoter of Taγ-VPE1 site 1 at −969 (Fig. 4B), which was bound by TaMYB13-1 even stronger than SynO2, an in vitro TaMYB13-selected binding sequence (Xue et al., 2011a), which was used as a positive control. In addition, TaMYB13-1 was also able to bind weakly to the motifs present in the upstream regulatory regions of TaMYB13-1, TaMYB13-2 and TaMYB13-3 (Fig. 4C). No binding activity of TaMYB13-1 for the motif present in the H2B promoter region was found (Fig. 4C).

Expression profiles of TaMYB13-1 and its target genes are positively correlated during stem development and in recombinant inbred lines

Because the expression of a positive regulator and its target genes is generally correlated, this work examined the relationship between the expression levels of TaMYB13-1 and the new target genes identified in this study (Ta1-FFT1 and Taγ-VPE1) in developing stems (5 days before anthesis to 10 days after anthesis). Similarly to TaMYB13-1, both Ta1-FFT1 and Taγ-VPE1 transcript levels were markedly upregulated in the top internode (peduncle) at the stem developmental period examined (data not shown). High correlations were observed between the expression levels of TaMYB13-1 and its target genes Taγ-VPE1 and Ta1-FFT1 (Fig. 5A and B). These high expression correlations are similar to those seen...
Table 3. TaMYB13 DNA-binding motifs found in the regulatory regions of TaMYB13-1 target genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession number</th>
<th>Seq length</th>
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<th>MBS Intron</th>
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<tr>
<td>Ta1-FFT1</td>
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<td>1425</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Ta1-SST</td>
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<td>2244</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Ta6-SFT1</td>
<td>HQ383531</td>
<td>850</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Ta6-SFT2</td>
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<td>4</td>
<td>ND</td>
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<tr>
<td>TaMYB13-1</td>
<td>Assembled from sequence data in CerealsDB</td>
<td>760</td>
<td>1</td>
<td>1</td>
</tr>
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<td>TaMYB13-2</td>
<td>Assembled from sequence data in CerealsDB</td>
<td>762</td>
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</tr>
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<td>TaMYB13-3</td>
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<td>1</td>
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<tr>
<td>Ubiquitin histone H2B</td>
<td>Assembled from sequence data in CerealsDB</td>
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<td>0</td>
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<tr>
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<tr>
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<td>4</td>
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<tr>
<td>IAA31-auxin-responsive</td>
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<tr>
<td>Aux/IAA family member</td>
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<td>1</td>
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<td>Assembled from sequence data in CerealsDB</td>
<td>1831</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
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<td>760</td>
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<td>1831</td>
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<td>3</td>
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</tbody>
</table>

*Previously published by Xue et al. (2011a). ND, not determined.

Fig. 4. In vitro DNA-binding assays of predicted TaMYB13 DNA-binding motifs present in the promoter regions of newly identified target genes and TaMYB13 genes. (A) Predicted TaMYB13-1 DNA-binding sites in the upstream regulatory regions of Ta1-FFT1, TaVPE1, TaH2B, and TaMYB13, based on the core TaMYB13-1-binding sequence (DTTHGGT, where D = A, G, or T; H = A, C, or T). SynO2 is an in vitro TaMYB13-1-selected motif. (B, C) In vitro DNA-binding assays determining the binding of TaMYB13-1 to the predicted motifs. Relative TaMYB13-1-binding activity was measured as fluorescence unit (FU) released from the cleavage of methylumbelliferyl β-d-cellulobioside by CELD fused to TaMYB13-1 after 3 h of incubation at 40 °C. Displayed values are means ± SD of three replicates. Control is an oligo that does not contain TaMYB13-1-binding motifs; SynO2 was used as a positive control. * P < 0.05; ** P < 0.01; n.s., not significant.
between TaMYB13-1 and Ta1-SST or Ta6-SFT genes (Xue et al., 2011a).

To further investigate the expression correlation between TaMYB13-1 and Ta1-FFT1 or Ta1-VPE1, the Affymetrix wheat genome array GSE9767 data, deposited at the NCBI GEO website by Xue et al. (2008b) was analysed using eight independent recombinant inbred lines with two field replicates per line (16 samples) to investigate their relationships. High correlations were found between the hybridization signal levels of the TaMYB13 probeset and the targets Ta1-VPE1 or Ta1-FFT (Fig. 5C and D).

Yield-related phenotypes of transgenic lines overexpressing TaMYB13-1 under mild water-deficit conditions

WSC levels in wheat are known to be positively associated with grain yield under terminal drought conditions (Aggarwal and Sinha, 1984; van Herwaarden et al., 1998b; Xue et al., 2008b). Therefore, this work examined the yield-related phenotypes of transgenic plants at T3 generation grown under mild water-deficit conditions in comparison with Bobwhite control plants. As shown in Table 4, the transgenic lines (a20, a21, and b2) had increased values for all measured traits, although not all increases were statistically significant. The most prominent increase was observed in the total grain weight per plant, but this increase was not statistically significant (P = 0.09). Significant increases were observed for the top spike weight, average spike weight, and grain weight per spike.

Discussion

Investigation of regulatory networks involved in controlling fructan synthesis associated genes, which include fructosyltransferases and their modification and processing enzymes, in plants is an important topic of research. It will lead to understand the regulatory pathways of fructan synthesis and the critical genes associated with the high fructan accumulation trait in temperate cereals, as well as to facilitate future genetic manipulation of fructan accumulation for human health benefit of higher fructan plant products (Roberfroid, 2005) and potential improvement of crop yield in abiotic stress-prone environments. TaMYB13-1 has been shown to be a transcriptional activator of Ta1-SST and Ta6-SFT in wheat using a transient transactivation system. This study showed that overexpression of TaMYB13-1 in transgenic wheat resulted not only in increased expression levels of the genes directly involved in or associated with fructan synthesis, but also in increased fructan and WSC concentrations in transgenic wheat lines, thus demonstrating that TaMYB13-1 mediates the coordinated regulation of a major set of genes involved in fructan synthesis.

Affymetrix array expression analysis revealed that 27 genes were upregulated at least 2-fold in the leaves of TaMYB13-1-overexpressing transgenic lines compared to Bobwhite control plants. All Ta1-SST and Ta6-SFT genes represented by the probesets in the Affymetrix wheat genome array (Ta1-SST2, Ta6-SFT1, and Ta6-SFT2) were found to be upregulated in the transgenic lines, which supports the proposed TaMYB13-1 regulatory role based on the transient transactivation data (Xue et al., 2011a). Most interestingly, two probesets that belong to Ta1-FFT genes, the third family of fructosyltransferases, were also upregulated in TaMYB13-1-overexpressing lines. This is one of the novel TaMYB13-1 target genes identified in this study, as the regulation of this family of fructosyltransferases by TaMYB13-1 was not investigated in the previous study (Xue et al., 2011a).

Correlation analysis also showed that the expression levels of these three families of fructosyltransferase genes (Ta1-SST, Ta6-SFT, and Ta1-FFT) were highly correlated with TaMYB13-1 expression among the sample set of transgenic lines and Bobwhite control plants, as well as among samples obtained from various stem developmental stages (Xue et al., 2011a; this study). It has been shown that the expression profiles of Ta1-SST and Ta6-SFT genes are positively correlated with that of TaMYB13-1 in recombinant inbred lines (Xue et al., 2011a). Correlation analysis of the Affymetrix data previously deposited by Xue et al. (2008b) showed that the expression of the Ta1-FFT probeset (Ta.3475.2.S1_at, which hybridizes with both Ta1-FFT1 and Ta1-FFT2) was also positively correlated with that of the TaMYB13 probeset in the stem of recombinant inbred SB lines. The correlation coefficients between TaMYB13-1 and Ta1-FFT1 in the flag leaf and stem in the datasets of the transgenic and control plants were very high. This high correlation was also observed during stem development. The fact that there were very high expression correlations between TaMYB13-1 and Ta1-FFT1 in various genetic backgrounds as well as during stem development makes it likely that Ta1-FFT1 is also a direct target of TaMYB13-1. To further support this, TaMYB13-1 was able to bind strongly to a motif present in the upstream regulatory region of Ta1-FFT1 in vitro DNA-binding assays.

In addition to genes that are directly involved in the fructan synthetic pathway, two genes (Ta1-VPE1 and TaFK1) were found to be upregulated in the leaf of TaMYB13-1-overexpressing lines and could be linked indirectly to this pathway. TaFK1 was upregulated about 2.4-times in the leaves of transgenic lines, indicating a role of TaMYB13-1 in the regulation of the fructose metabolism. Fructokinases (EC 2.7.1.4) catalyse the conversion of fructose to d-fructose-6-phosphate and have been shown to be induced upon the external application of fructose, glucose, and sucrose in tomato (Schaffer and Petreiko, 1997). Davies et al. (2005) have shown that in potato fructokinases are able to balance sucrose synthesis and metabolism in concert with sucrose synthase, which converts sucrose into fructose and UDP-d-glucose. Free fructose can also come from sucrose hydrolysis by invertase as well as from fructan exohydrolase trimming of fructans as a part of the fructan synthesis process (Bancal et al., 1992; Van den Ende et al., 2003; Lohier et al., 2007). The increased demand for sucrose in the cells
that express the elevated levels of fructosyltransferases might be partly offset by the increase in fructokinase, since the product of this enzyme, D-fructose-6-phosphate, is a substrate for sucrose-phosphate synthase. Therefore, an increase in fructokinase would favour carbon flow towards fructan accumulation. However, it is unclear if this regulation is direct or indirect, since this work was not able to find any TaMYB13-binding motifs in the upstream regulatory region of this gene. However, there were three TaMYB13-binding motifs present in the first intron of this gene, so the direct regulation of this gene by TaMYB13-1 cannot be excluded.

The expression of TaMYB13-1 and TaFK1 in the leaf correlates significantly in the dataset of the transgenic versus control plants, but was not statistically significant in the stem, which indicates that the expression of TaFK1 in the stem might be regulated predominantly by other transcription factors.

The other TaMYB13-1-upregulated gene that might be indirectly linked to the fructan synthesis is Taγ-VPE1, which may be involved in processing vacuolar fructosyltransferase proteins. VPE proteins are vacuolar cysteine proteases, known to cleave natural substrates in plants after asparagine residues and involved in processing the maturation or degradation of many vacuolar proteins (Yamada et al., 2005; Tsiatsiani et al., 2012). Known natural substrates of plant VPEs include storage proteins, vacuolar invertase, and carboxypeptidase Y (Hara-Nishimura et al., 1991; Shimada et al., 2003; Tsiatsiani et al., 2012). In Arabidopsis, four VPEs

Table 4. Yield-related phenotypes of TaMYB13-1-overexpressing transgenic wheat

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>TaMYB13-1 transgenic lines</th>
<th>Bobwhite control</th>
<th>P-value</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiller number/plant</td>
<td>11.7 ± 0.67</td>
<td>11.5 ± 0.44</td>
<td>0.74</td>
<td>1.7</td>
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<tr>
<td>Total spike weight/plant (g)</td>
<td>36.6 ± 3.83</td>
<td>32.9 ± 1.01</td>
<td>0.17</td>
<td>11.2</td>
</tr>
<tr>
<td>Total grain weight/plant (g)</td>
<td>28.8 ± 2.47</td>
<td>25.5 ± 0.44</td>
<td>0.09</td>
<td>12.9</td>
</tr>
<tr>
<td>Top spike weight (g)</td>
<td>4.22 ± 0.25</td>
<td>3.80 ± 0.04</td>
<td>0.05</td>
<td>11.1</td>
</tr>
<tr>
<td>Average spike weight (g)</td>
<td>3.14 ± 0.16</td>
<td>2.87 ± 0.05</td>
<td>0.05</td>
<td>9.4</td>
</tr>
<tr>
<td>Grain weight/spike (g)</td>
<td>2.47 ± 0.09</td>
<td>2.23 ± 0.07</td>
<td>0.02</td>
<td>10.8</td>
</tr>
<tr>
<td>Hundred grain weight (g)</td>
<td>4.80 ± 0.19</td>
<td>4.70 ± 0.18</td>
<td>0.51</td>
<td>2.1</td>
</tr>
<tr>
<td>Total grain number/plant</td>
<td>600 ± 67</td>
<td>547 ± 27</td>
<td>0.27</td>
<td>9.7</td>
</tr>
</tbody>
</table>
have been identified that are partially redundant in storage protein cleavage (Gruis et al., 2004). Some VPEs have also been reported to play a role in programmed cell death (Hayashi et al., 2001; Rojo et al., 2004; Harai-Nishimura and Hatsugai, 2011). Several groups have shown that fructosyltransferases in planta generally consist of two subunits generated from the cleavage of fructosyltransferase pre-proteins (Sprenger et al., 1995; Koops and Jonker, 1996; Van den Ende et al., 1996, 2000). Sprenger et al. (1995) reported that the cleavage of barley 6-SFT (Hv6-SFT) occurs between Asn and an EAD triplet. This cleavage site fits into the substrate specificities of VPEs. Wheat Ta1SST and Ta6SFT pre-proteins contain the cleavage site of NEAD, whereas Ta1FT pre-proteins have a similar site: NEVD. From the recent review on the natural substrates of plant proteases (Tsatsiani et al., 2012), it appears that only VPEs cleave the Asn–other amino acid residue bond in natural substrates known to date. Although the uncleaved version of a number of recombinant fructosyltransferases expressed in Pichia pastoris is functional (Lüscher et al., 2000; Altenbach et al., 2004; Van den Ende et al., 2006, 2011; Lasseur et al., 2011), some plant fructosyltransferases expressed in the yeast showed almost undetectable activities (Hisano et al., 2008; Lasseur et al., 2011). It is likely that this cleavage is necessary for increase in activity in planta or stability of fructosyltransferase proteins in the vacuole.

Analysis of the Affymetrix data of recombinant inbred lines showed that the expression of Taγ-VPE1 was highly correlated with the expression of TaMYB13-1 in recombinant inbred lines. Significant correlation was observed in the developing stem samples and in the transgenic/control plant datasets. Interestingly, the upstream regulatory region of this gene contains two motifs and one of them was bound very strongly by TaMYB13-1 in vitro DNA-binding assays, indicating that this gene is likely to be directly regulated by TaMYB13-1. Close expression correlations were found between Taγ-VPE1 and fructosyltransferase genes in the stem in all expression datasets. Close co-regulation of Taγ-VPE1 with fructosyltransferase genes within the TaMYB13-1 regulatory network together with the cleavage sites of fructosyltransferases fitting into the substrate specificity of Taγ-VPE1 point to the potential involvement of Taγ-VPE1 in processing the maturation of fructosyltransferases in the vacuole in wheat. These findings will lead to a new exciting research topic on the potential role of γ-VPE1 in modulating fructan accumulation by its ability in potential enhancement of fructosyltransferase activity as discussed above.

Although TaMYB13-1 is highly expressed in organs where fructans accumulate at high levels, it is also expressed in other organs where fructan synthetic activity is low, such as mature leaf. In particular, the highly homologous genes of TaMYB13 exist in non-fructan accumulating plant species such as Arabidopsis and rice. For example, the TaMYB13 homologue in Arabidopsis, AtMYB59, is involved in root growth and cell cycle (Mu et al., 2009). Therefore, it is likely that TaMYB13-1 also plays a role in regulation of other processes. Mu et al. (2009) published a list of upregulated genes in transgenic Arabidopsis that overexpresses AtMYB59. Although the promoters used for driving the expression of AtMYB59 and TaMYB13-1 were different (cauliflower mosaic virus 35S vs. Hv6-SFT) and the organ used in their study was also different from the organ used in this study (flag leaf vs. 12-day-old seedlings), this work was able to find nine genes that had an increased expression in both datasets (Supplementary Table S3). The upregulation of these common genes was relatively small, ranging from 1.21 to 2.42-times higher in TaMYB13-1-overexpressing lines than the control plants, but statistically significant (P < 0.05). This work was also able to find multiple core TaMYB13 DNA-binding motifs in the upstream regulatory region and/or introns of all these common targets in Arabidopsis (data not shown) except for At4g25630 and At5g60520. However, as the levels of the increase in the expression of most of these genes in the TaMYB13-1-overexpressing lines were low, it is likely that TaMYB13-1 plays only a minor role in the regulation of these genes. It is also interesting to see that TaMYB13-1 is able to bind to its own regulatory region, indicating a potential feedback loop, although its affinity to the motif was very low. Feedback loops have been reported previously for MYB transcription factors, such as CCA1 in Arabidopsis (Wang and Tobin, 1998).

It appears that an increase in the expression level of a single regulator has an impact on fructan and WSC accumulation. A significant increase in fructan and WSC concentrations was found in the leaf and top internode of TaMYB13-1-overexpressing lines, compared to wild-type control plants. The increase of fructans in these two organs was 2.2-fold in the flag leaf, and 15.4-fold in the top internode. WSC levels in these organs were also significantly increased in the transgenic lines. This increase was largely attributed to the enhanced accumulation of the fructan component of the WSCs. These data are in line with results that there is a high correlation between TaMYB13-1 expression levels and WSC or fructan levels in recombinant inbred lines (Xue et al., 2011a). In fact, in the datasets of the transgenic lines and control plants, very high correlations were observed between the levels of TaMYB13-1 mRNA and WSC or fructan in both the flag leaf and the top internode.

WSC levels and wheat yield under terminal drought conditions are known to be positively associated. The overexpression of TaMYB13-1 in transgenic plants resulted in an increase in spike weight and grain weight per spike under mild water-limited conditions. It is likely that the increased grain weight per spike in the transgenic plants is attributed to the enhanced accumulation of fructans in the stem, which supplies the increased amount of carbon reserve to the spike for grain filling. In addition, the grain weight per plant was increased by 13%, although the difference was not significant at the P-value level of 0.05. In view of that the contribution of the stored fructan to grain yield under terminal drought environments can also be attributed to the levels of fructan hydrolysis enzymes (fructan exohydrolases) during the fructan remobilization phase (Joudi et al., 2012), simultaneous manipulation of fructan exohydrolases...
may further improve grain yield through enhancing fructan remobilization.

In summary, this study clearly demonstrates that TaMYB13-1 can act as a positive regulator for regulation of all three families of fructosyltransferase genes (including Ta1-FFT), which are directly involved in the fructan synthetic pathway in wheat. TaMYB13-1 regulates the fructan synthetic pathway by direct binding to the regulatory regions of fructosyltransferase genes. This TaMYB13-1 regulatory network is upregulated by sucrose and stem developmental signal (Xue et al., 2011a), although besides sucrose what other factor is responsible for triggering fructan accumulation during stem development in wheat is still unclear. TaMYB13-1 is also involved in direct upregulation of Taγ-VPE1 through the binding to its promoter and Taγ-VPE1 may be involved in processing the maturation of fructosyltransferases for enhancing their activities. The currently proposed regulatory model of TaMYB13-1 is illustrated in Fig. 6. Together, these genes form a regulon for modulating fructan accumulation in the vacuole, which is mediated by the sucrose signalling pathway that involves protein kinases and phosphatases as previously reported (Martínez-Noël et al., 2001, 2009; Kusch et al., 2009; Ritsema et al., 2009). Whether involvement of these protein kinases and phosphatases in regulating the fructan synthesis is via the modification of TaMYB13-1 activity awaits future investigation, as the post-translational modification of transcription factors is a common mechanism in gene regulation, such as plant bZIP factors (Schütze et al., 2008). It appears that overexpression of TaMYB13-1 in transgenic wheat is sufficient to increase fructan concentrations in the leaf and stem. However, further studies are required to see whether an increase in fructan accumulation through overexpression of TaMYB13-1 is able to improve grain yield in terminal abiotic stress environments in the field, such as drought and heat stress at the reproductive stage of wheat.

**Supplementary material**

Supplementary data are available at *JXB* online.

**Supplementary Table S1.** Probesets that are downregulated at least 2-fold in TaMYB13-1-overexpressing transgenic lines compared to Bobwhite control plants.

**Supplementary Table S2.** Accession numbers of sequences used to assemble the genomic sequences of genes that are upregulated by TaMYB13-1 obtained by blast search in the wheat genome sequence database of CerealsDB.

**Supplementary Table S3.** Common upregulated target genes between TaMYB13-1 (this study) and AtMYB59-overexpressing transgenic plants.

**Supplementary Fig. S1.** Correlation in expression between Taγ-VPE1 and fructosyltransferase genes.

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