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A wheat *1-FEH w3* variant underlies enzyme activity for stem WSC remobilization to grain under drought

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Summary

- In wheat stems, the levels of fructan-dominated water-soluble carbohydrates (WSC) do not always correlate well with grain yield.
- Field drought experiments were carried out to further explain this lack of correlation. Wheat (*Triticum aestivum*) varieties, Westonia, Kauz and *c*. 20 genetically diverse double haploid (DH) lines derived from them were investigated.
- Substantial genotypic differences in fructan remobilization were found and the *1-FEH w3* gene was shown to be the major contributor in the stem fructan remobilization process based on enzyme activity and gene expression results. A single nucleotide polymorphism (SNP) was detected in an auxin response element in the *1-FEH w3* promoter region, therefore we speculated that the mutated Westonia allele might affect gene expression and enzyme activity levels. A cleaved amplified polymorphic (CAP) marker was generated from the SNP. The harvested results showed that the mutated Westonia *1-FEH w3* allele was associated with a higher thousand grain weight (TGW) under drought conditions in 2011 and 2012.
- These results indicated that higher gene expression of *1-FEH w3* and *1-FEH w3* mediated enzyme activities that favoured stem WSC remobilization to the grains. The CAP marker residing in the *1-FEH w3* promoter region may facilitate wheat breeding by selecting lines with high stem fructan remobilization capacity under terminal drought.

Introduction

Wheat is one of the major crops worldwide and increased grain yield is a major objective of wheat breeding. In recent decades, droughts have severely limited grain yields. Global warming is expected to further add to the severity and frequency of drought. Hence, drought stress becomes an increasing risk for wheat production in many parts of the world, especially those areas with a Mediterranean-type climate. Improvement of drought tolerance has been identified as a research priority in cereal breeding (Leflon *et al.*, 2005; Fleury *et al.*, 2010). High levels of stem water-soluble carbohydrates (WSC) have been implicated in drought tolerance in wheat (Volaire & Lelièvre, 1997; Wardlaw & Willenbrink, 2000; Foulkes *et al.*, 2007).

Stem WSC, mainly fructan together with glucose, fructose and sucrose, is one of the storage carbon sources for grain filling (Pheloung & Siddique, 1991; Kobata *et al.*, 1992; Schnyder, 1993). Fructans play important roles in grain filling and recovery from biotic and abiotic stress (Schnyder, 1993; Yang & Zhang, 2006; Valluru & Van den Ende, 2008). It was hypothesized that a mixture of high and low degree of polymerization (DP) fructans provides membrane protection by inserting at least part of the polysaccharide into the lipid head-group region of the membrane under water-deficit stress (Livingston *et al.*, 2009). In addition, during the phase of fructan degradation, released fructose residues may reduce osmotic potential and help to protect plants under drought stress (Valluru & Van den Ende, 2008; Livingston *et al.*, 2009) and under elevated carbon dioxide (Oliveira *et al.*, 2010; AbdElgawad *et al.*, 2014). Two novel emerging roles have been proposed for fructans. First, fructans may contribute to overall cellular reactive oxygen species (ROS) homeostasis by direct ROS scavenging mechanisms (Peshev *et al.*, 2013). Second, small fructans may act as phloem-mobile signaling compounds under stress (Van den Ende, 2013). Such antioxidant and signaling mechanisms may contribute to stress tolerance and disease prevention (Van den Ende, 2013).

Fructans are classified based on different linkage types between the fructosyl residues and the position of the glucose residue (Valluru & Van den Ende, 2008; Livingston *et al.*, 2009). Three types of fructans with a terminal glucose residue include the inulin-type

fructans with β–(2–1) linkages, levan-type fructans with linear β–(2–6) linkages and graminan-type fructans with both β–(2–1) and β–(2–6) linkages. Two types of fructans with internal glucose residue include the neo-inulin and neo-levan types. Graminan-type fructans typically accumulate in wheat, barley and other cereals. Bifurcose (or 1&6 kestotetraose) is a branched fructan and serves as a typical building block for graminan-type fructans.

Graminan-type fructans are synthesized by three types of fructan synthesizing enzymes in vegetative tissues of wheat (Kawakami & Yoshida, 2005; Gao *et al.*, 2010). 1-SST (sucrose: sucrose 1-fructosyltrasferase) transfers fructose from one sucrose to another, forming 1-kestotriose and glucose (Kawakami & Yoshida, 2002). 6-SFT (sucrose: fructan 6-fructosyltransferase) transfers fructose from a sucrose donor to 1-kestotriose as an acceptor, to form 1&6-kestotetraose (bifurcose) or to another fructan or sucrose to create new β–(2–6) linkages (Sprenger *et al.*, 1995; Kawakami & Yoshida, 2002). 1-FFT (fructan: fructan 1-fructosyltransferase) transfers fructose from one fructan to another fructan or to sucrose, forming β–(2–1) linkages (Jeong & Housley, 1992; Kawakami & Yoshida, 2005).

Acid invertases (INVs) hydrolyze sucrose. There are two types of acid INVs including cell wall (cw) and vacuolar INVs (Ruan, 2014). Overall, source–sink relationships are dynamic and continuously changing during development and in response to different biotic and abiotic stresses, and INVs fulfil crucial roles in these processes (Ruan, 2014). Under stress, sink strength increases are often associated with increased cw INV activities, which are considered to be pivotal enzymes at the integration point of metabolic, hormonal and stress signals (Proels & Roitsch, 2009). Vacuolar INVs may also function at the integration point of light, metabolic and hormonal signals (Rabot *et al.*, 2012).

Wheat fructans are degraded by fructan exohydrolases (FEHs) that only release terminal fructosyl units by using water as the acceptor. FEHs and cw INVs are extremely similar proteins, yet with a different functionality (Le Roy *et al.*, 2007, 2013). Several FEHs have been reported in wheat stems. 1-FEHs degrade β–(2–1) linkages (Van den Ende *et al.*, 2003; Van Riet *et al.*, 2006, 2008; Xue *et al.*, 2008). 6-FEHs (Van Riet *et al.*, 2006) degrade β–(2–6) linkages, while 6-KEHs (6-kestose exohydrolases) (Van den Ende *et al.*, 2005) and 6&1-FEHs (Kawakami *et al.*, 2005) hydrolyze 6-kestotriose and small graminans, respectively. In addition, a 6-FEH with intrinsic but low 1-FEH activity was able to degrade almost all graminans occurring in vegetative wheat tissues (Kawakami & Yoshida, 2012). It has been suggested previously that *1-FEH w3* (1-FEH-6B) is a key enzyme involved in stem WSC remobilization (Van Riet *et al.*, 2008; Zhang *et al.*, 2009), but 6-FEHs are also expected to play an important role (Joudi *et al.*, 2012). It can be assumed that the combined activities of all stem FEHs potentially contribute to enhance the utilization of stem WSC during grain filling under drought stress.

Soil drying after anthesis accelerates mobilization of stored carbohydrate reserves to the grain (Bidinger *et al.*, 1977; Austin *et al.*, 1980; Yang *et al.*, 2000; Yang & Zhang, 2006). The storage of WSC in the stem and subsequent remobilization to the grain can directly influence the harvest index, especially under post-anthesis drought stress (Mir *et al.*, 2012). It has been estimated that pre-anthesis reserves contribute up to 57% and 74% of the grain yield of wheat and barley (Gallagher *et al.*, 1976), respectively. However, there is also an inconsistent correlation between high stem WSC and grain yield, indicating that the key components of FEH-mediated WSC remobilization related to yield are not yet sufficiently defined (Užík & Žofajová, 2006; Dreccer *et al.*, 2009). The stem WSC remobilization clearly differs between genotypes (Evans & Wardlaw, 1996; Ehdaie *et al.*, 2006; Ruuska *et al.*, 2006; Ma *et al.*, 2014).

In this study, Westonia, Kauz and their double haploid (DH) lines were used in field drought experiments with two clear objectives. The first goal was to generate more precise information on FEH-mediated stem WSC remobilization to the grains. The second goal was to identify genes and enzymes related to stem WSC remobilization and develop molecular markers for efficient and accurate selection of stem WSC remobilization to the grains under terminal drought conditions.

Materials and Methods

Plant materials

Wheat (*Triticum aestivum* L.) varieties, Westonia, Kauz and their DH lines were used in this study. Westonia is developed in Western Australia and has a consistently high yield in medium and low rainfall regions of Western Australia. Kauz is developed by the International Maize and Wheat Improvement Center (CIMMYT, EI Batan, Mexico) (Butler *et al.*, 2005) and is considered to be drought tolerant (Rajaram *et al.*, 2002). Both varieties have high WSC levels in stems (*c.* 40%) after anthesis (Zhang *et al.*, 2009). DH lines were first selected based on their similar flowering time and height. The genetic diversity was investigated by using 195 single sequence repeat (SSR) markers and two *Rht-B1* and *Rht-D1* gene markers (Supporting Information Fig. S1) and this was used for further selection of an array of DH lines with substantially different genotypes. Nonmetric multidimensional scaling (MDS) of genetic dissimilarity was generated by using the Numerical Taxonomy System (NTsys) v2.2 and Plymouth Routines in Multivariate Ecological Research (PRIMER v6).

Field experiments in Merredin

The field drought experiments were carried out in Merredin (31.5°S, 118.3°E) field station, Western Australia in 2011 and 2012. Twelve neutron pressure bombs (1.5 m depth below surface) were distributed evenly in each drought and well-watered treatments for soil moisture measurement and the data were recorded fortnightly. Only data for 10, 30 and 50 cm depth were presented (Fig. S2) as the water content levels were similar between drought and well-watered below 50 cm depth.

In 2011, Westonia, Kauz and 22 DH lines were planted (5 m^2 per plot) randomly by two or three replicates on the 25 May 2011 in both drought and irrigated fields. In total, there were

143 plots with 70 and 73 plots for drought and irrigated treatments, respectively. The drought treatment was set up in rainout shelters and the irrigated treatment was outside. Drought treatment was initiated at anthesis. Besides 29 mm of rainfall, irrigated plants received 20 mm water on a weekly basis until 3 wk after anthesis. Soil water content was reduced significantly by 30% at 10 cm depth at 14 d post anthesis (DPA) in the drought treatment as compared with the irrigated plants (Fig. S2).

In 2012, seeds were sown on the 20 June 2012 because of the lack of rain in May. Twenty one DH lines (Fig. S1) and Westonia and Kauz were randomly sown by two replicates, resulting in 92 plots, with 46 plots for drought and irrigated treatments, respectively. The plot area was 1.8 m × 10 m. The drought treatment occurred under rainfall condition. Because of the late sowing, there was only 30.8 mm rainfall after anthesis until maturity. An additional 60 mm of water was irrigated by 15 mm week^{-1} for 4 wk after anthesis. In the drought treatment, soil water content was reduced significantly by 50–60% at 10 cm depth after 14 DPA (Fig. S2).

Plant harvest

Four main stems of each plot were sampled weekly between 11:00 and 17:00 h (Zhang *et al.*, 2008) from 1 week pre-anthesis to 6 wk post anthesis. The samples were immediately placed on dry ice and subsequently stored in a −20°C freezer. Frozen plant samples were chopped into <5 mm pieces and divided into two parts. One was stored at −80°C for enzyme and RNA extraction, and the other was stored at −20°C, freeze-dried, and then oven-dried at 75°C for WSC analysis. The thousand grain weight (TGW), grain number per spike (KN) and grain weight per spike (GW) from main stems were recorded at harvest.

Carbohydrate analysis

Sample preparation was as described in Zhang *et al.* (2008). WSC were extracted from the stem (sheath included) using boiling deionized water and quantified by colorimetry using the anthrone reagent (Fales, 1951; Yemm & Willis, 1954). A 200 µl aliquot of the same sample was passed through a 0.3 ml bed volume of Dowex®-50 H^+ and a 0.3 ml bed volume of Dowex®-1-acetate, followed by rinsing six times with 200 µl distilled water. The eluate was seven times diluted and then centrifuged at 13 000 ***g*** for 5 min. Twenty five microlitres of the diluted samples were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described (dos Santos *et al.*, 2013). The WSC components were quantified using the peak area with external standards for glucose, fructose, sucrose, 1-kestose, 6-kestotriose, neokestose, nystose and bifurcose. The total fructan concentration was calculated as the amount of the WSC concentration (as determined by the anthrone method) minus the concentration of glucose, fructose and sucrose. The total stem WSC concentration of selected samples was also determined by mild acid hydrolysis (Verspreet *et al.*, 2012), leading to identical results for mild acid hydrolysis and anthrone based methods (data not shown).

Protein extraction and enzyme activity measurements

Frozen samples were ground in liquid nitrogen with mortar and pestle and *c.* 150 mg was further homogenized in five volumes of 50 mM Na-acetate buffer (pH 5.0) also containing 1 mM phenylmethylsulfonyl fluoride, 1 mM mercaptoethanol, 10 mM sodium bisulfite, 0.02% (w/v) sodium azide and 0.1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged for 5 min at 13 000 ***g*** and 450 µl of the supernatant was taken and mixed with 252 mg ammonium sulphate. After overnight incubation on ice, samples were centrifuged (13 000 ***g***, 5 min) and precipitates were collected and washed with 600 µl of ice-cold 80% ammonium sulphate. The pellets were dissolved in 225 µl of 50 mM Na-acetate buffer (pH 5.0) containing 0.02% (w/v) Na-azide.

The protein solution was then incubated with different substrates for enzyme activity measurements. Sucrose as a single substrate at 200 mM was used for 1-SST, 6-SST and INV activity determinations, with quantification of the formed 1-kestose, 6-kestose and fructose, respectively. Fifty mM 1-kestose was used as substrate for 1-FFT and 1-FEH activity determinations, with quantification of the products nystose and fructose minus glucose for 1-FFT and 1-FEH activities, respectively. A combination of 50 mM sucrose and 50 mM 1-kestose was used for 6-SFT activity determinations and the level of bifurcose was quantified (Verspreet *et al.*, 2013). Five mM wheat stem fructan was used for 6-FEH activity measurement with quantification of the released fructose. Enzyme reaction mixtures (50 µl) were incubated at 30°C for 0, 20 or 30, 60, 90 or 180 min and a suitable time-point within the linear region of product formation was selected. The reactions were stopped by heating for 5 min (90°C) in a tenfold dilution with 0.04% (w/v) Na-azide and 20 µM mannitol as internal standard. Afterwards, a mixture of 30 µl of the reaction sample and 30 µl of 20 µM mannitol water were used, and 25 µl of the mixture was automatically injected onto HPAEC-PAD (Dionex, Sunnyvale, CA, USA), and products were quantified as described above.

RNA extraction and real time PCR

Total RNA extraction from the main stem (sheath included) and cDNA reverse transcription were performed as described (Zhang *et al.*, 2008). Quantitative reverse transcription-PCR (qRT-PCR) was carried out using the Corbert Rotor-Gene RG-3000 (Corbett Research, Brisbane, Queensland, Australia) as described (Zhang *et al.*, 2009). The gene expression levels of *1-FEH w1*, *w2* and *w3* were normalized against cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels (Wang *et al.*, 2010). Specific primer pairs were designed based on single nucleotide polymorphisms (SNPs) in exon 3 of the three *1-FEH* genes (Zhang, 2008). Gene expression was quantified using a relative standard curve method (as recommended by Corbett Research).

Database searches and sequence analysis

Wheat *1-FEH w1* (FJ184989), *w2* (FJ184991), and *w3* (FJ184990) are homologous genes on wheat chromosome 6A, 6D and 6B, respectively, and the gene structures were described in our previous study (Zhang *et al.*, 2008). The gene sequences are available from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). They were used as a query to obtain closely related wheat genome sequences available from the International Wheat Genome Sequencing Consortium (IWGSC) Survey Sequence Repository, for exploring the upstream and downstream regions of the respective genes. The retrieved DNA sequences were examined, edited and multiple alignments were carried out with BioEdit V.7.0.9 (Ibis Bioscience, Carlsbad, CA, USA) and Geneious V.6.1.3 (Biomatters Ltd, Auckland, New Zealand).

Primer design, PCR amplification of genomic DNA, cloning and enzyme digestion

Sequences of primers for *1-FEH w3* gene amplification have published before (Zhang *et al.*, 2008). Primers for promoter and downstream region amplification (Table S1) were designed based on the closest sequence from IWGSC and previously obtained sequences. For qRT-PCR, previously published gene specific primers were used to amplify a 100-bp fragment of exon 3 (Zhang *et al.*, 2008). Primer pair *GAPDHL* and *GAPDHR* were designed based on the gene sequence of *GAPDH* (NCBI number: AF251217.1). The PCR amplification and analyses followed Zhang *et al.* (2008). PCR products were cloned into a pGEM-T Easy Vector (Promega) and at least three independent clones from each PCR product were sequenced (ABI 377, Applied Biosystems, Foster City, CA, USA). The total volume of enzyme digestion reaction was 20 μl which included 10 μl of PCR products, 10 units of enzyme (1 μl), and 1 × CutSmart™ buffer (2 μl). The mixture was incubated at 37°C overnight and then heated at 80°C for 20 min to stop the reaction. The digested PCR products were run in a 2.5% agarose gel.

Marker analysis, genetic map construction and quantitative trait loci (QTL) mapping

Genomic DNA was extracted from a single plant for each DH line and their parental lines (Zhang *et al.*, 2008). A genetic map comprised of SSR, SNP and gene-based markers for *Rht-B1*, *Rht-D1*, *Vrn-A1a*, *Vrn-B1a*, *Vrn-D1a* and *1-FEH w3* CAP marker was constructed for the DH population using Map Manager (Manly *et al.*, 2001) and RECORD (van-Os *et al.*, 2005). SSR genotyping was performed using multiplex-ready PCR (Hayden *et al.*, 2008). Genotyping of 9000 SNPs and the amplification of *Rht-B1* and *Rht-D1*, *VRN1* (*Vrn-A1a*, *Vrn-B1a* and *Vrn-D1a*) were described previously (Zhang *et al.*, 2014). QTL mapping was performed with QTLNetwork 2.0 (Zheng *et al.*, 2013).

Statistical analysis

Phenotype data were analysed by multivariate analysis of variance (MANOVA) using the general linear model implemented in PASW v17 (California State University Information Services, Los Angeles, CA, USA). Wilks Lambda was used as the multivariate test statistic. Post hoc Tukey's Multiple Range tests were used to identify significant groupings. Pearson correlation coefficients between the level of stem WSC and yield components were calculated on a mean basis using Primer v6 (Clarke & Gorley, 2006). The Pearson correlation significance level was determined by bivariate analysis in PASW v 17.

Results

Genotype differences involved in the association between stem WSC and grain weight components under drought and irrigated conditions

Westonia and Kauz were investigated in the glasshouse before and it was observed that remobilization of stem WSC was associated with the level of *1-FEH w3* gene expression (Zhang *et al.*, 2009). In the present study, Westonia, Kauz and their DH lines were used in field drought experiments (2 years: 2011 and 2012) to further investigate possible genetic factors involved in wheat stem WSC remobilization. As expected, the statistical analysis confirmed that drought treatment caused a significant reduction in GW per main spike (Fig. S3). The KN per main spike was significantly lower under drought treatment, while the TGW were similar.

Under drought, GW was reduced in most, but not all DH lines (Fig. 1). As stem WSC are considered as a long storage carbon source for grain filling, they were recorded in a time course. Neither the maximal level of stem WSC, nor the total WSC at 17–20 DPA showed a significant correlation to GW, KN and TGW (data not shown), both in the main stem and in the harvest index cut. Some individual lines (e.g. lines 83, 277; Fig. 1) with average WSC levels achieved high GW under drought in 2011. Line 222, with the lowest stem WSC, achieved the highest GW in the irrigated condition, but not under drought. Line 207 showed the lowest GW and TGW but contained relatively high stem WSC under drought (Fig. 1). The levels of stem WSC remained similar in the Kauz parent line under drought and irrigated conditions. However, the GW of Kauz under drought was lower than that irrigated plants. The stem WSC level in Westonia was higher in drought treated plants compared with the irrigated ones. There was no reduction in GW of Westonia under drought (Fig. 1).

The decrease of bifurcose and increase of fructose under drought

The overall total stem WSC levels of Westonia and Kauz followed rather similar patterns post anthesis (Fig. 2A). To consider the changes of stem WSC in greater detail, the levels of specific stem WSC components were quantified (Fig. S4). The main WSC components include glucose, fructose, sucrose, 1-kestose, 6-kestose, bifurcose and mixed fructans (Fig. S4). Fructan levels increased up to 15 DPA and decreased thereafter (Figs 2B, S4). Fructan levels decreased faster under drought in comparison with irrigated conditions (Fig. 2B) and between 15–25 DPA they decreased slower in Kauz than in Westonia (Fig. 2B). Accordingly,

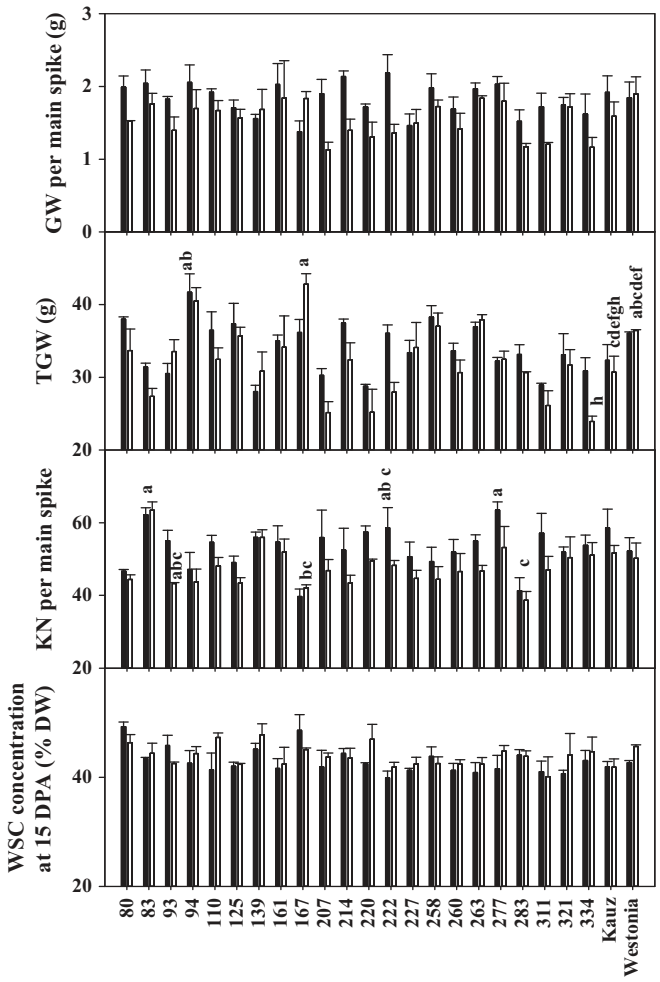

Fig. 1 The association between stem water-soluble carbohydrates (WSC) and core phenotypes, including grain weight (GW) per main spike, thousand grain weight (TGW) and kernel number (KN) per main spike in wheat *Triticum aestivum* varieties: Westonia, Kauz and their 22 double haploid (DH) lines under irrigated (closed bars) and drought conditions (open bars) in 2011. Error bars, + standard error (SE). Values with the same letter are not statistically different at $P = 0.05$. DPA, days post anthesis; DW, dry weight.

sucrose levels tended to remain higher in Kauz than in Westonia over this period (Fig. S5a), although statistical significance was only found with the Duncan test, not with the Tukey test. Between 20–30 DPA, the levels of bifurcose, a major and most persistent fructan in wheat stems, were significantly lower in both drought ($0.44 \pm 0.05\%$) and irrigated ($0.82 \pm 0.03\%$) plants in Westonia compared with Kauz (drought, $0.66 \pm 0.02\%$ and irrigated, $1.05 \pm 0.05\%$) (Fig. 3A, upper panel). The levels of fructose were higher in Westonia ($12.9 \pm 0.15\%$) than in Kauz ($10.1 \pm 0.90\%$) in drought treated plants over the same time period (20–30 DPA), although statistical significance was only found with the Duncan test, not with the Tukey test (Fig. 3A, lower panel).

1-FEH w3 is likely the main enzyme associated with β–(2–1) fructan degradation

In monocots, fructan levels are determined by the balance between fructan biosynthesis and degradation (Van den Ende *et al.*, 2003). The enzyme activities involved in fructan synthesis and degradation were determined on the same samples that were used for WSC analysis. Under drought conditions, the total 1-FEH activity was strongly induced and reached an earlier maximum in Westonia (28 DPA) as compared with Kauz (31 DPA) (Fig. 3B, upper panel). Bifurcose and 1-FEH enzyme activities correlated well over the 0–35 DPA period (Fig. S6a). Overall, there is a tendency that drought treatment reduces the enzyme activities involved in fructan synthesis, for example, 1-SST, 6-SST, 1-FFT and 6-SFT activities (Fig. 4). 6-SST and 6-SFT activities probably originate from one or more 6-SFT enzymes with intrinsic 6-SST activities. At 25 DPA, 1-SST and 1-FFT activities were significantly lower in Westonia plants compared with Kauz, both under irrigated and drought conditions (Fig. 4). Soluble acid INV activity was highly induced in Westonia under drought while it remained rather constant before 30 DPA and then increased sharply in Kauz under drought. 1-FEH activities are higher compared with the other enzymatic activities, including soluble acid INV activity (Figs 3, 4), although it should be noted that 6-FEH activities were tested at 5 mM substrate and 1-FEH activities at 50 mM. Adjusting both FEHs to the same millimolar levels suggests that both activities could be equally important.

Three isoforms of 1-FEH (1-FEH w1, w2 and w3) can be found in wheat (Van Riet *et al.*, 2008). To understand better which isoform contributes most to the total 1-FEH enzyme activity, the gene expression levels of three *1-FEH* genes were determined. The data show that the *1-FEH w3* expression was much higher than the expression levels of *1-FEH w1* and *w2*, suggesting that the 1-FEH w3 enzyme is the most prominent form contributing to the total 1-FEH activity involved in WSC remobilization under drought (Fig. 3B, lower panel). Drought strongly induced the *1-FEH w3* gene expression in Westonia and Kauz. Moreover, the level of *1-FEH w3* gene expression peaked much earlier in Westonia compared with Kauz under drought. Furthermore, the *1-FEH w3* gene expression level was significantly higher in Westonia than in Kauz under drought *c.* 15 DPA (Fig. 3, lower panel), preceding the 1-FEH activity optimum by *c.* 1 wk (Fig. 3A,B). This was also associated with faster bifurcose reduction and fructose increases in comparison with Kauz (Fig. 3A,B).

A CAP marker for *1-FEH w3*

Due to the putative importance of the *1-FEH w3* gene expression, the *1-FEH w3* gene sequences of Westonia and Kauz were more thoroughly investigated. For this purpose, the primer pairs FEH2F/FEHw3R and FEHw3F/FEH2151R were used to amplify the gene parts from both genotypes (Fig. S7a,b). Unfortunately, no SNP could be detected in the amplified 4.3 kb area (results not shown). The closest contig (6BS-ab-k71-contigs.fa.longerthan_200, 59, 674, 2935921) to the *1-FEH w3* gene was retrieved from IWGSC. This contig contained the downstream sequence of the *1-FEH w3* gene. A reverse primer (6BPR) was designed based on this information. The downstream region of *1-FEH w3* was isolated with the FEH4690F/6BPR primer pair

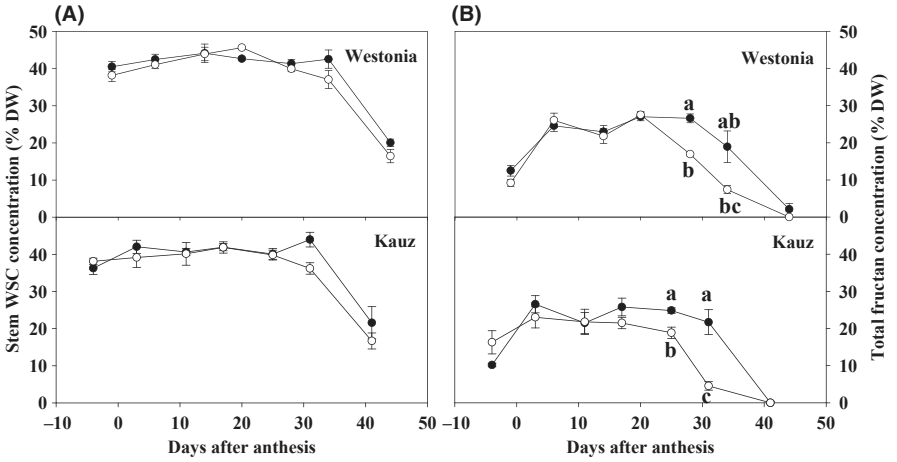

Fig. 2 (A) Stem water-soluble carbohydrates WSC and (B) fructan levels in wheat *Triticum aestivum* varieties, Westonia and Kauz under drought (open circles) and irrigated (closed circles) conditions at Merredin field station in 2011. The vertical bars represent standard error (SE). Values with the same letter are statistically not different at $P = 0.05$.

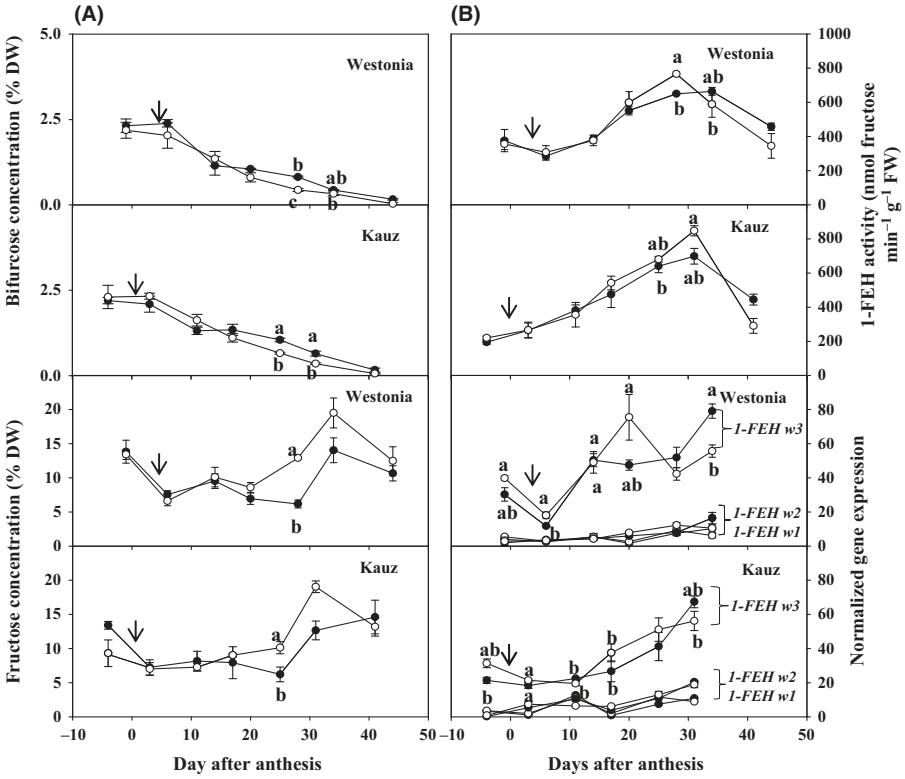

Fig. 3 (A) The profile of wheat stem (sheath included) bifurcose concentration (upper panel) and fructose concentration (lower panel) in wheat *Triticum aestivum* varieties, Westonia and Kauz under irrigated (closed circles) and drought (open circles) conditions. (B) Total 1-FEH enzyme activity (upper panel) and normalized gene expression patterns of *1-FEH w1*, *w2* and *w3* (lower panel). The vertical bars represent standard error (SE). Values with the same letter are statistically not different at $P = 0.05$. Arrows indicate the moment when the drought treatment was initiated.

(Fig. S7c). However, again no SNP could be found. Therefore, the *1-FEH w1* promoter region was isolated using the FEH13200F/6A630R primer pair, based on the promoter region of *1-FEH w1* available from the IWGSC (6AS-ab-k71-contigs.fa.longerthan_200, 207, 715, 4429358) (Fig. S8a,b). Several forward primers were designed based on the promoter sequence of *1-FEH w1* on 6A. A 6B genome specific primer 6B60R was designed based on previously obtained *1-FEH w3* sequences. The 6APF1/6B60R primer pair produced a 670 bp fragment of the promoter region of *1-FEH w3* (Fig. S8c,d). Fortunately, one SNP was detected between Westonia and Kauz (Fig. 5a, gene bank accession numbers KM262665 (Kauz) and KM262666 (Westonia)). A unique BsoB1 restriction enzyme (CYCGRG; detected with NEBcutter V2.0 software) cuts the SNP site in Westonia but not in Kauz. A size reduced and genome specific fragment (*c.* 250 bp) was amplified by the 6BPF2/6B60R primer pair (Fig. 5b,c). After overnight digestion with BsoB1, a 14 bp band difference was detected between Westonia and Kauz on a 2.5% agarose gel (Fig. 5d,e). This cleaved amplified polymorphic (CAP) marker was then used in the DH population of Westonia and Kauz and the *1-FEH w3* gene was mapped to the short arm of 6B, *c.* 1 cM away from the SSR marker wmc494 (Fig. S9).

Westonia alleles of the *1-FEH w3* gene in DH lines are associated with high *1-FEH w3* gene expression under drought

To further confirm whether the CAP marker is associated to the *1-FEH w3* gene expression level, four Westonia type

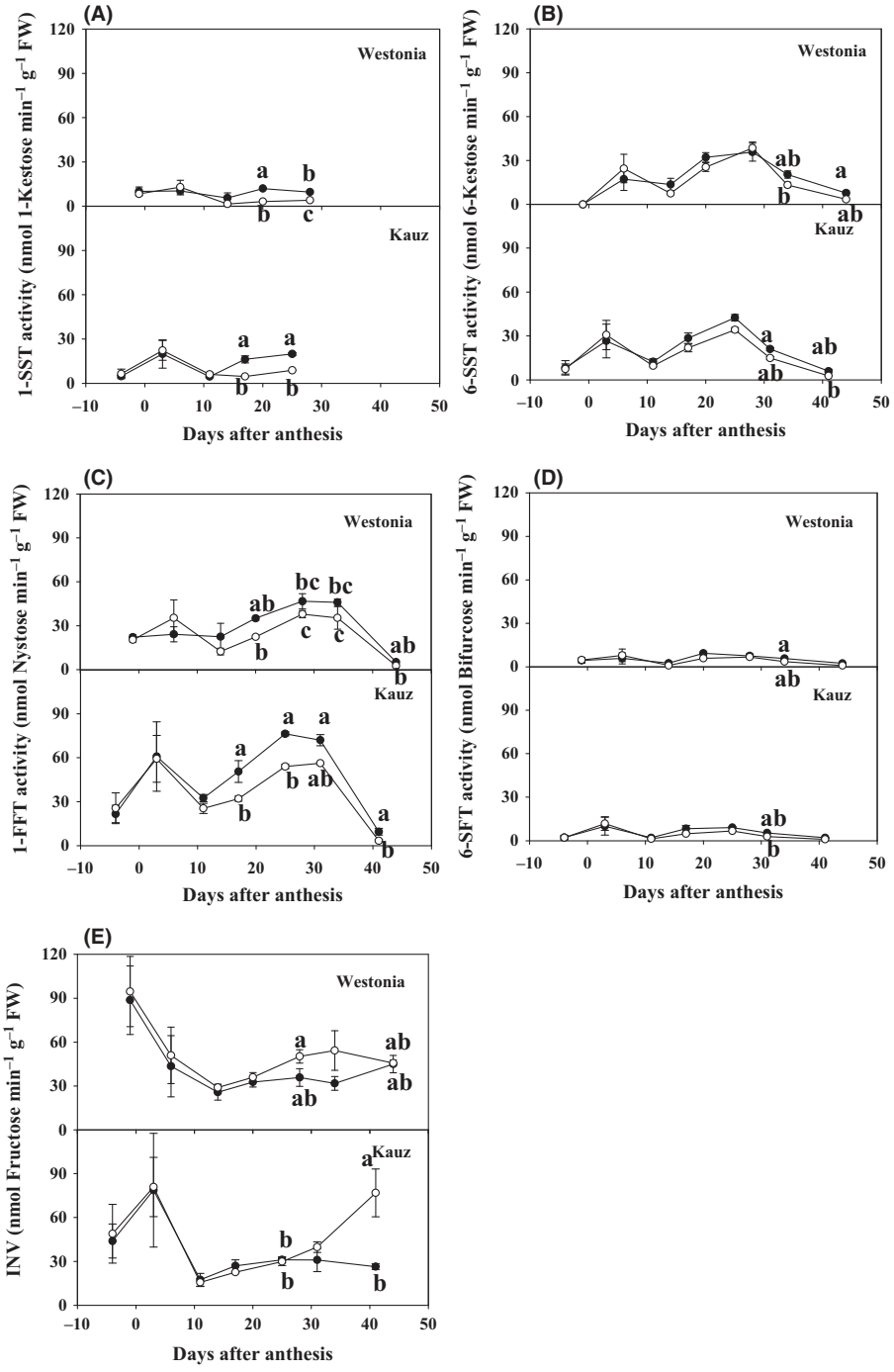

Fig. 4 Enzyme activities of (A) 1-SST, (B) 6-SST, (C) 1-FFT, (D) 6-SFT and (E) acid invertases (INV) in stems of wheat *Triticum aestivum* varieties, Westonia and Kauz under drought (open circles) and irrigated (closed circles) conditions in 2011. The vertical bars represent standard error (SE). Values with the same letter are statistically not different at $P = 0.05$.

DH lines (DH 321, DH 83, DH 139 and DH 167) and three Kauz type DH lines (DH 263, DH 311 and DH 125) were randomly selected and analysed for their *1-FEH w3* gene expression. DH 167, with an exceptionally low KN per spike (Fig. 1), showed no difference in *1-FEH* w3 gene expression under drought and irrigated conditions (data not shown). The mean of the three remaining Westonia type DH lines tended to show higher *1-FEH w3* gene expression levels at 15 DPA compared with lines containing the Kauz alleles under drought (Fig. 6). Under irrigated conditions, the levels of *1-FEH w3* gene expression in Westonia allele group were also higher than Kauz allele group at 30 DPA although the means at a certain DPA were insufficiently different to claim statistical significance. This result can at least partly be explained by the fact that all DH lines, like the Kauz and Westonia parents (Fig. 3), showed their own particular time-dependent *1-FEH w3* expression optima. However, when the 15, 25 and 30 DPA were pooled, the means and SE of Westonia and Kauz type under drought were 22.8 ± 2.9 and 15.7 ± 2.0, respectively.

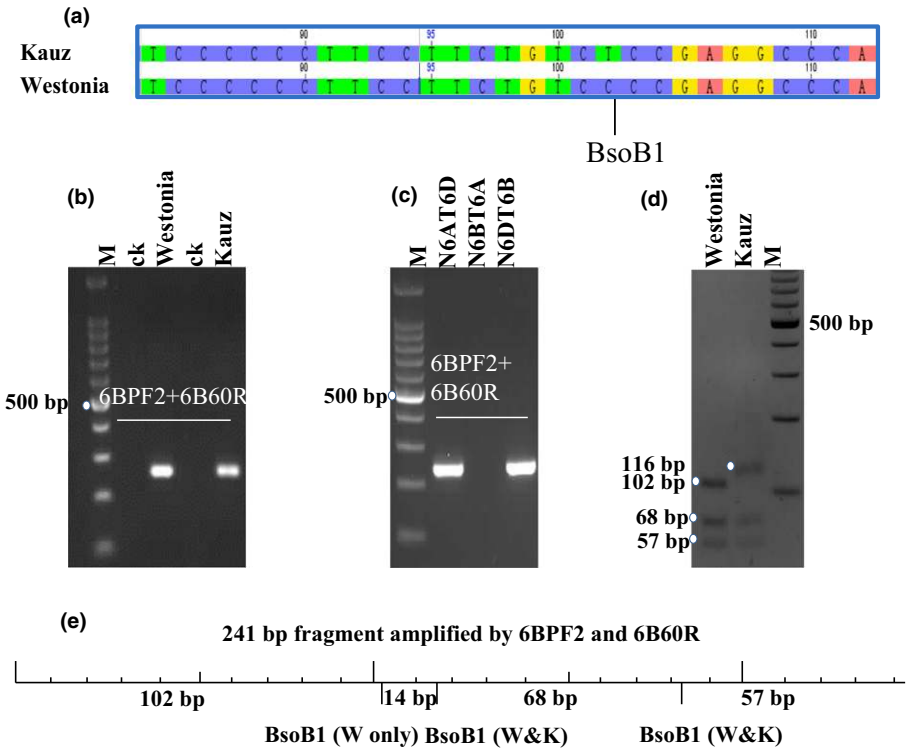

Fig. 5 Generation of a cleaved amplified polymorphic (CAP) marker. (a) One single nucleotide polymorphism (SNP) was located in the promoter region of *1-FEH w3*. (b) The SNP promoter region was amplified from *1-FEH w3* in wheat *Triticum aestivum* varieties, Westonia (W) and Kauz (K), and (c) from nulli (N)-tetra (T) lines N6AT6D, N6BT6A and N6DT6B. (d, e) A CAP marker was generated based on the 14-bp fragment length difference between Westonia and Kauz after overnight digestion on the *6BPF2/6B60R* amplification product with the restriction enzyme BsoB1 (CYCGRG). ck, negative control; M, standard marker.

The CAP marker of *1-FEH w3* correlates with a superior TGW under drought

The TGW QTL analysed under two well-watered and one early drought conditions was mapped 19.5 cM upstream of the chromosome 6B, *c.* 172 cM away from the above mentioned *1-FEH w3* locus using the developed CAP marker (Fig. S9) and the additive effect was contributed by Westonia, explaining 1.3% of the total phenotype (Zhang *et al.*, 2013).

In the drought experiments in Merredin, 22 and 21 DH lines were used in 2011 and 2012, respectively. In addition to the parental Westonia and Kauz lines, 29 and 23 plots for each *1-FEH w3* genotype per treatment were analysed in 2011 and 2012, respectively. The TGW in lines with Westonia type *1-FEH w3* alleles were significantly higher than those from lines with Kauz type *1-FEH w3* alleles under drought in 2011 and 2012. The level, of statistical significance for GW between both genotypes was only reached in 2011 (Fig. 7). There was no statistically significant difference in KN per spike between the different *1-FEH w3* genotypes under both drought and irrigated conditions.

The role of 6-FEH during stem fructan remobilization

Besides bifurcose, 6-kestose also represents a prominent fructan in wheat stems. The 6-kestose concentration was relatively stable between 3–3.7% of the stem dry weight before 20 DPA in Westonia, both under drought and irrigated conditions, while it gradually increased from 2.2% to 4% in Kauz (Fig. S10). Afterwards, it declined sharply in both lines. This degradation occurred faster under drought. At 20–30 DPA, the level of 6-kestose was significantly lower under drought in Westonia than in Kauz. At 25

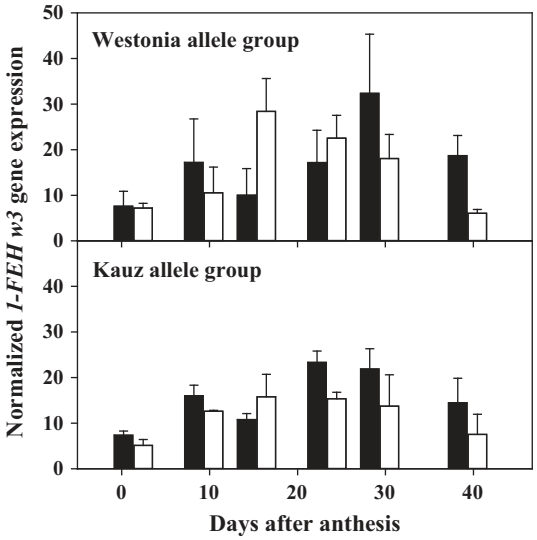

Fig. 6 Normalized *1-FEH w3* gene expression patterns in double haploid (DH) lines of wheat *Triticum aestivum* varieties, Westonia and Kauz using the same tissue sources that were used for stem water-soluble carbohydrate (WSC) analysis. Closed bars, irrigated conditions; open bars, drought conditions. Upper panel: the mean levels of Westonia alleles including DH 321, DH 83 and DH 139. Lower panel: the mean levels of Kauz alleles including DH 263, DH 125 and DH 311. The vertical bars represent standard error (SE).

DPA, the 6-FEH activity significantly increased under drought in Westonia compared with Kauz. Overall, 6-FEH activity increases were more extended under drought than under irrigated conditions in both lines (20–DPA; Fig. S10). The profile of 6-FEH activity could be closely correlated with the degradation

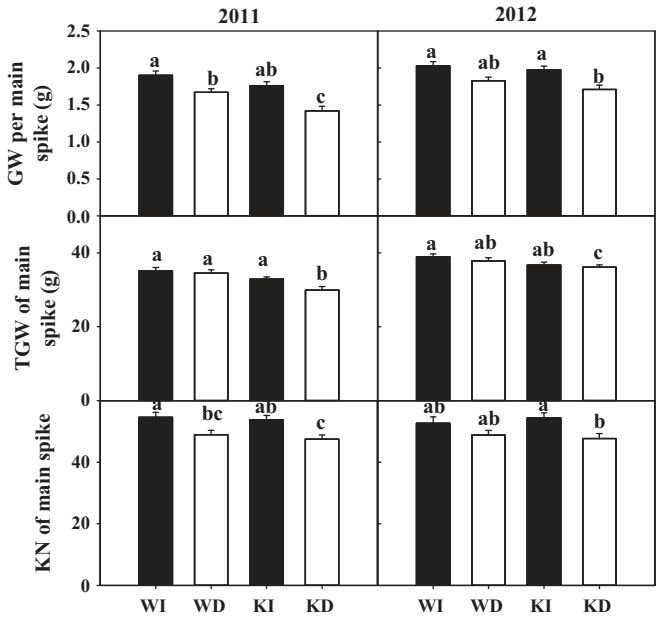

Fig. 7 The core phenotypes of grain weight (GW) per spike, thousand grain weight (TGW) and kernel number (KN) per spike associated with the cleaved amplified polymorphic (CAP) marker of *1-FEH w3* in selected double haploid (DH) lines and the parental lines of wheat *Triticum aestivum* varieties, Westonia and Kauz under irrigated (closed bars) and drought (open bars) conditions in 2011 and 2012. WI, *1-FEH w3* Westonia type under irrigated conditions; WD, *1-FEH w3* Westonia type under drought conditions; KI, *1-FEH w3* Kauz type under irrigated conditions; KD, *1-FEH w3* Kauz type under drought conditions. The vertical bars represent standard error (SE). Values with the same letter are not different at $P = 0.05$.

patterns of 6-kestose between 15–45 DPA (Fig. S6b), suggesting that 6-kestose could be one of the preferred substrates of wheat 6-FEH enzymes, warranting deeper investigations into 6-FEHs in wheat stems during carbon remobilization under terminal drought.

Discussion

Although there is generally an association between yield and high stem WSC at anthesis under terminal drought (Nicolas & Turner, 1993; Blum *et al.*, 1997; Snape *et al.*, 2007; Xue *et al.*, 2008), our field drought experiments demonstrated that high stem WSC levels were not necessarily associated with high GW. For instance, some lines with average stem WSC achieved high GW under drought conditions (Fig. 1). Such inconsistent correlations have been previously reported (Evans & Wardlaw, 1996; Ehdaie *et al.*, 2006; Ruuska *et al.*, 2006; Zhang *et al.*, 2009) and substantial genetic variations for stem WSC remobilization have been found in field and glasshouse experiments (Zhang *et al.*, 2009).

Here, carbohydrate dynamics, related enzymatic activities and FEH gene expression levels were followed in Westonia and Kauz and their DH lines. Typically, Kauz senesces earlier than Westonia (Zhang *et al.*, 2009). Overall, the 15–30 DPA period seemed to be crucial, with time-dependent differences between Westonia and Kauz. Between 15–25 DPA, sucrose and fructan levels remained higher in Kauz (Figs 2B, S5a), associated with higher 1-SST, 1-FFT and lower 1-FEH, 6-FEH and INV activities under drought (Figs 3B, 4E, S10b). In Westonia, *1-FEH w3* transcript levels peaked *c.* 1 wk before the maximal 1-FEH activity was reached (Fig. 3), confirming that 1-FEH w3 was most likely the main enzyme contributing to the 1-FEH enzymatic activity, as shown before by enzyme purification (Van Riet *et al.*, 2008). As wheat stem 1-FEH activities are known to be inhibited by sucrose at the post-translational level (Van den Ende *et al.*, 2003), the *in planta* 1-FEH activities in Kauz were likely to be even lower over this period. In accordance with these data, the major fructan bifurcose degraded faster and fructose rose quicker in Westonia between 15–28 DPA, indicating that fructans degraded and remobilized faster in Westonia compared with Kauz. The generated fructose can, at least partly, be used as a source to resynthesize sucrose by sucrose phosphate synthase and sucrose 6-phosphate phosphatase. Sucrose is the major transport compound in plants in general and in wheat in particular (Joudi *et al.*, 2012; Ruan, 2014). Although bifurcose can be broken down to a certain extent to 6-kestose and fructose by 1-FEHs (Van den Ende *et al.*, 2003), 6&1-FEHs and 6-FEHs likely degrade it too (Kawakami *et al.*, 2005). The different 6-FEH activity profiles between Westonia and Kauz may be caused by the different expression levels of *6-FEH* genes, but this requires further investigation. So far, only one 6-FEH has been cloned (Van Riet *et al.*, 2006), but this form is unlikely to be involved in vacuolar fructan degradation (Joudi *et al.*, 2012) and a plethora of similar *6-FEH* genes occur in wheat.

The situation clearly shifted during later developmental stages (> 25 or 28 DPA), with initially the discovery of more sharply increasing 1-FEH and 6-FEH activities in Kauz than in Westonia (Figs 3A, S10B), followed by strongly increased soluble INV activities in Kauz towards 40 DPA (Fig. 4E) associated with more strongly decreased sucrose levels in Kauz (Fig. S5a). It cannot be excluded that vacuolar INVs also contribute to the degradation of fructan oligosaccharides (De Coninck *et al.*, 2005; Van den Ende *et al.*, 2011), although the major function of increased soluble INV activity might be related to osmoregulation (Trouverie *et al.*, 2004). As a consequence of all these increased hydrolytic activities, fructan levels decreased faster in Kauz than in Westonia after 25–28 DPA (Fig. 2B), with fructan levels being completely depleted at 40 DPA in Kauz as an early senescent variety, even in the case of irrigated controls (Fig. 2B). The completely yellow Kauz stems under drought contained substantially higher glucose levels in stems (Fig. S5b), suggesting that glucose could not be longer used for remobilization or respiration, but only functioned as a compatible solute during the latest developmental stages.

We found a SNP in the promoter region of the *1-FEH w3* gene and the SNP occurred in an auxin response element (AuxRE: TGTCTC in the Kauz allele) located at −277 upstream of the ATG codon. An identical AuxRE motif was also found in the promoter region (−112) of chicory *1-FEH IIa* (Michiels *et al.*, 2004). The AuxRE can bind auxin response factors (ARFs). Such interactions activate or repress auxin-responsive genes (Guilfoyle & Hagen, 2007; Weiss & Ori, 2007). In *Arabidopsis*, five ARFs were classified as transcriptional activators, while the

others were considered transcriptional repressors (Ulmasov *et al.*, 1999; Guilfoyle & Hagen, 2007). However, it was noticed that an ARF classified as a repressor could function as an activator and vice versa, depending on cell type and environmental conditions (Guilfoyle & Hagen, 2007). The auxin level in plants may also influence the regulatory function of ARFs, although in general ARFs bind to TGTCTC AuxREs independent of the auxin status (Ulmasov *et al.*, 1999). The AuxRE binding site is modified to TGTCCC in the Westonia allele, preventing ARF binding. In general, when considered on a whole plant level, gibberellin acid (GA) and auxin (AUX) signaling counteract abscisic acid (ABA) signaling (Weiss & Ori, 2007; Du *et al.*, 2013). High levels of ABA lead to increased FEH activities in wheat stems under drought, with maximal ABA levels occurring *c.* 30 DPA (Yang *et al.*, 2004). Thus, GA and AUX signaling may counteract *1-FEH w3* gene expression in wheat stems. Such ARF-mediated inhibition of *1-FEH w3* might be absent in Westonia type, which means that it could be more tightly controlled by ABA signaling, typically associated with persistent drought (Weiss & Ori, 2007).

However, recent data suggest that AUX signaling may be of great importance under drought, consistent with a changed view that AUX and ABA signaling may not always act antagonistic to each other. In line with this view, *Arabidopsis* overexpression lines with increased IAA level exhibited enhanced drought stress tolerance, while mutant lines with lower endogenous IAA level showed decreased stress tolerance (Shi *et al.*, 2014). ABA accumulation under moderate drought stress modulates auxin transport in the root tip, enhancing proton secretion for maintaining root growth (Xu *et al.*, 2013). Moreover, it was recently found that pyrabactin resistance -like protein (PYL8), an ABA receptor, is required for the recovery of lateral root growth inhibition by ABA (Zhao *et al.*, 2014). This occurs independently of the ABA/Snf1 Related Kinase 2 (SnRK2) signaling pathway by enhancing the activities of several MYB factors that augment auxin signaling. This further confirms excessive crosstalk between ABA, AUX and sugar signaling pathways (Liu *et al.*, 2014; Van den Ende, 2014). Such mechanisms allow continued growth of certain plant parts (lateral roots, reproductive tissues) under drought stress, allowing continued water uptake and grain filling. It should however be noted that the exact stress level (mild vs heavy stress) might greatly determine the outcome of such reactions (Skirycz *et al.*, 2011).

In the case of drought stressed wheat, C skeletons (sucrose) from FEH-mediated breakdown of stem fructans and/or directly obtained from photosynthetic leaves might be used both for grain filling and export to the roots to stimulate lateral root formation. It can be speculated that Westonia and Kauz use different strategies in this respect, perhaps dictated by their differential ABA, AUX and sugar signaling networks, with the ARF-mediated *1-FEH w3* expression perhaps being of central importance. The data presented here suggested that the early senescing Kauz prioritizes on prolonged fructan accumulation in the stems, followed by rapid fructan degradation. Westonia, on the contrary, seems to initiate stem fructan degradation earlier, perhaps using part of this reserve to sustain lateral root growth formation to take up water and delaying overall senescence, resulting in a more extended period of grain filling. Both strategies are successful, although Westonia type showed superior TGW as compared with Kauz type (Fig. 7).

Recently, a complex relationship has been reported between AUX signaling, cw INV activity and cw INV inhibitors in developing rice kernels (French *et al.*, 2014). This fits well with the emerging view that sugars and sugar signaling control auxin levels in plants (LeClere *et al.*, 2010; Sairanen *et al.*, 2012; Wang & Ruan, 2013). Glucose produced by cw INV is believed to trigger hexokinase (HXK)-mediated signaling (LeClere *et al.*, 2010). In developing wheat grains, this is accompanied with temporal high levels of trehalose 6-phosphate, acting as an inhibitor of SnRK1, a central player in cellular energy homeostasis (Martínez-Barajas *et al.*, 2011). The T6P/SnRK1 module is believed to be involved in sucrose-specific signaling processes, including those that drive the expression of fructan biosynthesis genes (Van den Ende & El-Esawe, 2014), contributing, for instance, to temporal fructan accumulation during the milky stages in wheat grains (Verspreet *et al.*, 2013) and in flower parts (Ji *et al.*, 2011). Similarly, it can be speculated that sucrose-specific signaling processes might be relatively more important for fructan synthesis in Kauz stems than in Westonia stems over the 15–25 DPA period (Figs 2, S5a). However, it should be noted that sucrose can also act independently of SnRK1, by inhibiting the degradation of DELLA proteins, as recently established in *Arabidopsis* (Li *et al.*, 2014). Therefore, to fully understand whole plant's responses under drought, future research needs to focus on spatio-temporal differences in sugars and hormones in different plant parts (roots, leaves, stems, developing seeds), with particular focus on sugar and AUX signaling processes and crosstalk between them (Van den Ende, 2014).

From a practical point of view, the development of CAP marker for the *1-FEH w3* SNP, related to AUX signaling, could be very useful in wheat breeding focused on yield maximization under terminal drought. The CAP marker developed here (Fig. 5) was evaluated by random selection of seven DH lines of Kauz and Westonia type. Three out of four Westonia type lines showed high *1-FEH w3* gene expression levels between 15–30 DPA under drought, indicating that the CAP marker would be a useful indicator of the *1-FEH w3* gene expression level under drought (Fig. 6). However, the *1-FEH w3* gene expression in the randomly chosen DH 167 was similar between drought and irrigated conditions. This may be explained by the fact that the DH line had a very limited kernel number per spike. As explained above, it seems likely that the sink strength was lower as compared with other DH lines. Therefore, the speed of stem WSC remobilization and the related *1-FEH w3* gene expression might have been repressed in DH 167 under drought.

The harvest results showed that the TGW in Westonia type were consistently higher than in Kauz type (Fig. 7). A TGW QTL (Zhang *et al.*, 2013) contributed by Westonia was mapped to the short arm of 6B which was 173 cM away from *1-FEH w3,* indicating an association between TGW and the *1-FEH w3* gene. The QTL of grain filling efficiency in the DH population of two Chinese cultivars (Hanxuan 10 × Lumai 14) was mapped to the region of 6BS (Yang *et al.*, 2007). The TGW QTL was also detected on the 6B centromeric region in two populations of

recombinant inbred lines of tetraploid and durum wheat (Blanco *et al.*, 2002; Elouafi & Nachit, 2004). A yield QTL in the centromeric region of 6B was found using the Opata × Synthetic (ITMI: International Triticeae Mapping Initiative) population (Ayala *et al.*, 2002). These results indicate that the CAP gene marker of *1-FEH w3* links to the stem WSC remobilization efficiency in grain filling associated with high TGW under terminal drought conditions.

In conclusion, the levels of fructan-dominated WSC do not always correlate well with grain yield. High level of stem WSC combined with FEH-mediated remobilization efficiency may contribute to high TGW, especially under drought. The rate of the fructan degradation showed genotypic differences in fructan remobilization efficiency and underlying mechanisms were discussed. The gene expression data indicated that *1-FEH w3* was likely the main gene involved in the total 1-FEH enzyme activity. A CAP marker generated from the SNP in the promoter region distinguished two genotypes (Westonia and Kauz) with different levels of *1-FEH w3* gene expression. The Westonia genotype was linked to high gene *1-FEH w3* expression and high TGW indicating that the high gene expression of *1-FEH w3* contributed to the high levels of the stem WSC remobilization. The CAP marker of *1-FEH w3* may be useful for the selection for high stem WSC remobilization and high TGW in wheat breeding under terminal drought.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The nonmetric multidimensional scaling of 22 double haploid (DH) lines in the background of 225 DH lines of wheat *Triticum aestivum* Westonia and Kauz.

Fig. S2 Volumetric soil water content (v/v, %) at 10, 30 and 50 cm depth, respectively, in drought experiments at Merredin field station in 2011 and 2012.

Fig. S3 The average reduction in core phenotypes including grain weight per main spike (GW), thousand grain weight (TGW) and seed number per main spike (KN).

Fig. S4 The accumulation and degradation of stem water-soluble carbohydrate (WSC) components in wheat *Triticum aestivum* Kauz under drought and irrigated conditions from −4 to 41 d post anthesis (DPA).

Fig. S5 The patterns of stem (sheath included) sucrose and glucose concentrations in wheat *Triticum aestivum* Westonia and Kauz under irrigated and drought conditions.

Fig. S6 The correlation of stem (sheath included) bifurcose, 6-kestoseconcentration and the related 1-FEH, 6-FEH enzyme activities in wheat *Triticum aestivum* Westonia and Kauz, respectively, under irrigated and drought conditions.

Fig. S7 The *1-FEH w3* gene amplification including downstream 3′ terminal untranslated region from wheat *Triticum aestivum* Westonia and Kauz.

Fig. S8 The promoter region amplification of gene *1-FEH w3*.

Fig. S9 The quantitative trait loci (QTL) of height, thousand grain weight (TGW) and peduncle proportion detected on 6B.

Fig. S10 Evolution of stem (sheath included) 6-kestose concentrations and 6-FEH activities in wheat *Triticum aestivum* Westonia and Kauz under irrigated and drought conditions.

Table S1 Primers used for the amplification of wheat genomic DNA sequences and for qRT-PCR

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