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Transcript profiling of fructan biosynthetic pathway genes reveals association of a specific fructosyltransferase isoform with the high sugar trait in *Lolium perenne*

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SUMMARY

Lolium perenne cultivars with elevated levels of fructans in leaf blades (high sugar-content grasses) have been developed to improve animal nutrition and reduce adverse environmental impacts of pastoral agricultural systems. Expression of the high sugar trait can vary substantially depending on genotype × environment (G × E) interactions. We grew three potential high sugar-content and a control cultivar in three temperature regimes and quantified water soluble carbohydrates (WSCs) and the expression of all functionally characterised *L. perenne* fructan pathway genes in leaf tissues. We also analysed the distribution, expression and sequence variation of two specific isoforms of Lp6G-FFT (fructan: fructan 6G-fructosyltransferase). Our study confirmed a significant G × E interaction affecting the accumulation of fructans in the high sugar-content cultivar AberDart, which accumulated higher levels of high DP (degree of polymerisation) fructans in blades compared to the control cultivar only when grown at 20 °C (day)/10 °C (night) temperatures. The cultivar Expo on the other hand accumulated significantly higher levels of high DP fructans in blades independent of temperature. Fructan levels in pseudostems were higher than in blades, and they increased markedly with decreasing temperature, but there was no consistent effect of cultivar in this tissue. The expression of the high sugar trait was generally positively correlated with transcript levels of fructosyltransferases. Presence and expression of only one of the two known 6G-FFT isoforms was positively correlated with high fructan biosynthesis, while the second isoform was associated with low fructan concentrations and positively correlated with fructan exohydrolase gene expression. The presence of distinct 6G-FFT sequence variants appears to be associated with the capacity of high sugar-content grasses to accumulate higher fructan levels particularly at warmer temperatures. These findings might be exploited for the selection and breeding of 'warm-effective' high sugar-content grasses to overcome some of the limitations of current high sugar-content ryegrass cultivars.

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Introduction

Global meat and milk production depends to a high degree on the capacity of pastoral agriculture to provide forage plants such as perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Schedonorus arundinaceus* Schreb.) to meet animal feed requirements (Wilkins and Humphreys, 2003; Hopkins and Wilkins, 2006). Intensive pastoral systems require high input of mineral nitrogen (N) leading to high rates of nitrate leaching and emissions of the potent greenhouse gas nitrous oxide from pastures (Oenema et al., 1997; Müller and Sherlock, 2004). A high water soluble carbohydrate (WSC) content has been linked to the efficient utilisation of proteins in forage grasses such as perennial ryegrass (Humphreys, 1989; Wilkins and Humphreys, 2003). Typically, the WSC content of leaf blades

Abbreviations: AD, AberDart; AM, AberMagic; DP, degree of polymerisation; E, Expo; F, Fennema; 1-FEH, fructan 1-exohydrolase (EC 3.2.1.80); 1-FFT, fructan: fructan 1-fructosyltransferase (EC 2.4.1.100); 6G-FFT, fructan: fructan 6G-fructosyltransferase (EC 2.4.1.243); HMW, high molecular weight; INV, invertase (EC 3.2.1.26); LMW, low molecular weight; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; 6-SFT, sucrose: fructan 6-fructosyltransferase (EC 2.4.1.10); 1-SST, sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99); WSC, water soluble carbohydrates.

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('harvestable component') is low, and considerable efforts have been put into the development of forage grasses with increased sugar accumulation (mainly fructans) in blade tissues (Humphreys et al., 2006; Turner et al., 2006). These high sugar-content grasses can offer benefits to the dairy and meat industries through improving N use efficiency in grazing ruminants and potentially reducing N leaching and greenhouse gas emissions in the form of nitrous oxide from pastures (Ellis et al., 2011; Parsons et al., 2011a,b). More recently, high sugar-content grasses have also been proposed to be used as an alternative plant source for bioethanol production (Farrar et al., 2012).

The major reserve WSCs accumulating in temperate forage grasses are fructans, which are composed of linear or branched oligo/polymers of fructose attached to sucrose through glycosidic bonds of various linkage types (Vijn and Smeekens, 1999). Three types of fructans have been identified in perennial ryegrass, *i.e.* inulin series, inulin neoseries, and levan neoseries fructans, with the majority (up to 76%) belonging to the levan neoseries containing an internal glucose residue with a high proportion of β-(2,6) linked fructose residues (Pavis et al., 2001a,b).

Fructans are synthesised by a variety of enzymes belonging to the family of fructosyltransferases. Sucrose: sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) initiates *de novo* fructan synthesis by catalysing the formation of the trisaccharide 1-kestose (Edelmann and Jefford, 1968; Lüscher et al., 2000; Chalmers et al., 2003). Fructan: fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) produces fructans with β-(2,1) linkages (Edelmann and Jefford, 1968; Van den Ende et al., 1996), while the biosynthesis of neoseries fructans requires fructan: fructan 6G-fructosyltransferase (6G-FFT; EC 2.4.1.243) activity. 6G-FFT uses 1-kestose as a fructose donor and transfers the fructose unit to the C6 of the glucose moiety of sucrose or oligofructans *via* a β-(2,6) linkage (Vijn et al., 1997; Chalmers et al., 2005). Several sequence variants of 6G-FFT have been cloned and functionally characterised from perennial ryegrass and it has been shown that the encoded enzymes have both 6G-FFT and 1-FFT activity (Lasseur et al., 2006; Hisano et al., 2008). To date, no gene coding for an enzyme with exclusive 6G-FFT or 1-FFT activity has been isolated from perennial ryegrass. The trisaccharide neokestose (6G-kestotriose) is the shortest fructan of the neoseries fructans and can be elongated on either terminal fructose residue with β-(2,1) or β-(2,6) linked fructose units to form the inulin or levan neoseries fructans, respectively. The reaction leading to β-(2,6) linked fructans is catalysed by sucrose: fructan 6-fructosyltransferase (6-SFT, EC 2.4.1.10; Sprenger et al., 1995; Lasseur et al., 2011).

The regulation of fructan accumulation is also under the control of breakdown enzymes such as fructan exohydrolases (FEH, EC 3.2.1.80). Three types of exohydrolase are known, 1-FEH (6-FEH, and 6 and 1-FEH, preferentially degrading β-(2,1), β-(2,6), and both β-(2,1) and β-(2,6) linkages, respectively (Van den Ende et al., 2004; Kawakami et al., 2005). The only FEH cloned and functionally characterised from perennial ryegrass is Lp1-FEH (Lothier et al., 2007).

Both field and controlled environment studies have shown that the expression of the high sugar trait in high sugar-content grasses, and prospective benefits to pasture based animal production, can vary substantially and depends on environmental conditions and pasture management (Turner et al., 2006, 2008, 2010; Parsons et al., 2011a,b). Previously, we identified a strong interaction between the accumulation of high fructan levels in blades and growth temperature (Parsons et al., 2004). We discussed that it might be necessary to extend breeding strategies to select for perennial ryegrass genotypes which accumulate higher levels of WSCs specifically at warmer (night) temperatures to ensure trait expression in warmer climates *e.g.* in Southern regions of Europe, the dairy production areas of South-Eastern Australia, and in New Zealand. In the present study, we analysed genotypes from four perennial ryegrass cultivars for their potential to accumulate high levels of fructans in blades at a range of temperatures in a controlled environment study. So far the molecular mechanisms leading to high fructan accumulation in blades of high sugar-content grasses remain elusive; we therefore sought to determine whether high levels of fructans were associated with a differential regulation of fructosyltransferase and FEH expression in high fructan accumulating genotypes. We analysed transcript profiles of all functionally characterised perennial ryegrass fructan biosynthetic pathway genes using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and identified sequence variants of Lp6G-FFT potentially related to high fructan accumulation.

Materials and methods

Plant material and sample preparation

Seeds of four perennial ryegrass (*Lolium perenne* L.) cultivars, *i.e.* three potential high sugar-content cultivars (AberDart (AD), AberMagic (AM) – IGER (Institute of Grassland and Environmental Research, UK); Expo (E) – Grasslands Innovation Ltd., NZ), and one European control cultivar (Fennema (F)) were germinated and grown in pots filled with nutrient rich potting mix and grown for three months (Nov to Jan) in a glasshouse at AgResearch, Palmerston North, NZ. Plants were clonally replicated (each genotype to give three clonal replicates) and after three months three sets of 8 genotypes from each cultivar/population were transferred to controlled environment chambers (NZ Controlled Environmental Laboratory, Palmerston North, NZ). A total of 24 genotypes were grown in each climate chamber. Chambers were set to 14 h light (620 $\mu mol\ m^{-2}\ s^{-1}$), 10 h dark, and either 20 °C/20 °C, 20 °C/10 °C, or 10 °C/10 °C (light/dark temperature). Pots were watered with tap water and received 50 mL of half-strength Hoagland nutrient solution once a week. Plants were cut back fortnightly to 6 cm above ground.

Plants were destructively harvested after 10 weeks (14 d regrowth period) and separated into mature leaf blades (above the ligule) and pseudostems (sheaths plus enclosed immature leaves); dead material was discarded. The harvest took place 8 to 10 h after the start of the light period to minimise effects due to diurnal variation in sugar concentrations. The material was immediately frozen in liquid nitrogen and ground to a fine powder in liquid nitrogen. A subsample of the powder was freeze-dried under vacuum and subsequently stored at −20 °C until extracted for WSCs. The remaining powder was stored at −80 °C until DNA and RNA were extracted.

Carbohydrate concentrations

WSCs were extracted and quantified as described previously (Rasmussen et al., 2007). Freeze-dried, powdered plant material (25 mg) was extracted with 2 mL 80% ethanol (low molecular weight (LMW) WSC fraction) and subsequently with 2 mL water (high molecular weight (HMW) WSC fraction). The ethanol extracted LMW WSC fraction contains a mixture of glucose, fructose, sucrose and low degree of polymerisation (DP) fructans (Prud'homme et al., 1992) with sucrose being the dominant carbohydrate in this fraction (see Supplementary Table S2). Glucose, fructose, and sucrose concentrations were determined in the LMW WSC fraction using enzymatic assays following the procedures described in Rasmussen et al. (2008). The water extracted HMW WSC fraction contains mainly high DP fructans (DP > 8) and is referred to in the following as high DP fructans.

To estimate total carbohydrate concentrations in both the LMW and HMW WSC fractions, we used the anthrone method

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