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RESEARCH PAPER

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 glycoside hydrolase 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) (Lüscher *et al*., 1996; Müller *et al*.,

2000; Kawakami and Yoshida, 2002, 2005; Ritsema and Smeekens, 2003b).

Fructans are accumulated when the production of photoassimilates exceeds the need (Pollock, 1984). In wheat, stems are the major storage sites of fructans (Ruuska *et al.*, 2008). Fructans are the major component of water-soluble carbohydrates (WSCs) in the fructan accumulation phase in wheat stems (Ruuska *et al.*, 2006). WSCs in the stem in the peak accumulation phase, which are mainly fructans, can account for over 40% of the stem dry weight (Blacklow *et al.*, 1984) and are important carbon reserve for grain filling in temperate cereals. The contribution of stored WSCs to grain filling ranges from 20 to 45%, depending on the incorporation of respiration and other carbon sinks (Austin *et al.*, 1977; Bonnett and Incoll, 1992, 1993; van Herwaarden *et al.*, 1998a; Wardlaw and Willenbrink, 2000). WSCs in stems become even more important under drought stress conditions where the contribution of stem reserves to grain yield increases significantly (Aggarwal and Sinha, 1984; van Herwaarden *et al.*, 1998b). Positive association of stem WSC concentration with grain weight or yield in wheat has been observed in many studies, particularly under water-limited environments (Foulkes *et al.*, 2002; Asseng and van Herwaarden, 2003; Xue *et al.*, 2008b, 2009; McIntyre *et al.*, 2011, 2012). This positive association is mainly attributed to the fructan component of the stem WSCs.

Besides being important for temporary carbon storage, fructans have also been reported to protect cells from drought and cold stress. Pilon-Smits *et al.* (1995) have shown improvement of drought tolerance of transgenic tobacco plants that express a bacterial fructosyltransferase, and Kawakami *et al.* (2008) have found the increased cold tolerance of rice plants expressing *1-SST* or *6-SFT* from wheat. This increased tolerance is likely due to their stabilizing effect on the membranes of the stressed cells and serving as an antioxidant (Demel *et al.*, 1998; Hincha *et al.*, 2000; Kawakami *et al.*, 2008; Valluru and Van den Ende, 2008, 2009; Livingston *et al.*, 2009; Bolouri-Moghaddam *et al.*, 2010).

The coding sequences of many fructosyltransferases have been isolated from various plant species. Sprenger *et al.* (1995) reported the isolation of the *6-SFT* cDNA from barley, followed by the publication of many other fructosyltransferases from different species (de Halleux and Cutsem, 1997; Hellwege *et al.*, 1997; Vijn *et al.*, 1997, 1998; Van der Meer *et al.*, 1998; Van den Ende *et al.*, 2000, 2006; Kawakami and Yoshida, 2002, 2005; Lasseur *et al.*, 2006, 2011). Enzyme activities and the expression of the genes encoding these enzymes have been shown to be regulated by various signals. For example, sucrose, besides being a substrate for the fructan synthesis, has been suggested as a signalling molecule that is able to upregulate the expression of genes in the fructan synthetic pathway (Wagner *et al.*, 1986; Müller *et al.*, 2000; Koroleva *et al.*, 2001; Martínez-Noël *et al.*, 2001, 2009, 2010; Nagaraj *et al.*, 2001, 2004; Lu *et al.*, 2002; Ruuska *et al.*, 2008, Ritsema *et al.*, 2009; Xue *et al.*, 2011a, 2013). Other factors that play an important role in the fructan synthetic pathway are protein kinases and phosphatases (Martínez-Noël *et al.*, 2001, 2009, 2010; Kusch *et al.*, 2009; Ritsema *et al.*, 2009).

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 the manufacturer's instruction.

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 *TaRP15*, RNA polymerases I, II, and III 15 kD subunit (Xue *et al.*, 2008b). Primers used for the amplification of *Ta1-SST1*, *Ta1-SST2*, *Ta6-SFT1*, *Ta6-SFT2*, *Ta1-FFT1*, *Ta1-FFT2*, *TaRP15*, and *TaRPII36* were as published by Xue *et al.* (2008a, b, 2011a). The following real-time PCR primer pairs were used for the quantification of *Taγ-VPE1* and *TaFK1* mRNA levels: 5′-CGAGCTGATTGGAAACCTTCT-3′ (forward) and 5′-AGCGAGCCACATTGTGATTCAA-3′ (reverse) for *Taγ-VPE1*; 5′-TCTTGGAGATCAAGGATGCAAGT-3′ (forward) and 5′-CACCAGCACCTGTCGTATCAAC-3′ (reverse) for *TaFK1*.

Transformation of wheat with TaMYB13-1 *construct*

TaMYB13-1 expression construct (Hv6-SFT:TaMYB13-1:ricerbcS3′) 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 act1: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 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:rice rbcS 3′ 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 1000 bp 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 µl^{-1} shared herring sperm DNA, 0.3 mg ml^{-1} bovine serum albumin, 10% glycerol, and 0.025% Nonidet P-40. For each assay, 40 000 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-cellobioside 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 T_0 transgenic wheat

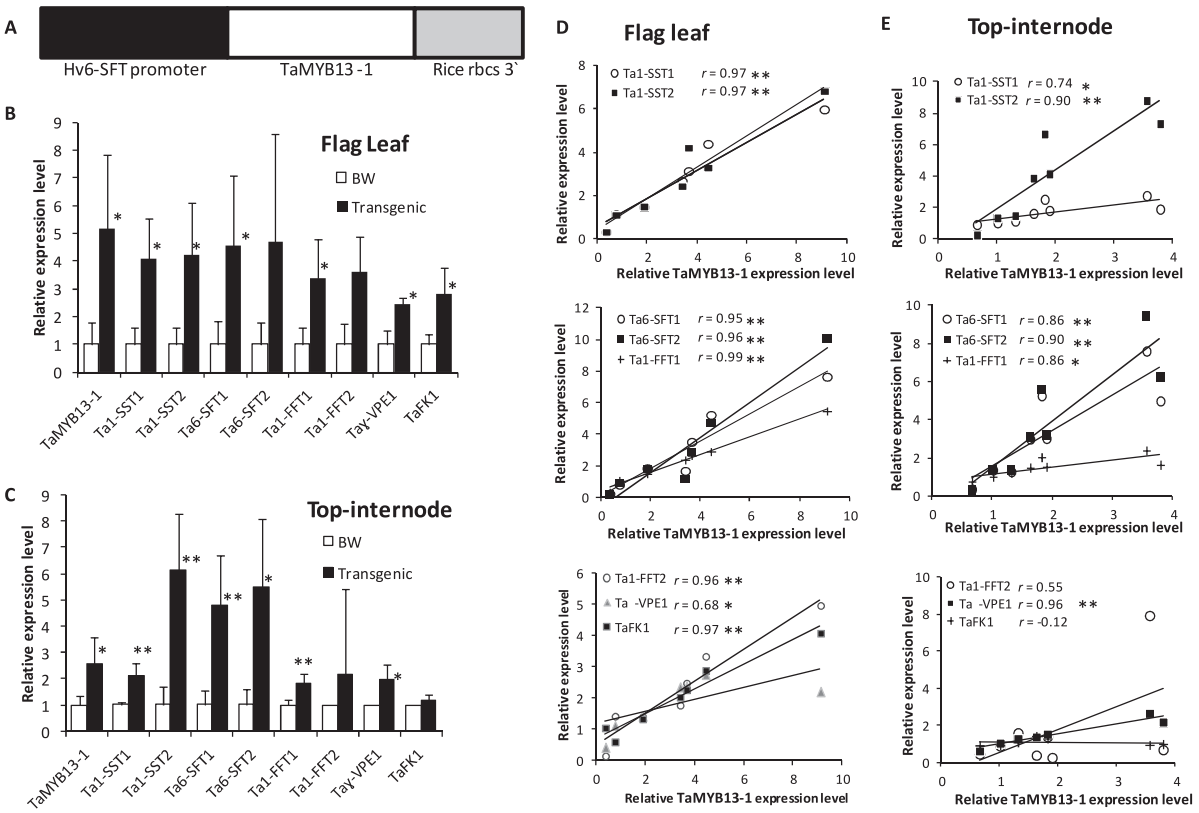

Fig. 1. Expression levels of *TaMYB13-1* and its target genes in *TaMYB13-1* transgenic lines and Bobwhite (BW) control plants. (A) *TaMYB13-1* expression cassette. (B, C) Relative expression levels of *TaMYB13-1* and its target genes in the flag leaf (B) and top internode (peduncle) (C) of the plants at anthesis; values are means ± SD of 4–5 independent T_2 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. * $P < 0.05$; ** $P < 0.01$.

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 T_1 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 T_2 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.

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 *Hv6-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*

Values are expression ratio (EXPR) of the mean of three independent transgenic lines to the mean of three biological replicates of Bobwhite.

Probeset	Description	EXPR	*P*-value
Ta.24195.1.A1_at	Unknown protein	17.17	0.02
TaAffx.71942.1.A1_at	Putative retro-element	5.42	0.00
Ta.3475.2.S1_at	Ta1-FFT2 and Ta1-FFT1	5.32	0.04
Ta.3475.1.A1_at	Ta1-FFT2	4.35	0.02
TaAffx.83540.1.S1_at	Unknown protein	4.60	0.00
Ta.2788.1.A1_at	Ta1-SST2	4.20	0.02
Ta.2789.1.S1_a_at	Ta6-SFT2	3.92	0.03
Ta.2789.2.S1_x_at		3.31	0.03
Ta.2789.2.S1_at		2.74	0.03
Ta.2789.1.S1_at	Ta6-SFT1	3.11	0.02
Ta.12834.1.S1_s_at	TaMYB13-1, TaMYB13-2, and TaMYB13-3	3.73	0.05
Ta.12258.1.A1_at	Unknown protein	3.38	0.00
Ta.7378.5.A1_at	Histone H2B	3.10	0.04
Ta.13965.1.S1_at	Ubiquitin-protein ligase	2.88	0.04
Ta.30798.3.S1_at	Vacuolar processing enzyme 3 (Taγ-VPE1)	2.85	0.02
Ta.15881.1.S1_at	Eukaryotic translation initiation factor	2.61	0.02
TaAffx.19583.2.A1_at	Microtubule-associated protein	2.61	0.00
TaAffx.59883.1.S1_at	Putative amino acid permease	2.50	0.05
Ta.10107.2.S1_a_at	Fructokinase (TaFK1)	2.50	0.01
Ta.10107.2.S1_x_at		2.49	0.02
Ta.10107.1.S1_at		2.18	0.02
Ta.25919.1.S1_at	MYB transcription factor	2.45	0.04
Ta.23069.1.S1_at	IAA31-auxin-responsive Aux/IAA family member	2.42	0.05
TaAffx.43914.1.S1_s_at	Unknown	2.41	0.02
Ta.1549.1.S1_at	Aspartyl protease family protein	2.40	0.00
Ta.20658.1.S1_a_at	Putative lipase	2.36	0.01
Ta.10838.1.S1_at	CCT motif family protein	2.31	0.03
Ta.10838.1.S1_x_at		2.17	0.04
Ta.10772.1.A1_at	Nodulin-like protein	2.26	0.03
Ta.28063.1.S1_x_at	Glycine-rich protein	2.21	0.01
Ta.20658.2.S1_x_at	Esterase precursor	2.16	0.03
Ta.27746.1.S1_at	Unknown protein	2.05	0.03

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*.,

2008b), 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

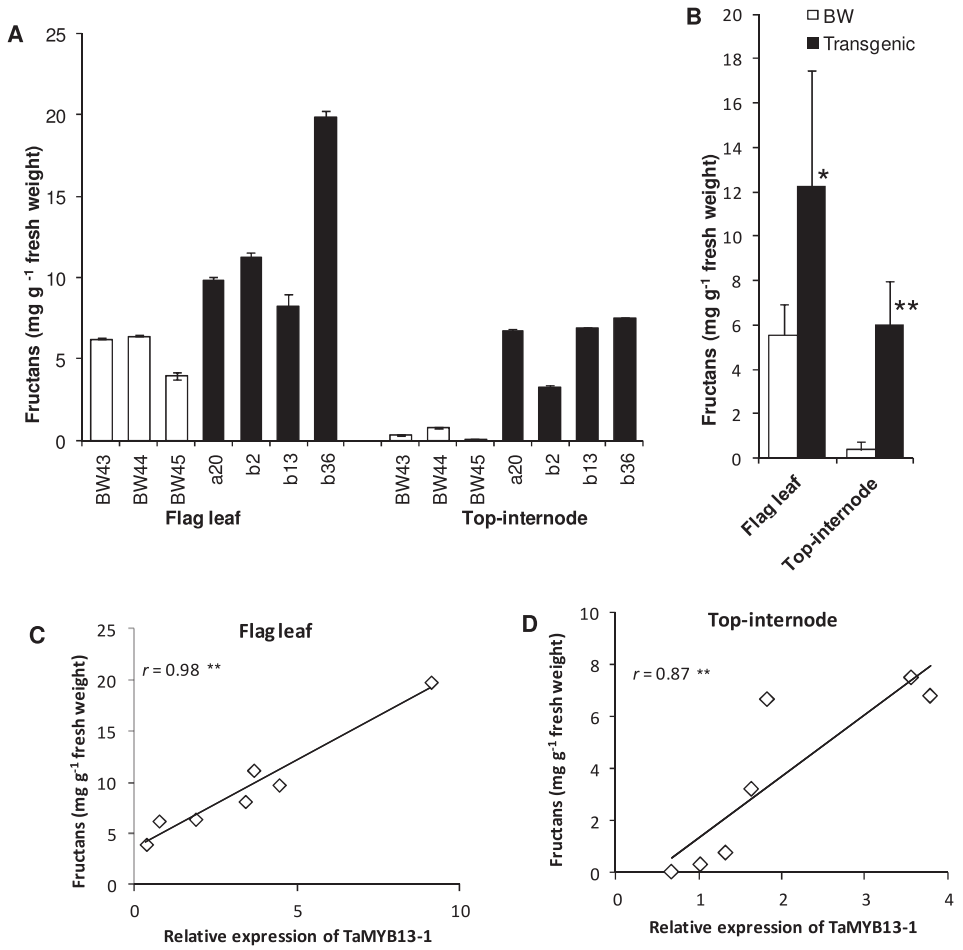

Fig. 2. Fructan concentrations in the flag leaf and top internode of transgenic plants overexpressing *TaMYB13-1* and relationship between *TaMYB13-1* mRNA levels and fructan levels. (A) Fructan concentration changes in individual transgenic lines in comparison with Bobwhite control plants (BW43/44/45); 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. * $P > 0.05$; ** $P > 0.01$.

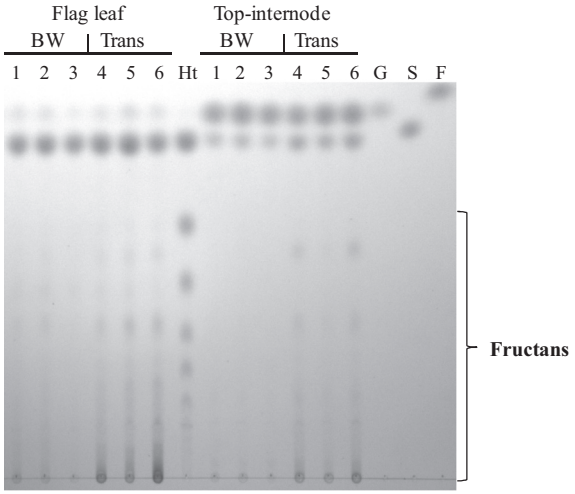

Fig. 3. Thin-layer chromatogram of WSC extracts from the flag leaf and top internode of Bobwhite (BW) control plants and transgenic lines (Trans) overexpressing *TaMYB13-1*. Increase in fructan levels in transgenic lines is apparent both in the flag leaf and top internode. Lanes 1–3, BW43/44/45; lanes 4–6, *TaMYB13-1* transgenic lines (a20, b2, and b36); lane Ht, WSC extract from *Helianthus tuberosus*. F, Fructose; G, glucose; S, sucrose.

Table 2. *Water-soluble carbohydrate concentrations in the flag leaf and top internode at anthesis from Bobwhite control plants and TaMYB13-1-overexpressing transgenic plants*

Values are means ± SD of three biological replicates of Bobwhite or five independent *TaMYB13-1* transgenic lines (a20, a21, b2, b13, b36).

	WSC in flag leaf (mg (g freshweight)$^{-1}$)	WSC in top internode (mg (g freshweight)$^{-1}$)
Bobwhite	18.20±2.59	32.03±2.80
Transgenic	24.73±5.15*	37.58±2.90*
% increase	35.9	17.3

WSC, water-soluble carbohydrate. * $P < 0.05$.

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*

Seq Length is the length of the upstream regulatory region sequence that this study was able to identify. MBS URS displays the number of predicted TaMYB13 DNA-binding motifs found in the upstream regulatory region and MBS Intron displays the number of predicted TaMYB13 DNA-binding motifs in introns. Accessions of sequences assembled from sequence data in CerealsDB can be found in Supplemental Table S2.

Target	Accession number	Seq length	MBS URS	MBS Intron
Ta1-FFT1	FJ361762	1425	3	0
Ta1-SST	FJ228689	2244[a]	2[a]	ND
Ta6-SFT1	HQ738531	850[a]	3[a]	ND
Ta6-SFT2	HQ738530	1001[a]	4[a]	ND
TaMYB13-1	Assembled from sequence data in CerealsDB	760	1	1
TaMYB13-2	Assembled from sequence data in CerealsDB	762	1	1
TaMYB13-3	Assembled from sequence data in CerealsDB	752	1	1
Putative histone H2B	Assembled from sequence data in CerealsDB	960	1	0
Ubiquitin-protein ligase	Assembled from sequence data in CerealsDB	622	0	1
Tay-VPE1	Assembled from sequence data in CerealsDB	1140	2	5
Putative amino acid permease	Assembled from sequence data in CerealsDB	1061	0	5
TaFK1	Assembled from sequence data in CerealsDB	1209	0	4
MYB transcription factor	Assembled from sequence data in CerealsDB	1918	1	0
IAA31-auxin-responsive Aux/IAA family member	Assembled from sequence data in CerealsDB	1172	0	1
CCT motif family protein	Assembled from sequence data in CerealsDB	1831	1	3

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

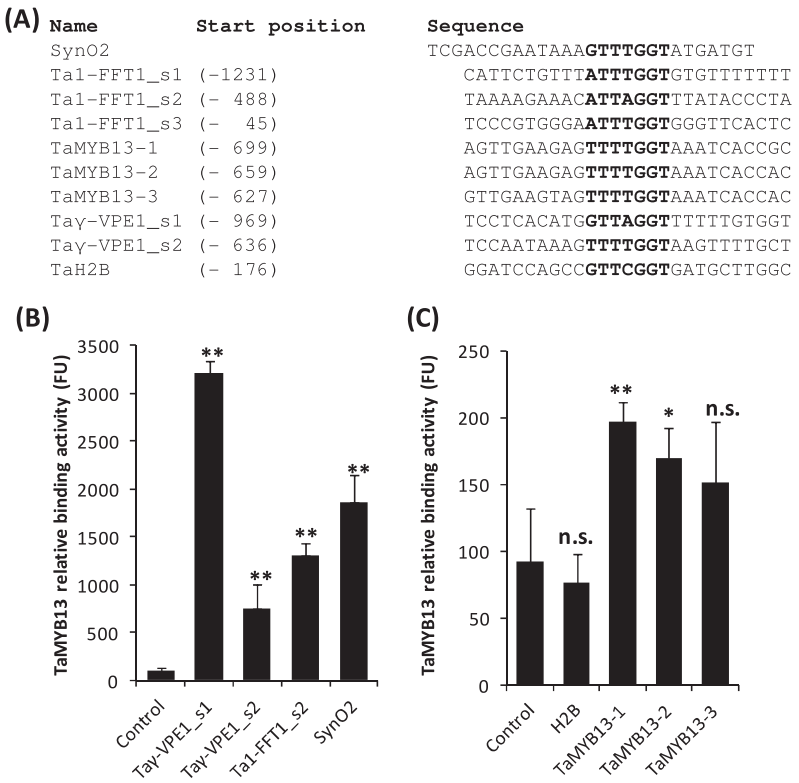

Fig. 4. *In vitro* DNA-binding assays of predicted TaMYB13 DNA-binding motifs present in the promoter regions of newly identified target genes and *TaMYB13* genes. (A) Predicted TaMYB13-1 DNA-binding sites in the upstream regulatory regions of *Ta1-FFT1*, *Tay-VPE1, TaH2B*, and *TaMYB13*, based on the 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 *Taγ-VPE1*, the Affymterix 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 *Taγ-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 T_5 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, 2007) 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 *in vitro* DNA-binding assays.

In addition to genes that are directly involved in the fructan synthetic pathway, two genes (*Taγ-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 Petreikov, 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; Lothier *et al*., 2007). The increased demand for sucrose in the cells

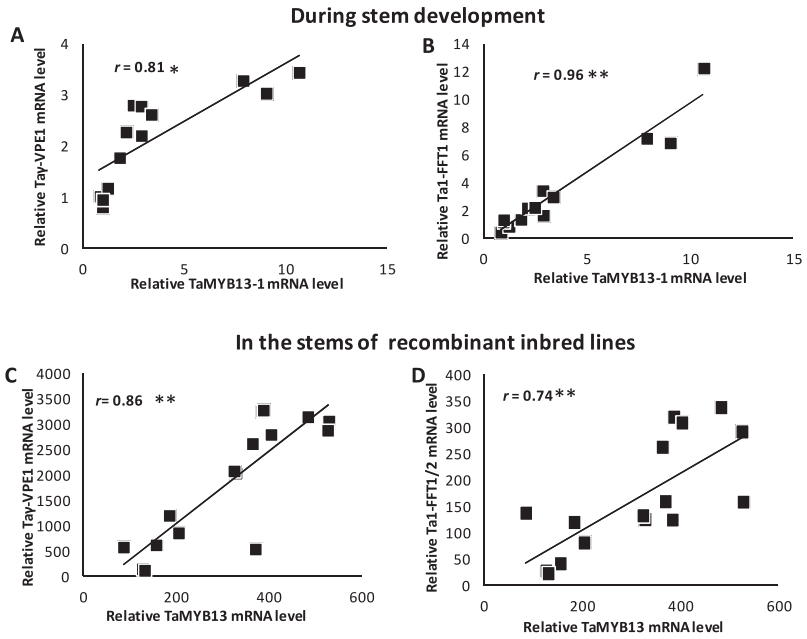

Fig. 5. Correlation in expression between *TaMYB13-1* and *Ta1-FFT* or *Taγ-VPE1* in developing stems and in the stems of recombinant inbred lines. (A, B) Expression correlation in developing stems analysed by quantitative reverse-transcription PCR. (C, D) Expression correlation in the stems of recombinant inbred lines from Affymterix wheat genome array GSE9767 data, deposited at the NCBI GEO website by Xue *et al*. (2008b). The probesets used for analysis are: Ta.30798.3.S1_at (*Taγ-VPE1*), Ta.3475.2.S1_at (*Ta1-FFT1* and *Ta1-FFT2*), and Ta.12834.1.S1_s_at (*TaMYB13-1, TaMYB13-2* and *TaMYB13-3*). Values are Affymetrix array hybridization signals.

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*

Values shown are means ± SD (n = 3). Pots of 14.3-l capacity with a 29-cm top diameter were used for growing transgenic plants (three pots) and wild-type Bobwhite (three pots). Each pot grew six plants. The plants were grown under a mild water-deficit regime. Individual phenotypic data derived from each pot were used for calculation of the mean values of each group. For *TaMYB13-1* transgenic lines, mean values are derived from three independent transgenic lines (a20, a21, and b2) at the T_5 stage.

Phenotype	TaMYB13-1 transgenic lines	Bobwhite control	*P*-value	% increase
Tiller number/plant	11.7 ± 0.67	11.5 ± 0.44	0.74	1.7
Total spike weight/plant (g)	36.6 ± 3.63	32.9 ± 1.01	0.17	11.2
Total grain weight/plant (g)	28.8 ± 2.47	25.5 ± 0.44	0.09	12.9
Top spike weight (g)	4.22 ± 0.25	3.80 ± 0.04	0.05	11.1
Average spike weight (g)	3.14 ± 0.16	2.87 ± 0.05	0.05	9.4
Grain weight/spike (g)	2.47 ± 0.09	2.23 ± 0.07	0.02	10.8
Hundred grain weight (g)	4.80 ± 0.19	4.70 ± 0.18	0.51	2.1
Total grain number/plant	600 ± 67	547 ± 27	0.27	9.7

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; Hara-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 specificity of VPEs. Wheat Ta1-SST and Ta6-SFT pre-proteins contain the cleavage site of NEAD, whereas Ta1-FFT pre-proteins have a similar site: NEVD. From the recent review on the natural substrates of plant proteases (Tsiatsiani *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 *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

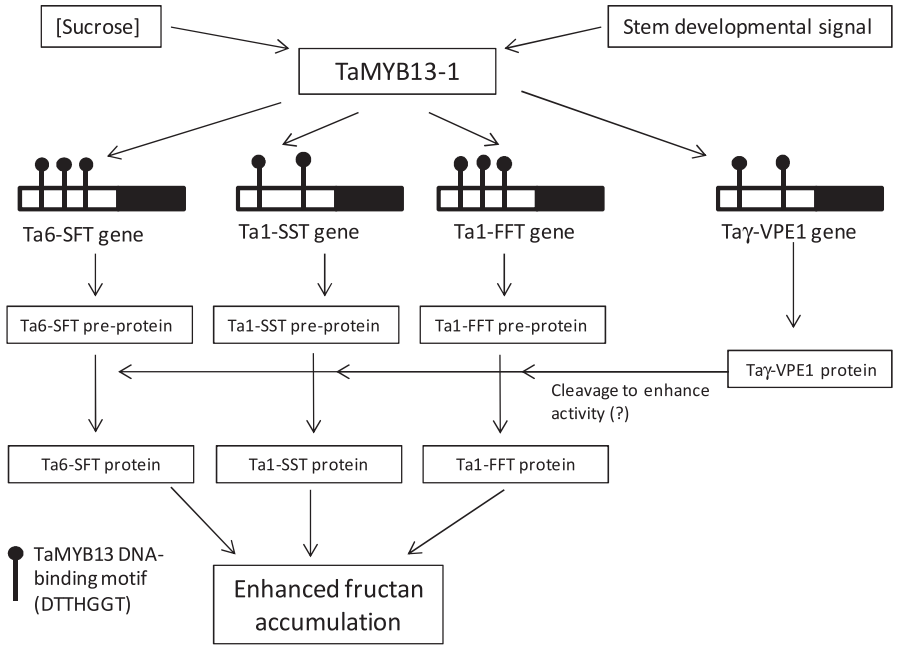

Fig. 6. Illustration of the proposed regulatory model of TaMYB13-1 for fructan synthesis in temperate cereals. The TaMYB13-binding sites in *Ta6-SFT1*, *Ta1-SST*, *Ta1-FFT1*, and *Taγ-VPE1* promoters are indicated. The γ-VPE protein might be involved in the maturation of fructosyltransferases in the vacuole by cleavage of the fructosyltransferase pre-proteins into two subunits (mature fructosyltransferases).

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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