

RESEARCH ARTICLE

Fructan biosynthesis gene expression upon cold acclimation in orchardgrass (*Dactylis glomerata* L.)B. Shaun Bushman¹  | Joseph G. Robins¹ | Xinxin Zhao² | Guangyan Feng³ | Xinquan Zhang³ | Linkai Huang³ | Matthew D. Robbins¹¹USDA-ARS Forage and Range Research Laboratory, Logan, Utah, USA²Institute of Urban Agriculture, Chinese Academy of Sciences, Chengdu National Agricultural Science and Technology Center, Chengdu, Sichuan, China³Sichuan Agricultural University, Chengdu, Sichuan, China

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Abstract

Background: Fructan content and flux in temperate forages can benefit the grasses through increased cold hardiness, increased drought tolerance, and improved forage quality. Orchardgrass (*Dactylis glomerata* L., or cocksfoot) produces relatively long and unbranched levan-type fructans, but the genes involved in their biosynthesis are uncharacterized.**Methods:** Through the evaluation of five orchardgrass cultivars and breeding lines that differ in their cold hardiness and freezing tolerance, we tested fructan and monosaccharide accumulation upon cold acclimation. The glycoside hydrolase-32 (*GH32*) gene family members involved in fructan biosynthesis were identified and grouped with homologous genes from *Triticum aestivum* and *Lolium perenne*.**Results:** In each of four *GH32* gene families, there were specific genes with high transcript levels and no deletions in *GH32* motifs. The candidate for sucrose-fructan 6-fructosyltransferase (*6-SFT*) exhibited the highest transcript levels of any *GH32* gene in this study and was induced upon cold acclimation. Conversely, three invertase and two fructan exohydrolase genes, with roles in sucrose and fructan hydrolysis, had reduced transcript levels upon cold acclimation.**Conclusions:** These data provide putative roles of *GH32* genes in orchardgrass, and show that *6-SFT*, vacuolar invertase (*VI*), and fructan exohydrolases (*FEH*) genes play a role in fructan biosynthesis and metabolism for cold acclimation in orchardgrass.

KEYWORDS

freezing tolerance, fructosyltransferase, glycoside hydrolase, RNAseq, water soluble carbohydrate

INTRODUCTION

Up to 15% of flowering plants use fructans as carbohydrates to store energy and form carbon skeletons (Hendry, 1993). In these plants the fructans are mainly stored in vacuoles (Lasseur et al., 2011), and accumulate mainly in elongating leaf sheaths, leaf bases, and crowns (Pavis et al., 2001). Fructans are protectants against various abiotic stresses (Valluru & Van den Ende, 2008) and involved in cell wall metabolism (Lammens et al., 2009). Cold hardening of temperate grasses is accompanied by increased expression of fructan biosynthesis and catabolism gene transcripts (Kawakami & Yoshida, 2002), increased enzyme activity (Hisano et al., 2008), and increased fructan content (Livingston

et al., 2009). Indeed, *Lolium perenne* L. transformed with a *Triticum aestivum* L. fructan biosynthesis gene had increased fructan content and freezing tolerance (Hisano et al., 2004). Fructans in forage grasses are induced by sucrose, light, drought, and cold temperatures (Gallagher et al., 2007; Maleux & Van den Ende, 2007), and fructan biosynthesis gene transcriptional regulation is tied to fructan levels (Hisano et al., 2008).

Enzymes responsible for catalyzing the biosynthesis and degradation of fructans are in the glycoside hydrolase 32 (*GH32*) family (Lammens et al., 2009). In plants, *GH32* enzymes include invertases, fructosyltransferases, and fructan exohydrolases (*FEH*). Invertases are a group of β -fructofuranosidases that can be categorized as cell wall (*CWI*) and vacuolar (*VI*) types (De Coninck et al., 2005);

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and predominantly hydrolyze sucrose and saccharides with a low degree of polymerization (DP) (Le Roy et al., 2007). There are several conserved amino acid motifs for binding and catalysis of *GH32* encoding genes: WMNDPNG, WSGSAT, RDP, and the ED motifs (Lammens et al., 2009; Lasseur et al., 2011). The WMNDPNG, or sucrose binding box, is conserved in invertases and *FEH* genes but can be variable in fructosyltransferase genes (Ritsema et al., 2005). Acid residues at all four motifs are critical for carbohydrate binding and catalysis (Lammens et al., 2009), and mutations of single amino acids can change the activity and kinetics of *GH32* enzymes (Le Roy et al., 2007; Ritsema et al., 2006; Schroeve et al., 2008).

The first step in the process of long DP fructan biosynthesis is the conversion of sucrose to 1-kestotriose or 1-kestose (Chalmers et al., 2003), which is catalyzed by sucrose:sucrose 1-fructosyltransferase (*1-SST*; E.C.2.4.1.99). Thereafter, the elongation of the polymer through the addition of fructose molecules is catalyzed by an F-type fructan:fructan 1-fructosyltransferase (*1-FFT*; E.C. 2.4.1.100) that produces β -2,1 linkages, an S-type sucrose:fructan 6-fructosyltransferase (*6-SFT*; E.C. 3.2.1.26) that produces β -2,6 linkages (Lasseur et al., 2011), or other possible F-type fructosyltransferases (e.g., *6G-FFT*) that contribute to neoseris fructans (Hisano et al., 2008; Lasseur et al., 2009; Ritsema et al., 2005). Genes encoding fructosyltransferases are most homologous to, and evolved from, *VI* genes with just a few mutations (Ritsema et al., 2006; Vijn & Smeekens, 1999). Long DP fructans can in turn be degraded from terminal ends with fructan exohydrolases (*FEH*; E.C. 3.2.1.153); with *1-FEH* preferentially hydrolyzing β -2,1 linkages (Van Den Ende et al., 2003), *6-FEH* preferentially hydrolyzing β -2,6 linkages (Van Riet et al., 2006), and other *FEH* genes hydrolyzing neoseris fructans (Kawakami & Yoshida, 2005). These *FEH* enzymes can act in trimming and turnover as well as degradation of fructans (Lothier et al., 2007; Pollock, 1986; Van Den Ende et al., 2003); and *FEH* genes evolved from *CWI* genes with few mutations (De Coninck et al., 2005; Le Roy et al., 2007).

Fructans with β -(2,1) linked inulin types are predominant in dicotyledons while β -(2,1) and β -(2,6) linked levan types predominate in temperate grasses, bacteria, and fungi (Vijn & Smeekens, 1999). *Brachypodium distachyon* (L.) P. Beauv., an excellent genetic model for grasses, does not synthesize long DP fructans and only has β -fructofuranosidase and other invertase gene functions that act on short DP saccharides (Lammens et al., 2009; Li et al., 2012). Triticeae cereals appear to have the broadest array of fructan biosynthesis genes and enzyme activities, with *T. aestivum* containing branched inulin and levan type fructans with both β -(2,1) and β -(2,6) linkages (Kawakami & Yoshida, 2005). As a result, a broad array of *T. aestivum* *GH32* genes have been cloned and characterized (Kawakami & Yoshida, 2005; Van den Ende et al., 2005; Van Riet et al., 2008). Within the forage grasses, *L. perenne* produces both low DP levan series and higher DP β -(2,6) levan neoseris with internal glucose molecules (Pavis et al., 2001). The high DP levan neoseris in *L. perenne* results from activity of a *6G-FFT* enzyme rather than *1-FFT* (Lasseur et al., 2009). Orchardgrass (*Dactylis glomerata* L.), was hypothesized as an ideal

model for fructan biosynthesis because of its unbranched and long DP β -(2,6) levan series (Chatterton et al., 1993; Livingston et al., 2009; Maleux & Van den Ende, 2007), but due to its auto-tetraploid genome it was unsuitable as a genetic model. Nevertheless, with a genome assembly available (Huang et al., 2019), characterizing orchardgrass genes involved in fructan biosynthesis is now feasible.

Because of the relationship between winter hardiness and fructan accumulation, we measured the fructan content of five orchardgrass germplasm sources considered to differ in winter hardiness. We hypothesize that fructan accumulation upon cold acclimation increase and cause orchardgrass plants to become more freezing tolerant, and that fructan biosynthesis gene transcripts are induced upon cold acclimation while fructan degradation gene transcripts are reduced. Within the context of recent genomic resources, we have identified *GH32* candidate genes with likely invertase, fructosyltransferase, and exohydrolase gene functions based on sequence similarity to functionally characterized genes in Triticeae and *L. perenne*. We show that orchardgrass does indeed contain fewer fructosyltransferases but more *FEH* and *CWI* gene families than Triticeae or *L. perenne*, and pinpoint highly expressed genes for each gene family.

MATERIALS AND METHODS

Plant materials

Five orchardgrass germplasm sources were used: “AC Killarney,” “Grasslands Tekapo” (Tekapo), “Potomac,” PI 372621, and a UTWH-102 breeding line. AC Killarney is a cold tolerant cultivar developed in Alberta, Canada while Tekapo is a more winter active and cold intolerant cultivar from New Zealand (Rumball et al., 1997). In internal pilot studies, PI 372621 produced higher water-soluble carbohydrate levels under control conditions than other cultivars and accessions and was thus hypothesized to carry cold tolerance. The UTWH-102 breeding line was collected in high elevation and freezing-stressed sites in Utah (Robins et al., 2018), and persisted under freezing conditions with little snow cover. Finally, Potomac was included as a commonly grown cultivar (Bushman et al., 2011) with no expectations of cold tolerance. These germplasm sources were intended to exemplify a range of differences in winter hardiness and freezing tolerance.

Field winter survival test and growth chamber freezing tolerance assessment

To assess winter hardiness, winter survival at a Utah field site and freezing tolerance in a growth chamber were tested. For winter survival, nine seedlings of each germplasm source were transplanted in a completely randomized design at the Utah State University Panguitch research farm (37.869° N, 112.436° W, 1994 m a.s.l.) in fall 2017. Winter temperatures and snowfall were recorded at the Panguitch farm weather station, and a plant vigor score was recorded on June 1 of 2018. The

vigor score ranged from 0 to 5, with 0 equivalent to plant mortality and 5 to healthy mature plants. The winter survival data were analyzed using a simple general linear model in the lme4-R package (Bates et al., 2015), with genotype as fixed effects and replications as random effects. Means and standard errors were shown in the results. To assess freezing tolerance, 50 plants of each entry were germinated in 50-cell flats in a randomized design, and grown to the five-tiller stage. Seedlings were then placed in a growth chamber at 4°C with 8 h of low light at 150 $\mu\text{mol s}^{-1} \text{m}^{-2}$ (PAR) for 14 days. After cold acclimation, eight seedlings of each germplasm source were randomized into new flats and subjected to one of four freezing temperatures: -6°C, -9°C, -12°C, and -15°C. The ramping from 4°C to the target freezing temperature occurred at a rate of 1.2°C each hour (Skinner & Garland-Campbell, 2014), and the seedlings remained at each final freezing temperature for 12 h in the dark. After the freezing treatments, plants were returned to 4°C at the same ramping rate, and maintained at 4°C in a growth chamber for 8 h with low light at 150 $\mu\text{mol s}^{-1} \text{m}^{-2}$. The seedlings were then placed in a greenhouse at 25°C/15°C (day/night) with natural light for 30 days and the survival of each entry was recorded. Analysis of the median lethal temperature for 50% of the plants to die (LT₅₀) was accomplished using a probit analysis with the binomial family function in R (Bates et al., 2015), with the following model:

$$P(y_{ijk} = 1) = \Phi(B(0) + B(g_i) + B(t_j) + B(gt_{ij})),$$

where $P(y_{ijk} = 1)$ is the probability of plant death, Φ is the cumulative distribution function of the standard normal distribution, $B(0)$ is the intercept, $B(g_i)$ is the fixed effect of genotype (i), $B(t_j)$ is the fixed effect of temperature (j), and $B(gt_{ij})$ is the interaction effect between genotype (i) and temperature (j).

The results were imported in MASS library using the p.dose command to estimate LT₅₀ values and standard error values for each entry (Venables & Ripley, 2002). Concomitantly, additional seedlings were germinated in 50-cell flats and maintained in a greenhouse at 25°C/15°C (day/night) with natural light for 30 days before sampling as controls.

Carbohydrate quantification and gene expression profiling

Eighteen of the 50 cold-acclimated crowns from each germplasm source, in three-crown bulks, were quickly washed, separated from other plant tissues, and flash frozen in liquid nitrogen. These crown samples were used for carbohydrate quantification and gene expression profiling, with three replicates used for the former and three for the latter. Similarly, bulked crowns from control samples were quickly washed, separated from other plant tissues, and flash frozen in liquid nitrogen. For water-soluble carbohydrate estimates, HPLC was used to quantify glucose, fructose, sucrose, and fructan on the control and cold-acclimated crowns. For each bulked sample, crowns were lyophilized and 10 mg of dried and ground tissue was

placed in 50 mL deionized water and incubated for 60 min at 65°C with gentle rocking. Of this suspension, 1 mL of the water extract was filtered through 45 μm syringe filters and placed in the sample vials. With MilliQ ultra-pure water as the flow-through, the samples were resolved on a CarboSep CHO 682 column (Chrom Tech Inc., Apple Valley, MN, USA) with an Adsorbosphere XL SCX guard column (Dr. Maisch GmbH, Ammerbuch, Germany), and a 1260 refractive index detector (Agilent, Santa Clara, CA, USA). Column temperatures were 85°C, 20 μL was injected from each sample, and a standard curve of fructose was used to estimate quantities of all four carbohydrate peaks. Differences, means, and standard errors were calculated using a general linear model in R (Bates et al., 2015), with genotype, treatment, and genotype $\times$ treatment as fixed effects. The values are reported as mg g^{-1} of dried tissue. Glycoside hydrolase family 32 sequences were obtained from gene annotations of an orchardgrass reference genome assembly (Huang et al., 2019) using BlastX searches from model organism genes and an E-value cutoff of 1e^{-5} . These included: *T. aestivum* 6-SFT (ABO29887); *T. aestivum* 1-SST (ABO29888); *T. aestivum* 1-FFT (ABO88409); *T. aestivum* 6&1-KEH (ABO89269); *T. aestivum* 6-KEH (ABO89270); *T. aestivum* CWI (AF030420); *L. perenne* FT1 (AF481763); *L. perenne* 6G-FFT (AF492836); *L. perenne* 6-SFT (AF494041); *Festuca arundinaceae* 1-SST (AJ297369); *T. aestivum* 1-FEH w1 (AJ516025); *Hordeum vulgare* L. 1-SST (AJ567377); *H. vulgare* 1-FEH (AJ605333); *H. vulgare* VI (AJ623275); *T. aestivum* VI (AJ635255); *T. aestivum* 6-FEH (AM075205); *L. perenne* VI (AY082350); *L. perenne* 1-SST (AY245431); *B. distachyon* INV (Bradi1g52210); *B. distachyon* VI (Bradi3g00910); *B. distachyon* FFase (Bradi5g16900); *L. perenne* 1-FEH (DQ018712); *L. perenne* FEH (DQ073968); *L. perenne* CWI (DQ073969); *H. vulgare* 1-FFT (JQ411253); *L. perenne* 6-FEH (KY554803); *H. vulgare* 6-SFT (X83233). Once identified, the orchardgrass fructan biosynthesis genes were translated and aligned to the model grass amino acid sequences using MAFFT in Geneious Prime v2025 software (Auckland, New Zealand). Unrooted gene relationship trees were generated using a neighbor joining distance method in Geneious Prime. Basal gene expression values of each *GH32* gene were extracted from the RNAseq data along with log2 fold changes and adjusted probability values (p_{adj}). Reference map positions were extracted from the published assembly annotation.gff file (Huang et al., 2019).

For gene expression profiling, tissue from the control and cold-acclimated crowns was harvested and collected as listed above. Total RNA was extracted from these 30 control and cold-acclimated crowns using the DirectZol RNA extraction kit (Zymo, Tustin, CA, USA), and 20 million PE.150 reads (Illumina, San Diego, CA, USA) were sequenced from each sample at Novogene (Sacramento, CA, USA). These transcripts are held in the National Center for Biotechnology Information Short Read Archive, under BioProject PRJNA1157851. Transcript reads were quality (SLIDINGWINDOW:4:20) and end trimmed using Trimmomatic v0.39 software (Bolger et al., 2014), mapped to an orchardgrass reference genome assembly (Huang et al., 2019) using HiSat2 v2.2.1 software (Kim et al., 2019), and read counts generated using HTseq v2.0.8 software in union mode to produce transcript counts

(Putri et al., 2022). The counts were filtered to require at least 10 counts in at least five samples. DeSeq2 v1.38.3 software (Love et al., 2014) was used for normalization and differential expression analysis with genotype, treatments, and genotype $\times$ treatment as fixed effects in the model. Differentially expressed genes (DEGs) were called if the false discovery rate probability value (p_{adj}) was less than 0.05 and at least a twofold difference occurred between transcript counts. Functional and Gene Ontology annotations were created for each gene using OMICS Box software (BioBam Bioinformatics S.L., Valencia, Spain), with tBlastX, Blast2GO, and InterPro pipelines employed. A grouping of gene expression profiles with four carbohydrate content patterns was made with WGCNA (Langfelder & Horvath, 2008), with a clade cutoff at 0.2 to determine module numbers and membership.

RESULTS

Field winter survival test and growth chamber freezing tolerance assessment

The Panguitch field site recorded a winter low temperature of -22°C , an average winter temperature from October through March of -10°C , and an average snowcover from December through March of 7 mm. Although significant mortality occurred at the Panguitch site for all five entries, AC Killarney had the lowest mortality as shown by the highest overall vigor scores, while Tekapo had the highest mortality and lowest vigor scores (Figure 1a). Likewise, in the freezing study, AC Killarney had an LT_{50} lower than -11°C while Tekapo had an LT_{50} at -8°C (Figure 1b). For both measurements, UTWH-102 was second in freezing tolerance and winter survival while Potomac and PI 372621 were not significantly different from each other and lower than AC Killarney and UTWH-102 (Figure 1a,b).

Carbohydrate quantification

Overall sucrose and glucose levels were detected at less than 5 mg g^{-1} dried tissue, fructose at between 5 and 10 mg g^{-1} , and fructans at $15\text{--}36\text{ mg g}^{-1}$. Both glucose and fructose levels increased in cold-acclimated crowns,

significantly so for most entries (Figure 2). Sucrose levels were inconsistent across the entries; and not significantly different between control and cold-acclimated crowns for three of the five entries. Fructan levels, however, were induced by cold in all five entries, with the cold-hardy AC Killarney showing the highest induction and the highest overall cold-acclimated level and Tekapo having the lowest cold-acclimated level (Figure 2). PI 372621 crowns, which had the highest fructan content under control temperatures, exhibited a smaller induction of fructans upon cold acclimation in comparison to AC Killarney. Likewise, UTWH-102, second to AC Killarney in winter-hardiness and freezing tolerance, exhibited a relatively small induction of fructans in cold-acclimated tissues.

GH32 gene family cluster analysis

Twenty-one *GH32* genes were identified and aligned to related genes from *B. distachyon*, *T. aestivum*, *H. vulgare*, and *L. perenne* (Figure 3); and separated into the four major fructan catabolism gene clades. One of the 21 orchardgrass genes grouped together with a *B. distachyon* β -fructofuranosidase (FFase) gene, and both were proximal to the *VI* gene clade. Four other orchardgrass genes were annotated as both β -fructofuranosidase and *FEH* genes, and aligned closest to a *T. aestivum* 6-*FEH* gene in a sub-clade separate from other *FEH* genes (Figure 3).

Seven orchardgrass genes grouped with *L. perenne* or *T. aestivum* *CWI* sequences, and these were proximal to the *FEH* clades. Aside from the four orchardgrass genes that grouped with *T. aestivum* 6-*FEH*, four other orchardgrass genes were in the *FEH* clade, some grouping closer to 1-*FEH* and others to 6-*FEH* genes from *L. perenne* or Triticeae sequences. Similar to the relationship between *CWI* and *FEH*, the two orchardgrass genes that grouped with *VI* genes were proximal to the fructosyltransferase clade. Since orchardgrass mainly synthesizes β -(2,6) linkages (Chatterton et al., 1993), the identification of a 6-*SFT* was of primary interest. The DG2G04963 gene grouped together with other 6-*SFT* genes into a distinct sub-clade, which was then anchored to other related fructosyltransferase (1-*SST* and *FFT*) genes. The two other fructosyltransferase genes in that clade likely have 1-*SST* and *FFT* activity.

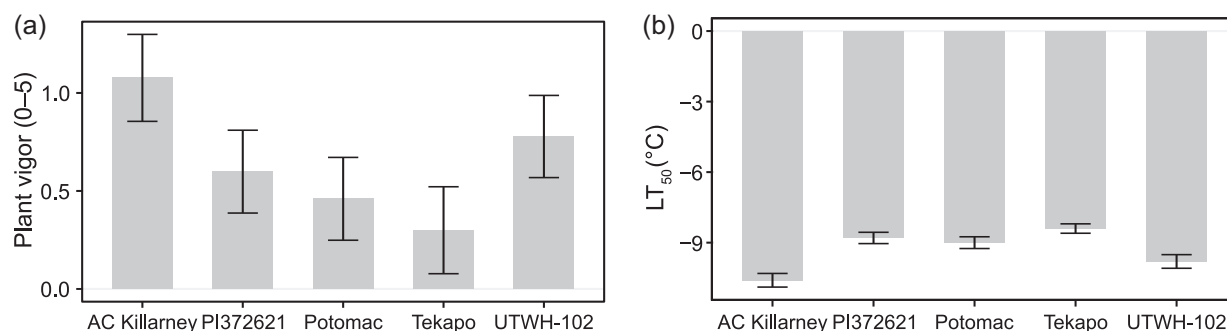


FIGURE 1 (a) Means and standard errors of plant vigor after the 2018 winter season at Panguitch research farm, Utah for five orchardgrass entries. (b) Means and standard errors of the mean lethal temperature for 50% of the plants to die (LT_{50}) values of five orchardgrass entries subjected to sequential freezing conditions after 14 days of cold acclimation. Overlapping standard errors were not significantly different at $p < 0.05$.

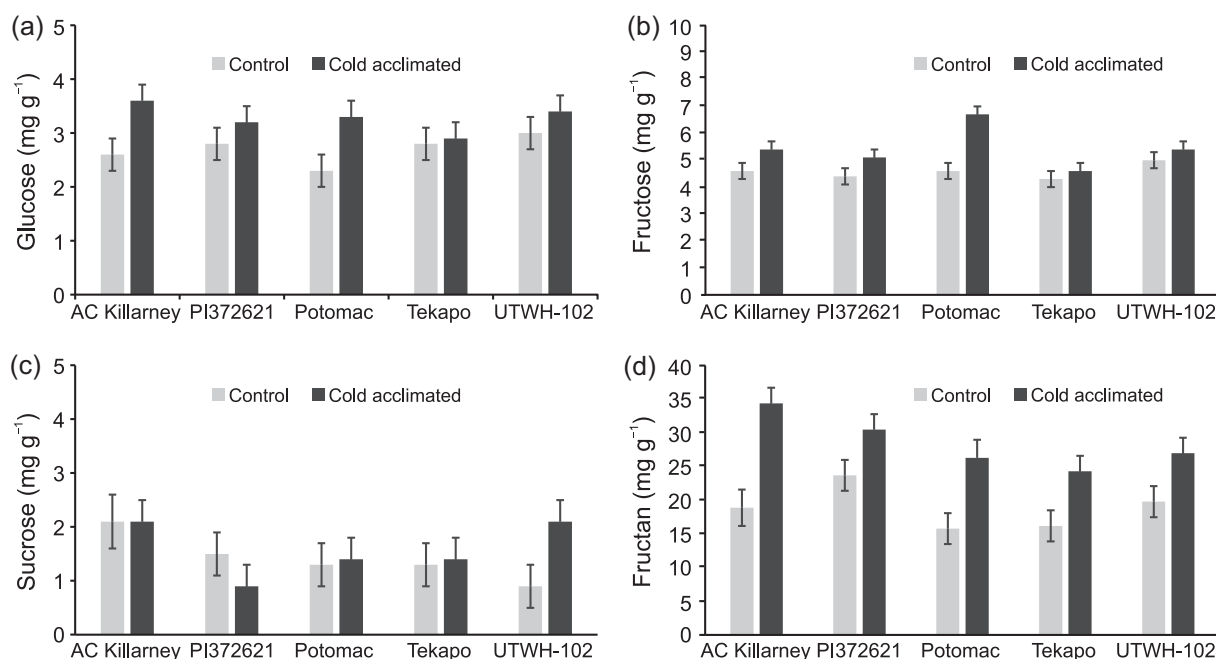


FIGURE 2 Glucose, fructose, sucrose, and total fructan levels across five germplasm sources and upon cold-acclimation in *Dactylis glomerata* crown tissues. Overlapping standard errors were not significantly different at $p < 0.05$.



FIGURE 3 Unrooted gene dendrogram showing homology based on amino acid sequence similarity for four main clades of GH32 genes in *Dactylis glomerata* L. (DG) and related genes from temperate grasses. Bolded *D. glomerata* gene names correspond to differentially expressed genes (DEGs) induced upon cold acclimation while italicized *D. glomerata* gene names correspond to DEGs reduced upon cold acclimation.

Gene expression profiling

To determine which fructosyltransferase genes were expressed and induced by cold-acclimation, we utilized a transcriptome approach to sequence genes expressed in control and cold-acclimated crowns. Of the 42 333

genes in the *D. glomerata* annotation data set (Huang et al., 2019), 25 026 had expression evidence in at least one of the entries and treatments and 5123 were DEGs upon cold-acclimation (Table S1). Transcript levels were induced in 1903 and reduced in 3220, and DEGs clustered into eight modules of expression according to

their association with the four carbohydrate levels (Figure 4). Of those eight expression modules, modules 2 and 7 showed the highest correlations with fructan content. Module-7 included the vast majority of DEGs, was characterized by equal levels of induction/reduction in transcript levels across all five germplasm sources and upon cold-acclimation, and included seven of eight *GH32* DEGs (Table S1). The remaining *GH32* DEG in module-2 (DG2G04963) also showed an approximately equal change in transcript level in all five germplasm sources upon cold-acclimation, similar to module-7, but with an outlying UTWH-102 control transcript level that was significantly higher than the others.

Eighteen of the 21 orchardgrass *GH32* genes had expression support in control or cold-acclimated tissues, and eight were DEGs (Table 1 and Figure 3). Of the seven *CWIs*, two had no expression evidence and none were differentially expressed with a twofold change. A cluster of two *CWI* genes on chromosome-3 included one gene with high basal expression level and intact *GH32* motifs while the other showed no expression evidence and had a deletion in the WMNDPNG motif (Table 1). All eight putative *FEH* genes had expression evidence, with three showing greater than a twofold increase or decrease in transcript levels upon cold-acclimation. Three *FEH* genes were clustered on chromosome-3 and three were clustered on chromosome-4; coincident with sub-clades from Figure 3. In both clusters a dominant gene had high basal expression

levels and no deletions in the WMNDPNG motif (Table 1); DG2G00215, DG2G00400, and DG4G00207. One of the sub-clades was characterized by a Glutamine in the WMNDPNG motif while the other sub-clade had Methionine or Isoleucine in that position. The DG3G00215 orchardgrass *FEH* gene that grouped with *T. aestivum* 6-*FEH* (Figure 3) was induced upon cold-acclimation while DG3G00400 and DG3G00402 in the same sub-clade showed reduced transcripts upon cold-acclimation.

The β -fructofuranosidase-1 and both *VI* genes had reduced transcript levels upon cold-acclimation while two of the fructosyltransferases, including the putative 6-*SFT* (DG2G04963), were induced (Table 1). The DG2G04963 gene had a phenylalanine in the WMNDPNG motif, and exhibited the highest basal level of transcript abundances of all *GH32* genes in this treatment and study (Table 1). A third fructosyltransferase gene had no expression evidence and unique sequences at the two *GH32* motif sites. These data are consistent with increased fructan content upon cold acclimation caused by an induction to fructan synthesis by fructosyltransferase genes and a reduction in hydrolysis of fructans by *FEH* and *VI* genes.

DISCUSSION

Although orchardgrass is grown in cool temperate climates, winters are often characterized by insulating snow cover. Thus winter hardiness can be correlated with factors such as snow mold (*Typhula incarnata* Lasch, Epicr. (1838)) resistance rather than freezing tolerance (Sanada et al., 2010). However, in freezing climates where snow cover is unpredictable, winter hardiness exemplifies freezing tolerance rather than snow mold resistance. In the high-elevation Panguitch location in this study, the average snow cover over the winter season was 7 mm, which was insufficient to cover crowns of orchardgrass plants, such that the winter hardiness results were more characteristic of freezing tolerance. Combining the Panguitch winter hardiness and freezing tolerance phenotypic responses, the Canadian cultivar AC Killarney showed the highest trait values of both, and was significantly better than Grasslands Tekapo, Potomac, and PI 372621. UTWH-102, initially selected near the Panguitch site, also had high winter hardiness and freezing tolerance and was similar to AC Killarney.

Most of the water-soluble carbohydrates increased upon cold acclimation in all five germplasm sources. Glucose and fructose feed into sucrose synthesis, and all three are involved in multiple biological processes besides fructan accumulation and winter hardiness. Nevertheless, fructose and glucose increased upon cold acclimation, as a trend in some germplasm sources and significantly in other germplasm sources. Sucrose levels varied upon cold acclimation for the five germplasm sources, indicative of its dynamic role in plant growth and development rather than a strict source for fructan biosynthesis (Winter & Huber, 2000). In context of freezing tolerance, sucrose levels were shown to fluctuate over time during cold acclimation (Gallagher et al., 2007) and were instrumental in fructan induction or turnover through the regulation of the VI and 1-SST enzyme

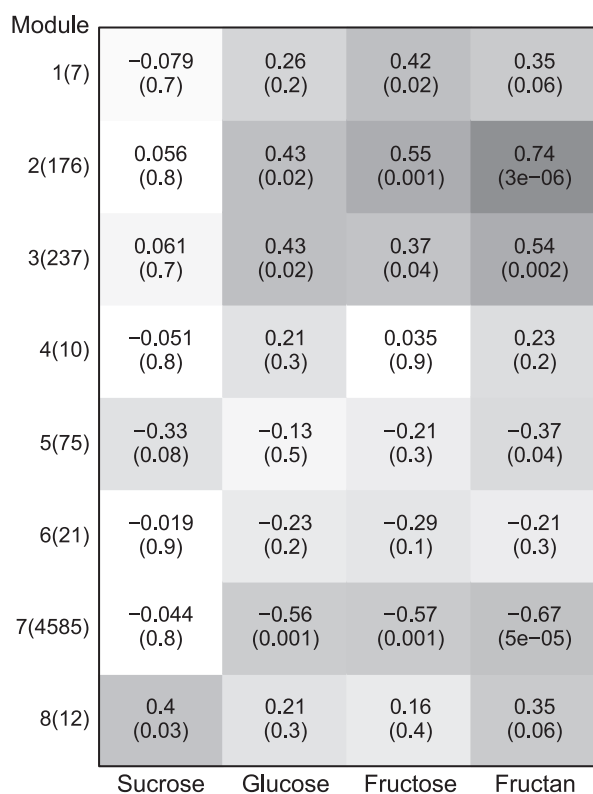


FIGURE 4 Differentially expressed genes grouped into eight modules based on expression patterns across two treatments and five *Dactylis glomerata* L. germplasm sources. The numbers of differentially expressed genes (DEGs) are listed in parentheses next to each module, and a heatmap of correlations and (*p* values) between modules and content of each of the four measured carbohydrates is shown.

TABLE 1 Glycoside hydrolase 32 (*GH32*) genes from *Dactylis glomerata* L. and their homology group, chromosome position, expression values, adjusted probability values (p_{adj}), and major amino acid motif sequences.

<i>GH32</i> gene	<i>GH32</i> family	Chromosome: position	Base count	Log ₂ fold change	p_{adj}	WMNDPNG motif	WSGSAT motif
DG2G01456	CWI	Chr2:57297102-57300843	771.5	-1.49	0.025	WINDPNG	WSGSTT
DG3G02665	CWI	Chr3:95208157-95210751	—	—	—	WI---NG	WSGSAT
DG3G02666	CWI	Chr3:95245903-95249567	1441.3	-1.02	0.921	WINDPNA	WSGSAT
DG5G02922	CWI	Chr5:159620551-159623856	—	—	—	WINDPNG	WSGSAT
DG5G03161	CWI	Chr5:167773401-167775320	36.7	1.29	0.825	WINDPNG	WSGSAT
DG5G05140	CWI	Chr5:234944233-234955227	28.6	1.31	0.203	WMNDPNA	WSGSAT
DG6G02005	CWI	Chr6:115278723-115284518	139.8	1.04	0.778	-----G	WSGSAT
DG1G05145	FEH	Chr1:238158453-238161861	849.4	1.82	<0.001	WINDPSG	WTGSTT
DG3G00215	FEH	Chr3:9828843-9841514	1827.7	7.59	<0.001	WQNDPNG	WSGSAT
DG3G00395	FEH	Chr3:15227181-15229413	33.9	1.02	0.948	WQ---NG	WSGSAT
DG3G00400	FEH	Chr3:15314359-15318637	1573.6	-37.51	<0.001	WQNDPNG	WSGSAT
DG3G00402	FEH	Chr3:15390179-15401974	10.0	-112.62	<0.001	WQ---NG	YSGSAT
DG4G00207	FEH	Chr4:6289104-6296490	1460.2	1.82	<0.001	WMNDPSG	WTGSTT
DG4G00208	FEH	Chr4:6310066-6316451	189.6	-1.05	0.849	—	WTGSAT
DG4G00212	FEH	Chr4:6478098-6481546	118.0	-1.48	0.001	WIN---G	WTGSAT
DG3G02142	FFase	Chr3:76569274-76574899	201.7	-6.71	<0.001	WMNDPNG	WTGSAT
DG2G00095	VI	Chr2:3453877-3460955	74.9	-3.86	<0.001	WMNDPNG	WSGSAT
DG2G04966	VI	Chr2:222270036-222273950	381.4	-15.10	<0.001	WMNDPNG	WTGSAT
DG2G04962	FT	Chr2:221997545-222000705	1602.1	3.20	<0.001	YMNDPNG	LTGSIT
DG2G04963	FT	Chr2:222055303-222058920	22204.4	8.27	<0.001	FMSDPNG	LSGSIT
DG2G04965	FT	Chr2:222260375-222262852	—	—	—	YM---NG	MTGSTT

activities (Winter & Huber, 2000). In this study, fructan levels were induced in all five germplasm sources upon cold acclimation for 4 weeks as predicted. Although too few germplasm sources were examined in this study for statistical correlations, the level of induction or the fructan content was highest for AC Killarney and lowest for Tekapo (Figure 2).

Much of the identification of genes involved in fructan biosynthesis predated genomic assemblies and were based on BAC or PCR gene cloning. Thus the numbers of genes, their relationship with each other, and their relative transcription is not well understood in the temperate forage grasses. Furthermore, orchardgrass chromosome numbers were assigned by Huang et al. (2019) by decreasing length while reference *L. perenne* chromosomes were assigned based on synteny to Triticeae. As orchardgrass produces a relatively simple unbranched levan type β -(2,6) fructan profile, like *Phleum pratense* L. (Tamura et al., 2009), we hypothesized that it carries fewer fructosyltransferases than *T. aestivum* or *L. perenne*. Indeed, in the reference genotype sequenced by Huang et al. (2019), only three fructosyltransferases were detected and one of them had no expression evidence (Table 1). These three each carried four exons, as well as unique WMNDPNG and WSGSAT motifs indicative of fructosyltransferases (Francki et al., 2006; Ritsema

et al., 2006). The DG2G04963 gene grouped with 6-*SFT* genes in other grasses, which was of particular interest as it would encode gene activity that elongates kestotriose to longer DP β -(2,6) fructans in orchardgrass. With the largest basal transcript level, and induction upon cold acclimation in the five orchardgrass germplasm sources, this gene is a likely candidate for 6-*SFT* fructan biosynthesis in orchardgrass. Although it was grouped in module-2 in the transcriptome results, indicative of equal transcript induction in all five germplasm sources, its role in abiotic stress tolerance, forage regrowth, and forage quality poses it as a candidate for further study.

Where orchardgrass does not synthesize appreciable amounts of F-type fructans (Chatterton et al., 1993), the DG2G04965 gene, with no expression evidence in this experiment, might encode a pseudogene product with minimal *FFT* activity. The remaining fructosyltransferase gene, DG2G044962, showed homology to 1-*SST* and 6*G-FFT* in *L. perenne*; which themselves are 83% identical at the amino acid level (Lasseur et al., 2011). DG2G044962 exhibited relatively high basal expression in our study and was induced upon cold acclimation. It mapped together with the other two fructosyltransferase genes and a *VI* gene in a cluster on orchardgrass chromosome-2, similar to a syntenic map location on *L. perenne* chromosome-7 (Chen et al., 2024) and a

cluster of homologous genes in *H. vulgare* (Huynh et al., 2012). This gene could function as *1-SST*, but as the primary DP3 fructan in orchardgrass is a 6-kestotriose rather than a 1-kestotriose (Chatterton et al., 1993), more experimental evidence would be necessary to confirm its role.

Three orchardgrass genes were found to be related to *VI* genes, with DG3G02142 most similar to a β -fructofuranosidase. All three grouped with respective *B. distachyon* β -fructofuranosidase or invertase genes (Figure 3) and exhibited transcript reductions upon cold acclimation in this study (Table 1). Both β -fructofuranosidase and *VI* genes have functions to hydrolyze sucrose to glucose and fructose, and their reduction would increase the pool of sucrose that could be fed into fructan biosynthesis (Winter & Huber, 2000). The DG2G00095 gene grouped closest to *VI* genes from other temperate grasses and the DG2G04966 gene was colocated with the three fructosyltransferases in a gene cluster on chromosome-2 (Table 1). In contrast to *VI* genes, seven *CWI* genes were identified, consistent with a *CWI* gene family expansion in orchardgrass (Huang et al., 2019). The DG3G02666 *CWI* gene, with the highest transcript levels, was clustered on chromosome-3 near another *CWI* with no transcript evidence. None of the *CWI* genes were differentially expressed, consistent with their diverse roles in other plant biological processes (Winter & Huber, 2000).

A larger number of *FEH* genes were grouped into two sub-clades with four genes each (Figure 3). In one sub-clade, DG4G00207 and DG1G05145 grouped with a *L. perenne* 6-*FEH*, DG4G00208 grouped with a *L. perenne* 1-*FEH*, and DG4G00212 grouped with a *L. perenne* unspecified *FEH* (Figure 3). These four *FEH* homologs were characterized by a Threonine rather than a Serine in the WSGSAT motif, and three were clustered together on chromosome-4 (Table 1). Unlike *L. perenne*, which produces branched neoseries of fructans and exhibits both 1-*FEH* and 6-*FEH* activities (Lothier et al., 2007; Marx et al., 1997), orchardgrass fructans are characterized by β -(2,6) linkages such that orchardgrass is not predicted to have appreciable 1-*FEH* activity. In this study, the putative 1-*FEH* gene (DG4G00208) had a relatively low basal transcript count of 190 while the DG4G00207 and DG1G05145 genes, which grouped with *L. perenne* 6-*FEH*, had basal expression counts of 1460 and 849, respectively (Table 1). Since the two putative 6-*FEH* genes also showed slight increases in transcript abundances (Table 1), they may have roles in turnover steps during fructan biosynthesis. The second sub-clade of *FEH* genes included DG3G00215, DG3G00395, DG3G00400, and DG3G00402. These were characterized by a Glutamine rather than the Methionine in their *GH32* WMNDPNG motif, were annotated as β -fructofuranosidases rather than fructan exohydrolases by Blast2Go algorithms (Table S1), but were also grouped with a *T. aestivum* 6-*FEH* gene (Figure 3). Three of the four were DEGs, with DG3G00215 highly induced and DG3G00400 and DG3G00402 highly reduced upon cold acclimation (Table 1). Three of the four were also located in a gene cluster on chromosome-3, with DG3G00400 as the gene with the highest basal transcript levels in that cluster. The *FEH* genes whose expression was reduced upon cold acclimation may contribute to winter hardiness by

preventing the loss of fructans throughout winter, which in turn may provide freezing tolerance and snow mold (*Typhula ishikariensis* S. Imai (1930)) resistance (Yoshida et al., 1998; Yoshida, 2021). Further experimentation, to distinguish the expression of these genes upon a broader array of abiotic stress and defoliation responses, would be necessary to differentiate their function from the other *FEH* genes.

CONCLUSIONS

In this study, 21 *GH32* genes were identified and characterized in relationship to cold acclimation and freezing tolerance. Consistent with the relatively uncomplicated fructan pattern of orchardgrass, few fructosyltransferase and *VI* genes were identified. In contrast seven and eight *CWI* and *FEH* genes, respectively, were identified. An expression pattern is shown where fructan biosynthesis genes were induced while key *VI* and *FEH* genes were reduced upon cold acclimation, highlighting specific genes involved in fructan synthesis and their role in orchardgrass' freezing response.

AUTHOR CONTRIBUTIONS

B. Shaun Bushman: Conceptualization; formal analysis; investigation; project administration; resources; supervision; validation; writing—original draft; writing—review and editing. **Joseph G. Robins:** Conceptualization; formal analysis. **Xinxin Zhao:** Investigation. **Guangyan Feng:** Investigation. **Xinquan Zhang:** Resources; supervision. **Linkai Huang:** Conceptualization; resources; supervision. **Matthew D. Robbins:** Data curation; formal analysis; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the NCBI under BioProject PRJNA1157851.

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