Fructan biosynthesis

in *Lolium perenne*

Tissue, cultivar and temperature effects on gene expression and protein accumulation profiles

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ABSTRACT

Cultivars of *Lolium perenne* with high concentrations of water soluble carbohydrates (WSCs) offer opportunities to mitigate greenhouse gas emissions (nitrous oxides) from grazed pastures and improve meat and milk production in livestock. Our previous studies demonstrated that fructan accumulation in the blades of high WSC grasses involves a strong gene x environment interaction. To identify the temperature effects on the expression of high sugar trait in the high sugar cultivars, we conducted a pot trial in climate chambers with temperature regimes set at 10/10, 20/10 and 20/20°C (day/night), respectively. Water soluble carbohydrate concentrations, the expression of the key genes and proteins: 1-SST (sucrose: sucrose 1-fructosyltransferase), 1-FFT (fructan: fructan 1-fructosyltransferase), 6G-FFT (fructan: fructan 6G-fructosyltransferase) and 1-FEH (1-fructan exohydrolases) involved in the fructan biosynthetic pathway of *L. perenne* were compared in blades and sheaths of three selected high sugar cultivars (P, A and H) and a common cultivar (F) grown under the three temperature regimes. We found that amongst the selected 3 high sugar cultivars, high molecular weight (HMW) WSC content was significantly higher in P and A cultivars, regardless of the temperature regimes. As expected, sheaths contained significantly higher concentrations of HMW WSCs (fructans) compared to leaf blades. The highest WSC contents in both leaf and sheath tissues accumulated at 10/10°C while the lowest accumulated at 20/20°C. Gene expression profiles demonstrated that all four genes studied were more significantly expressed in sheaths compared to blades, and the expression levels were highly correlated with fructan accumulation in this tissue. Low temperature resulted in significant up-regulation of 1-SST in sheaths, but not in blades. 1-FFT was highly expressed in blades of A and P cultivars. Unexpectedly, 6G-FFT was expressed more significantly in the control F cultivar, but not in the high sugar cultivar P. Protein expression profiles showed that 1-SST protein accumulated to high levels in sheaths, whereas protein levels of 1-FFT and 1-FEH were higher in blades. 1-SST protein levels in both blades and sheaths generally increased in plants grown at low temperatures, whereas 1-FFT protein was not affected by low temperatures in blades and sheaths, furthermore, in both tissues there was no consistent effect observed between the different cultivars and temperature regimes on 1-FEH protein levels.

ABBREVIATIONS

1-FEH:	1-fructan exohydrolases
1-FFT:	Fructan: fructan 1-fructosyltransferase
1-SST:	Sucrose: sucrose 1-fructosyltransferase
6G-FFT:	Fructan: fructan 6G-fructosyltransferase
6-SFT:	Sucrose: fructan 6-fructosyltransferase
AP:	Ammonium persulfate
bp:	Base pair
cDNA:	Complementary deoxyribonucleic acid
C_T**:**	Threshold cycle
C-terminus:	Carboxyl-terminus
DEPC:	Diethylpyrocarbonate
DM:	Dry mass
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
dNTPs:	Dinucleotide triphosphates
DP:	Degree of polymerisation
EDTA:	Ethylenediaminetetraacetic acid
FEHs:	Fructan exohydrolases
***g*:**	Relative centrifuge force
h:	Hour(s)
His:	Histidine
HMW:	High molecular weight fructans
HSD:	Honestly Significant Different test
HSG:	High sugar grass
IPTG:	Isopropyl-β-D-thiogalactopyranoside
kDa:	Kilodaltons
LB:	Luria-Bertani
LMW:	Low molecular weight
mA:	Milli ampere
min:	Minute(s)
NCBI:	National Center for Biotechnology Information

Ni-NTA:	Nickel-nitrilotriacetic acid
PBS:	NaH_2PO_4- NaCl buffer
PCR:	Polymerase chain reaction
PMSF:	Phenyl methyl sulfonyl fluoride
PVDF:	Polyvinylidene difluoride
qPCR:	Quantitative polymerase chain reaction
RNA:	Ribonucleic acid
RNase:	Ribonuclease
rpm:	Revolutions per minute
rRNA:	Ribosomal RNA
RT-PCR:	Reverse transcription polymerase chain reaction
Rubisco:	Ribulose 1,5-bisphosphate carboxylase
SDS:	Sodium dodecyl sulfate
SDS- PAGE:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sec:	Second(s)
TAE:	Tris-acetate-EDTA buffer
Taq:	*Thermus aquaticus*
TBST:	Tris-HCl-NaCl-Tween 20 buffer
TEMED:	N,N,N',N'-tetramethylethylenediamine
Tris:	Trishydroxymethylaminomethane
UV:	Ultraviolet light
V:	Voltage
WSCs:	Water soluble carbohydrates
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE

Fructan biosynthesis in *Lolium perenne* - A Literature Review

Perennial ryegrass (*Lolium perenne* L.) is one of the most important temperate pasture grasses in the world and it is grown on more than 7 million hectares of pastoral land in New Zealand (Siegal et al., 1985). Perennial ryegrass cultivars with high concentrations of water soluble carbohydrates (WSCs) have recently been advocated for the New Zealand dairy industry because they offer benefits through: (i) improving milk yield per cow by increasing digestible dry matter intake per day, whether from the direct benefits of a greater digestibility of a high WSC diet, or indirectly through the effects of this on total intake (see review papers of Edwards et al., 2007a; Edwards et al., 2007b); (ii) enhancing nitrogen (N) supply (from the ruminant intestine) for milk production through improved efficiency of protein capture in the rumen (Miller et al., 2001a); and (iii) reducing the proportion of N eaten that is returned to the soil in urine, thereby reducing N leaching and greenhouse gas emissions in the form of nitrous oxide (Di and Cameron, 2002).

High sugar grass (HSG) cultivars, selected and bred in the United Kingdom by IGER, are supposed to accumulate high levels of fructans, the major storage carbohydrates of ryegrass and other temperate grasses, in the blades. These HSG cultivars have shown consistently high levels of WSC in a range of UK environments (Jones and Roberts, 1991; Munro et al., 1992), resulting in significant improvements in ruminant productivity, boosts in milk and meat production, and reductions in nitrogen releases into the environment (Miller et al., 1999; 2001b; Lee et al., 2002). Unfortunately, the full expression of the high WSC trait of these HSG cultivars is not reliably achieved under field conditions in New Zealand (Parsons et al., 2004); but low temperatures, either low night temperatures or a preceding period of sustained cold, did induce full expression of the high WSC trait in herbage of HSG cultivars in a controlled

environment study (Parsons et al., 2004). However, specific molecular mechanisms of the increased accumulation of fructans in blades of high sugar *L. perenne* cultivars, especially at low temperatures, are poorly understood.

In silico promoter analysis of genes coding for enzymes of the fructan biosynthetic pathway has shown the presence of putative sucrose-, light-, stress- and cold-related motifs (Chalmers et al., 2005). Individual enzymes, such as 1-SST (sucrose: sucrose 1-fructosyltransferase), 1-FFT (fructan: fructan 1-fructosyltransferase), 6G-FFT (fructan: fructan 6G-fructosyltransferase) and FEHs (fructan exohydrolases), are suggested to be involved in the fructan biosynthetic pathway of *L. perenne* (Pavis et al., 2001a; Chalmers et al., 2005). However, the specific effects of low temperature on the expression of the high sugar trait in high sugar cultivars, on gene and protein expression need to be studied in detail at the transcriptional and translational level to reveal how particular genes and/or enzymes play a key role under varying environmental conditions. These studies might offer critical insights beyond the current knowledge and lead to novel approaches to develop HSG cultivars.

This chapter briefly reviews high sugar cultivar-related studies and mainly focuses on studies on the biosynthesis of fructans in plants and fructan metabolism in *L. perenne*.

1.1. Practical importance of high sugar grass cultivars in agriculture

Water soluble carbohydrates (WSCs), mainly in the forms of low molecular weight (LMW) sucrose and high molecular weight (HMW) fructans, are the primary source of readily available energy and vital for the breakdown of proteins in grass ingested by grazing ruminants (Humphreys, 1989a). The WSC content has been proposed to affect the efficiency with which animals use the protein in herbage and determines the quality of the feed (Wilkins and Humphreys, 2003). In ruminants, N conversion is a microbial process taking place in the foregut. An abundant supply of readily available energy as fermentable sugar, presented simultaneously ('in synchrony') with N, is critical for microbial growth and efficient ruminant nutrition (Miller et al., 1999). High levels of WSCs in herbage of grass can therefore increase the efficiency of protein breakdown in the rumen, leading to improved animal performance (Van Vuuren et al., 1993). However, the WSC content is often relatively low in herbage of conventional grasses,

resulting in poor efficiency of grass protein utilisation, with only about 25% of the feed protein being incorporated into milk. As a consequence of this, the majority protein is excreted in faeces and urine (Beever et al., 1986). This is not only financially costly but is also harmful to the environment. Feeding fresh forage cultivars with high WSC concentrations has been proposed as a way to address imbalances in energy and protein availability for microbial synthesis (Beever et al., 1986; Dove and Milne, 1994). Hence, an increased supply of energy to the rumen, leading to an improved capture of dietary N into rumen microbial biomass, increases supply of protein to the ruminant in the form of greater microbial protein flow to the duodenum (Miller et al., 2001a).

The grass varieties of perennial ryegrass with high WSCs in the blades have been produced by conventional plant breeding in the UK by IGER and shown increased concentrations of WSC in the harvested component (20–40% of DM) compared to other commercial varieties (10–15% of DM) (Humphreys, 1989a; b; Radojevic et al., 1994; Smith et al., 1998). Moreover, in grazing experiments, these varieties have demonstrated up to 2.5 litres more milk per cow per day (Miller et al., 1999; 2001a), up to 20 percent increases in liveweight gain of suckling lambs (Lee et al., 2001), and increased palatability, while about 24 percent less fed nitrogen was lost to the environment (Miller et al., 2001b) when compared to conventional grass varieties. In addition, WSCs are associated with various advantageous characters in *Lolium perenne*, such as cold, drought and salt tolerance (Levitt, 1980; Munns and Weir, 1981; Gorham et al., 1981), increased tiller survival (Thomas and Norris, 1981), enhanced persistence (Arcioni et al., 1985), and good regrowth after cutting or grazing (Alberda, 1966).

1.2. Physiology of fructan biosynthesis in plants

Fructans are synthesized from sucrose and are an alternative reserve carbohydrate to starch. They are a class of water-soluble polysaccharides composed of linear or branched oligo/polymers of fructose attached to sucrose through glycosidic bonds of various linkage types. They are present in approximately 15% of the flowering plant species, including several economically important species such as wheat, barley and

forage grasses (*Poaceae*), chicory and Jerusalem artichoke (*Asterales*), and some bulb forming plants e.g. onion and tulip (*Liliales*). Fructans are mainly localised in vacuoles of both photosynthetic and storage cells (Frehner et al., 1984; Wiemken et al; 1986), but their presence in phloem sap (Wang and Nobel, 1998) and the apoplastic space has also been reported (Livingstone and Henson, 1998; Van den Ende et al., 2005b). In addition to the well-known role of fructans as reserve carbohydrates, there are several physiological advantages of fructans in plants: (i) regulation of temporary partitioning of assimilates by controlling sucrose concentration in the cell and thereby preventing sugar-induced feed-back inhibition of photosynthesis (Pollock, 1986; Hendry, 1993); (ii) facilitation of sucrose unloading from the phloem and maintenance of osmotic potential to ensure cell enlargement in the elongation zone (i.e. osmoregulation) (Pavis et al., 2001b; Van den Ende et al., 2000); and (iii) protection of plants during drought (Pilon-Smits et al., 1995) and cold stress (Chatterton et al., 1989; Olien and Clark, 1993; Konstantinova et al., 2002; Hisano et al., 2004a), probably by stabilizing plant membranes (Vereyken et al., 2001; Hincha et al., 2002).

In general, fructan accumulating plants are perennials and they are most abundant in areas where growth is restricted to certain seasons within an annual cycle (Cairns et al., 1997), such as in regions with seasonal patterns of drought or low temperatures. In the fructan containing plants, large quantities of fructans commonly accumulate in specialized organs. They are, for example, abundant in the leaf sheaths of grasses, the taproot of chicory (*Cichorium intibus*), the tubers of dahlia (*Dahlia variabilis*) and the bulb of tulip (*Tulipa gesneriana*) and onion (*Allium cepa*). In these specialized organs fructans are frequently present as a heterogeneous mixture with different degree of polymerization (DP) and structures, but the presence of a specific type of fructan has been found to be species specific (Pollock and Cairns, 1991; Housley and Pollock, 1993).

The content and structure of fructans in fructan accumulating plants are highly influenced by environmental conditions and the developmental stage of plants (Sims et al., 2001; Sims, 2003), facilitated by flexible polymerisation and depolymerisation processes. Polymerisation and accumulation of fructans can usually be induced in

plants by conditions that reduce plant growth and carbon translocation rates more than photosynthetic rates, such as low temperatures (Livingston, 1991), nutrient limitation (Wang and Tillberg, 1996), water stress (Volaire and Lelievre, 1997), and high CO_2 concentrations (Smart et al., 1994), whereas depolymerisation and mobilisation of fructans can be triggered by energy-demanding activities such as regrowth after defoliation (Amiard et al., 2003), during grain filling (Yang et al., 2004), sprouting (Machado and Dietrich, 1993), and inflorescence development (Cairns et al.,1999). Physiological studies of these processes are abundant, however, molecular mechanisms behind the accumulation and degradation are still unclear, and key genes and enzymes of fructan biosynthesis responding to different growth stages and certain environmental conditions have not been fully identified.

1.3. Biosynthetic pathway of fructans in plants

Fructan structures and sizes vary in plants. The chain of fructans can be linear or branched (Fig.1.1); lengths (degree of polymerization, DP) can be as short as three or as long as up to a few hundred fructose units; linkages between adjacent fructose residues can be all identical or mixed; and the position of the glucose residue can be internal or external to the molecule (Ritsema and Smeekens, 2003). However, the varieties of structures present in any species are not random and each species appears to accumulate a characteristic set of fructans (Chalmers et al., 2005).

Fructans are derived from an initial fructosyl transfer to one of the primary hydroxyl groups of sucrose, resulting in three basic trisaccharides: 1-kestose, 6-kestose and 6G-kestose. These three trisaccharides subsequently are further elongated by adding β (2-1) or β (2-6) linked fructosyl units to form fructans with different structures and sizes (Van den Ende et al., 2004) (Fig.1.1). 1-kestose contains a β (2-1) linkage between the C2 of one fructose residue and the C1 of the fructose residue of sucrose. 6-kestose consists of a β (2-6) linked fructose. Its C2 is attached to the C6 of the fructose residue of sucrose. While 6G-kestose has two β (2-1) linked fructoses attached to the sucrose starter unit; one is linked to the C1 of the fructose residue (as is also the case in inulin) and the other to the C6 of the glucose residue. This type of fructan polymer has a

fructose chain on both ends of the glucose molecule, i.e. the glucose residue is internal to the molecule.

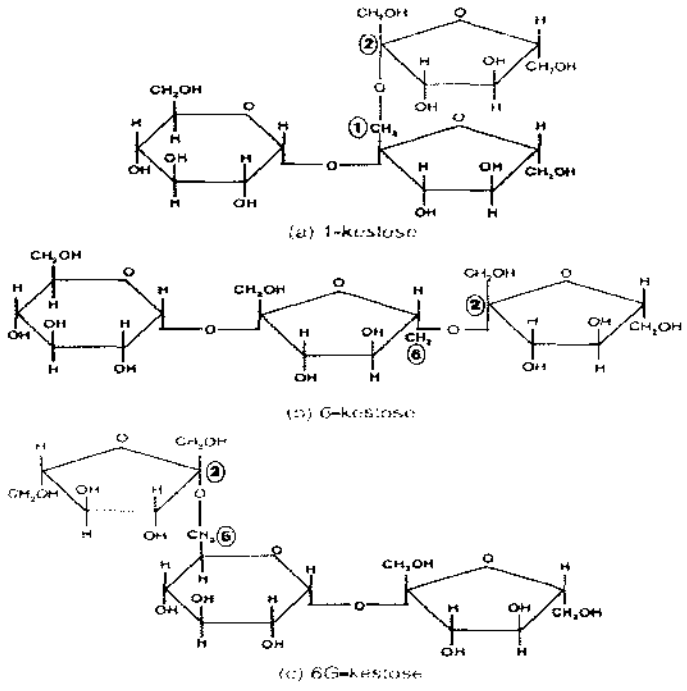


Fig.1.1. Molecular structures of the three trisaccharide precursors of plant fructans. The β(2-1) and β(2-6) linkages are indicated.(Cited from Chalmers et al. 2005)

Five major classes of fructans have been characterised in higher plants based on the fructosyl linkages: inulin series, levan series, graminan, inulin neoseries, and levan neoseries. The inulin series fructans are based on 1-kestose, the shortest inulin molecule, and are predominantly found in the taproot or tubers of dicotyledonous species such as *Cichorium intybus*, *Helianthus tuberosum*, and *Taraxacum officinale* (Asterales) (Edelman and Jefford, 1968). This type of fructan consists almost entirely of β (2-1)-linked fructose residues connected to a terminal sucrose moiety (Edelman and Jefford, 1968). 6-kestose is the simplest levan molecule and can be considered as the parent structure of the levan type (also called phleins) of fructans. The basic structure of this fructan type is a linear β (2-6)-linked fructose polymer which is found mostly in grasses such as *Triticum aestivum*, *Dactylis glomerata*, and *Bromus tectorium*, where it is primarily stored in the leaf bases (Bancal et al., 1992; Chatterton

et al., 1993a; 1993b). 6G-kestose is the first residue of the inulin and levan neoseries. The linkage on fructose residues can be β (2-1) or β (2-6), respectively. Many of the fructan molecules from *Avena* (Livingston et al., 1993) and *Lolium* species (Sims et al., 1992; Pavis et al., 2001b) belong to the levan neoseries, while some fructans from *Liliales* such as onion and asparagus belong to the inulin neoseries (Shiomi, 1981, Vijn et al., 1997; Ernst et al., 1998). In graminaceous monocotyledonous plants such as wheat and barley, fructans called graminans are structurally more complex and often consist of β (2-6)-linked fructose residues with β (2-1) linkage branches (Wiemken et al., 1995; Kawakami et al., 2005). 6- kestose forms the backbone of this class of fructans. The smallest graminan is the molecule bifurcose which contains both β (2-1) and β (2-6) linkages.

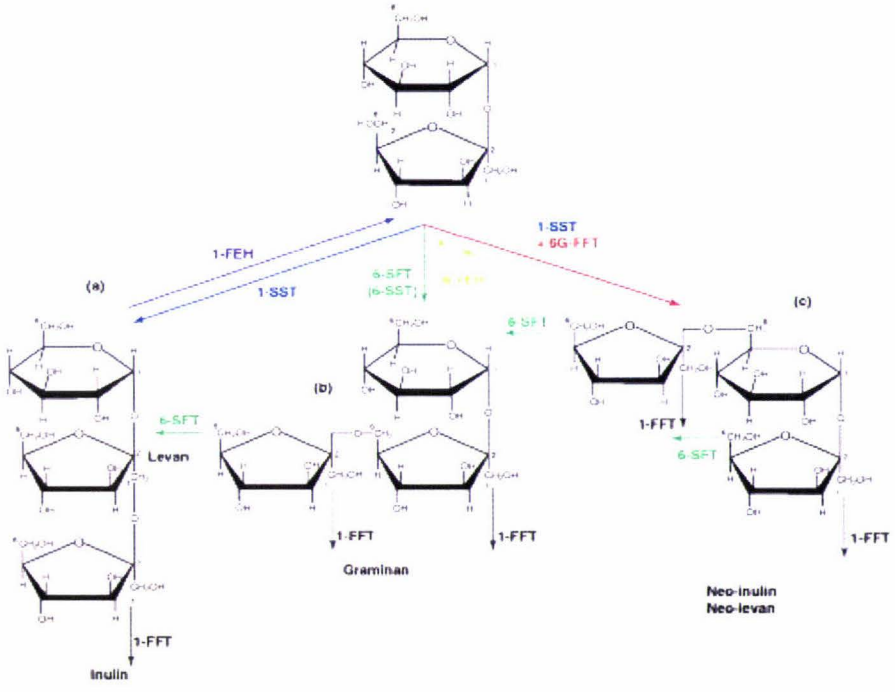


Fig.1.2. Model of the enzymology of fructan biosynthesis and breakdown in plants. (Cited from Van den Ende et al., 2004)

The species and even tissue-specific variability in fructan structure and DP in plants has been attributed mainly to differences in fructosyl transferases (Hellwege et al., 2000; Pavis et al., 2001a; Vergauwen et al., 2003) According to the model for plant fructan biosynthesis (Van den Ende et al., 2004) (Fig.1.2), inulin is synthesised from

sucrose by the combined action of two fructosyl transferases, i.e. sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan: fructan 1-fructosyltransferase (1-FFT). Firstly, 1-SST initiates *de novo* fructan synthesis by catalysing fructosyl transfer from one sucrose molecule to the fructosyl residue of another sucrose molecule via a β (2-1) linkage, resulting in the formation of the trisaccharide 1-kestose, the simplest and shortest inulin. 1-FFT then uses 1-kestose or fructans with a higher DP as fructose donors and transfers the fructose to a variety of pre-existing fructans or sucrose resulting in fructans with β (2-1) linkage only, but of varying chain length (Edelman and Jefford, 1968; Koops and Jonker, 1996; Lüscher et al., 1996; Van den Ende and Van Laere, 1996). The validity of the 1-SST/1-FFT model *in vivo* was supported by simultaneous expression of heterologous 1-SST and 1-FFT in potato (Hellwege et al., 2000) and sugar beet (Sévenier et al., 1998).

In addition to the role of chain elongation, 1-FFT is implicated in the determination of the length of inulin synthesised *in planta*. Expression of the genes encoding 1-FFT from Jerusalem artichoke and globe artichoke (*Cynara scolymus*) in tobacco protoplasts showed that the inulin produced by globe artichoke 1-FFT was longer than that produced by Jerusalem artichoke 1-FFT (Hellwege et al., 1998). Vergauwen et al. (2003) and Van den Ende et al. (2005a) also reported that both the purified (native) and recombinant globe thistle (*Echinops ritro*) 1-FFT protein preferred longer inulin chains as acceptors while the chicory 1-FFT had a higher affinity for short carbohydrates (sucrose, fructose and 1-kestose) as acceptor substrates. These results coincided with the *in vivo* length distribution of inulin in these plants, and it has been hypothesised that the size of the fructosyl polymers produced by a plant depends mostly on the enzymatic activity of their respective 1-FFTs (Van den Ende et al., 2005a), although a role for exohydrolases in defining the final size of the fructosyl chain in plants cannot be excluded.

Sucrose: fructan 6-fructosyltransferase (6-SFT, EC 2.4.1.10) has been proposed to be the key enzyme for the formation and extension of β (2-6) fructosyl linkages in the levan and mixed levan fructans. 6-SFT catalyses the formation of bifurcose (1&6 kestotriose), using sucrose as fructosyl donor and 1-kestose, produced by 1-SST, as fructosyl acceptor, thus introducing a branch (Wiemken et al., 1995), which can be elongated by either 1-FFT or 6-SFT, resulting in branched and mixed-type levans. If

only sucrose is available as a substrate for 6-SFT, it has been suggested that 6-SFT catalyses the formation of the trisaccharide 6-kestose, which then can be elongated by 6-SFT again to produce higher DP β (2-6) linked levans (Duchateau et al., 1995). The function and activitiy of this enzyme has been characterised and confirmed by expressing the barley 6-SFT gene in tobacco and chicory; transgenic plants produced bifurcose fructans and β (2-6) linked levans (Sprenger et al., 1997).

The best-characterised enzyme responsible for biosynthesis of neoseries fructans is fructan: fructan 6G-fructosyltransferase (6G-FFT), which initiates the formation of the 6G-linked chain (Shiomi, 1989). 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 (Shiomi, 1989; Vijn et al., 1997). The trisaccharide neo-kestose (6G-kestotriose) produced is the shortest fructan of the inulin neoseries and can be elongated on both terminal fructose residues with β (2-1) or β (2–6)-linked fructose units to produce the inulin neoseries or levan neoseries fructans, respectively. Vijin et al. (1997) demonstrated that fructans of the inulin neoseries are synthesized in transgenic chicory plants harbouring the onion 6G-FFT gene. Weyens et al. (2004) introduced 1-SST and 6G-FFT from onion into sugar beet and the transgenic sugar beet produced inulin and inulin neoseries fructans, a fructan profile closely resembling that from native onion. Furthermore, studies based on onion and *L. perenne* have suggested that 6G-FFT in these two species is bi-functional (Fujishima et al., 2005; Lasseur et al., 2006): it not only has 6G-FFT activity, but also fulfils 1-FFT functions catalysing the formation of β (1–2) linkages in fructans of the inulin neoseries. These results question the need for a separate 1-FFT activity in these two species. 6G-FFT is probably also needed for the synthesis of complex graminans with a fructosyl chain attached to the glucose residue of sucrose, although this has not been reported from species which accumulate this type of fructans.

Sprenger et al. (1995) first reported the cloning of a plant gene of the fructan biosynthetic pathway: 6-SFT from *Hordeum vulgare*. Since then, several genes encoding enzymes associated with fructan biosynthesis have been cloned from various plants, e.g. 1-SST from *Cichorium intybus* (de Halleux and Van Cutsem, 1997), *Cynara scolymus* (Hellwege et al., 1997; 1998), *H. tuberosum* (van der Meer et al., 1998), and *H. vulgare* (Nagaraj et al., 2004); 1-SST and 6G-FFT from *Allium cepa*

(Vijn et al., 1997; 1998); 1-SST from *Festuca arundinacea* (Lüscher et al., 2000); 6-SFT from *Agropyron cristatum* (Wei and Chatterton, 2001) and *Poa secunda* (Wei et al., 2002); 1-SST, 6-SFT and 1-FFT from *Triticum aestivum* (Kawakami and Yoshida, 2002; Kawakami et al., 2005); and 1-SST, FT1 and 6G-FFT from *L. perenne* (Lidgett et al., 2002; Chalmers et al., 2003; Lasseur et al., 2006). Their enzyme activities have been verified with either purified native and/ or recombinant proteins. Some fructan genes have been transferred into other plants such as chicory (Vijn et al., 1997), potato (Hellwege et al., 1997; 1998; 2000), sugar beet (Sèvenier et al., 1998), and tobacco (Pilon-Smits et al., 1995; Konstantinova et al., 2002). In most instances, transgenic plants had increased amounts of fructans. Some synthesised fructans with novel structures in addition to their native fructans (Hellwege et al., 2000; Sévenier et al., 1998; Sprenger et al., 1995; 1997; Weyens et al., 2004), while others also demonstrated increased levels of tolerance to environmental stresses such as cold, drought and freezing (Pilon-Smits et al., 1995; 1999).

The net fructan accumulation in plants is controlled not only by fructan biosynthetic enzymes, but also by fructan exohydrolases (FEHs), which degrade fructan reserves whenever energy supplies are limited (Van den Ende et al., 2001; Morvan-Bertrand et al., 2001) or under stress (Van den Ende and Van Laere, 1996). Fructan exohydrolases are able to remove fructose monomers from the end of the fructan chains using water as acceptor, resulting in the release of free fructose (Henson and Livingston, 1996; Marx et al., 1997a; b; Van den Ende et al., 2005b). Most plant FEHs lack invertase activity and generate one sucrose molecule as an end product (Verhaest et al., 2007). Based on the linkage type [β (2-1) vs β (2-6)] that is preferentially hydrolysed, fructan exohydrolases are classified into two types of enzymes: 1-FEHs (inulinases, E.C. 3.2.1.153) and 6-FEHs (levanases, E.C. 3.2.1.154). So far, 1-FEH has been characterised and cloned from *Cichorium intybus* (Van den Ende et al., 2000; 2001), *L. perenne* (Lothier et al., 2007), onion bulbs (Benkeblia et al., 2005) and wheat (Van den Ende et al., 2003a); 6-FEH from sugar beet (Van den Ende et al., 2003b) and wheat (Van den Ende et al. 2005b); and 6 &1-FEH, which is able to hydrolyse both β (2-1) and β (2-6) linkages, and 6-KEH (which preferentially hydrolyses 6-kestose) from wheat (Van den Ende et al., 2003b, 2005b; Van Riet et al., 2006; Kawakami et al., 2005). In addition, from temperate grasses, three 6-FEH (Henson and Livingston, 1996; Marx et al., 1997b; Van Riet et al., 2006) and three 1-FEH (Van den Ende et al.,

2003a) enzymes have been purified to homogeneity, including one 6-FEH from *L. perenne* (Marx et al., 1997b).

In perennial grasses, FEHs are implicated in the fast mobilisation of fructans immediately after defoliation (Yamamoto and Mino, 1985; Morvan-Bertrand et al., 2001) in order to deliver carbon to leaf growing cells (Amiard et al., 2003). It has also been reported that, in *L. rigidum* leaves (Smouter and Simpson, 1991), barley leaves (Wagner and Wiemken, 1986), and perennial ryegrass stubble (Prud'homme et al., 1992; Marx et al., 1997b), high *in vitro* FEH activities have been measured, not only during periods of fructan breakdown, but also during periods of fructan biosynthesis. It was proposed that FEHs might be also involved in fructan biosynthesis in these plants (Lothier et al., 2007), possibly playing an important role in determining the *in vivo* fructan patterns (Bancal et al., 1992; Van den Ende et al., 2003a).

In addition to fructan exohydrolases, the enzymes involved in fructan biosynthesis have also been implicated in fructan degradation. For example, 1-FFT can shorten fructan polymers by transferring fructosyl units from fructans with higher DP to those with lower DP (Edelman and Jefford, 1968; Van den Ende, et al., 2000).

1.4. Fructan metabolism in *Lolium perenne*

By using model systems, in the past 20 years several aspects of fructan physiology, biochemistry and enzymology have been studied widely, which led to progressive understanding of fructan biosynthesis as outlined above. This section focuses on the literature specific to fructan metabolism in *L. perenne*.

1.4.1. Fructan accumulation in Lolium *spp.*

Fructans are the principal WSCs in grasses like *L. perenne*. In general, mean concentrations of fructans can reach 10 – 20 mg g^{-1} dry mass in blades in the field (Humphreys, 1989a; Turner et al., 2006). In addition to the difference between cultivars, fructan content in ryegrass also varies as a complex function of a number of environmental factors, such as irradiance, temperature and water availability. Among these environmental factors, low temperature is one of the best-known factors to

induce accumulation of fructans in plants. Generally, fructan concentrations are greatly elevated in blades and sheaths of plants experiencing cool conditions (Chatterton et al., 1989; Schnyder and de Visser, 1999). For example, fructan concentrations in all plant parts of crested wheatgrass did undergo a 3 to 10-fold increase when the plants were transferred from warm (20°C) to cold (5°C) temperatures (Chatterton et al., 1987). It has also been reported that cold stress (5°C at night) induced fructan accumulation in leaf blades of barley (*Hordeum vulgare*) and wheat seedlings (Wagner and Wiemken, 1987). In *L. temulentum*, exposure to low temperature (5°C) induced fructan accumulation, especially in the meristematic regions of developing leaves (Pollock, 1984). In addition to an increase in fructan content, an increased degree of polymerization (DP) of fructans has been observed in timothy grass during cold treatment (10°C at day; 5°C at night time) (Thorsteinsson et al., 2002). Kawakami and Yoshida (2002) also found that the levels of 1-SST and 6-SFT transcripts in winter wheat during cold hardening increased concomitantly with fructan content. Fructan accumulation reflects the general concept that fructans are accumulated in cool temperate perennial *Lolium* spp. when photosynthetic production (source activity) is greater than consumption (sink activity) of carbohydrates for growth (Thorsteinsson and Tillberg, 1990). However, there is little information available on the molecular regulation of fructan metabolism in grasses by low temperatures.

Like other temperate grasses, there is a marked seasonal pattern of accumulation of WSCs in *L. perenne*: total WSCs accumulate throughout summer and autumn, leading to the highest total accumulated during winter (Pollock and Jones, 1979), whereas in early spring WSC levels fall, coinciding with flowering. WSC concentrations also vary during the day, with increasing amounts towards the end of the photoperiod (Gordon, 1986).

1.4.2. Fructan distribution in L. perenne

In *L. perenne*, blades and sheaths are serving as fructan storage compartments with fructans predominantly present in the basal part of the elongating leaf and mature sheaths and smaller amounts in expanded leaf blades (Guerrand et al., 1996; Pavis et al., 2001b; Morvan-Bertrand et al., 2001). In both elongating leaves and in sheaths, fructan content was found to be heterogeneously distributed along the longitudinal

axis, preferentially accumulating in the base part of these tissues, and being the highest in the base part and lowest in the differentiation zone or near the ligules, respectively (Lüscher and Nelson, 1995; Schnyder and Nelson, 1987; Roth et al., 1997; Pavis et al., 2001b).

The preferential accumulation of fructans in the different tissues has been suggested to reflect the respective physiological function of these tissues. Fructans in elongating leaves have been proposed to serve several functions: unloading of sucrose from phloem, maintenance of osmotic potential, and short-term or long-term carbohydrate storage (Volenec, 1986). The basal region of elongating leaves comprises most of the meristematic growth zone (Wilhelm and Nelson, 1978). Leaf growth in *L. perenne*, in common with many other cool temperate grasses, is confined to this region (Volenec and Nelson, 1981; Schnyder et al., 1990). Growing leaves are strong sinks for sucrose (Schnyder and Nelson, 1987) and it is thought that during the cell proliferation phase, sucrose arising from photosynthesis of either expanded blades or emerging parts of expanding leaves is transported to the leaf base, and used as substrate for the formation of fructans and cell division (Allard and Nelson, 1991). In differentiating cells, which exert lower sink strength for imported carbon than dividing and elongating cells, endogenous fructan hydrolysis provides the carbon and energy required to support biosynthetic processes like lignin formation and secondary cell wall deposition (Allard and Nelson, 1991).

Mature sheaths contain higher concentrations of fructans than elongating leaves, they can accumulate up to 70% of fructans stored in the vegetative part of grass (Morvan-Bertrand et al., 2001), and have a greater proportion of high DP fructans than expanded blades or expanding leaves (Guerrand et al., 1996; Pavis et al., 2001b). It has been suggested that sheaths function metabolically as reservoir of non-structural carbohydrates in plant species devoid of storage organs *per se* (Housley and Volenec, 1988; Pavis et al., 2001b). Under the conditions in which carbon is fixed in excess relative to the overall plant demand, assimilates are unloaded along the path and stored mainly as fructans in sheaths, with reserves being mobilised in response to factors such as the onset of spring growth, reproductive growth or re-growth after defoliation (Labhart et al., 1983). The spatial gradient of distribution of fructans along the

longitudinal axis of the sheaths has been suggested to have a role in facilitating photosynthesis in blades and emerging leaves to proceed as it prevents sugar induced feedback inhibition of the photosynthetic system (Pavis et al., 2001b). It has also been reported that in tall fescue the concentration of fructans in sheaths depends on the position of the sheath in the tiller, i.e. on their age. The younger the sheath was, the higher the fructan content (Volenec, 1986).

Mature leaf blades also contain fructans but in much smaller amounts, only about one fifth of the fructan level in the elongating leaf base and one fourth of the sheath base (Pavis et al., 2001b) and it has been observed that fructans start to accumulate in leaf blades when the storage capacity of both leaf sheaths and bases have already decreased (Guerrand et al., 1996).

1.4.3. Fructan metabolism-related genes and enzymes in L. perenne

The composition of fructan isomers in *L. perenne* has been investigated and three types of fructans have been identified in *L. perenne*: inulin series, inulin neoseries, and levan neoseries fructans (Sims et al., 1992; Bonnett et al., 1994; Pavis et al., 2001 a; b). The most abundant trisaccharides found in *L. perenne* are 1-kestotriose and 6G-kestotriose, with 6- kestotriose present in significantly smaller amounts. The tetra-, penta- and hexa-saccharide fractions were reported to comprise 1,1-kestotetraose, 1&6G-kestotetraose, 6G,6-kestotetraose, 1,1&6G-kestopentaose, 6G,6,6-kestopentaose, and 1,1,1&6G-kestohexaose, respectively (Pavis et al., 2001b). Notably, bifurcose (1&6 kestotetraose), commonly found in barley and wheat, has never been identified in *L. perenne* (Guerrand, 1996; Pavis et al., 2001b). The majority (76%) of high molecular weight fructans (DP > 8) contains an internal glucose residue with a high proportion of β (2-6) linked fructose residue, 70 times more than the β (1-2) linked (Pavis et al., 2001b).

Fructosyltransferase activities in specific tissue are consistent with the concentration of fructans in these tissues (Guerrand et al., 1996; Pavis et al., 2001a). For example, when

plants were subjected to fructan accumulating conditions, 1-SST and 6G-FFT activities were highest in expanding leaves, lowest in mature leaf blades and intermediate in mature leaf sheaths,; activities of 1-SST and 6-G-FFT were greatest in the basal segment of both elongating leaves and mature leaf sheaths (Pavis et al., 2001a). In plants experiencing re-growth after defoliation, sheaths lost up to 96% of their fructans during the first 6 d of regrowth (Morvan-Bertrand et al., 1999). Concomitantly, the activities of 1-SST and 1-FFT decreased in all parts of the plants (Guerrand et al., 1999), and the activity of FEH increased 3.5-5 fold in elongating leaf bases and 2-3 fold in sheaths (Morvan-Bertrand et al., 2001).

Based on these biochemical studies, a model for *L. perenne* fructan biosynthesis has been proposed (Pavis et al., 2001a; Chalmers et al., 2003) (Fig.1.3), suggesting that at least four enzymes are responsible for the production of the full spectrum of fructan species found in *L. perenne*, i.e. 1-SST, 1-FFT, 6-G-FFT and 6-SST or 6-FFT. The combination of 1-SST activity with 1-FFT and 6-G-FFT for net biosynthesis of the inulin series and inulin neoseries fructans has been confirmed and characterized in crude enzyme extracts as well as with expressed recombinant proteins (Pavis et al., 2001a; Chalmers et al., 2003; Lasseur et al., 2006). However, which enzymes are responsible for the synthesis of the β (2-6) linkage in the levan neoseries fructans still remains unclear. Currently, the only known enzyme capable of synthesising β (2-6) linkages in grasses is 6-SFT (Sprenger et al., 1995; Kawakami and Yoshida, 2002), but the role of 6-SFT is questioned here, as bifurcose, the product of 6-SFT activity, is not present in *L. perenne*. Guerrand et al. (1996) and Pavis et al. (2001a) suggested the existence of 6-SST or 6-FFT-like enzymes that might prefer fructans other than 1-kestose (e.g. neokestose or 6-kestose) as acceptor substrates to produce the β(2-6) linkage in the branched levan neoseries in the absence of 6-SFT. However, so far these kinds of enzyme have never been fully characterized in any target species. Moreover, fructosyltransferases generally exhibit more than one activity. For example, the recombinant 6G-FFT protein demonstrated both 6G-FFT and 1-FFT activity (Lasseur et al., 2006). The recombinant 6G-FFT is not only able to catalyse the formation of β(2-1) linkage onto fructans of the inulin neoseries, but also capable of elongating the β (2-1) linked chain of inulin simultaneously, providing evidence that a separate 1-FFT protein in *L. perenne* is not needed. However, the sequence of a putative 1-FFT

homologue has been submitted to the NCBI database (AB188920). To answer the question if an independent 1-FFT exists in *L. perenne*, or if fructans present in *L. perenne* are synthesised by a three enzyme system: 1-SST, 6G-FFT together with 6-(S/F) FT, requires further investigations.

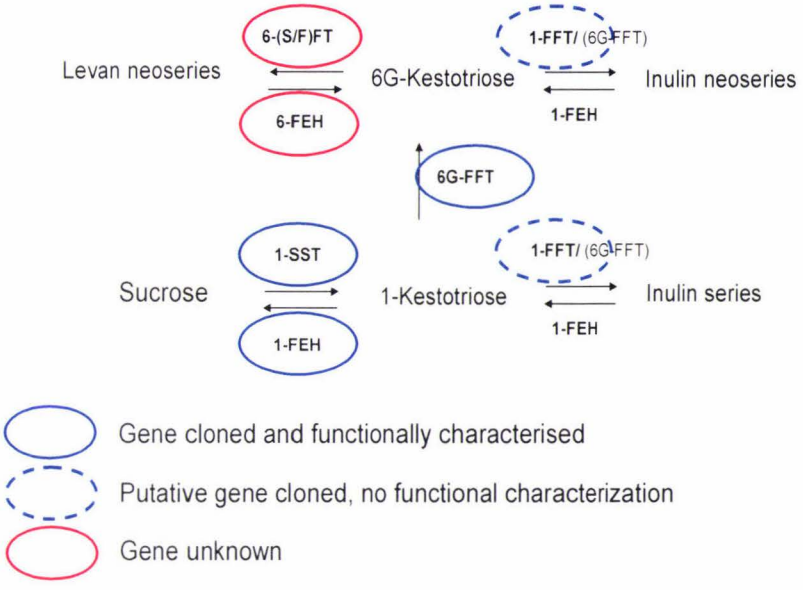


Fig.1.3 Hypothetical pathway of fructan synthesis in *L. perenne* and proposed activities of the enzymes implicated. (Modified from: Pavis et al. (2001b) and Chalmers et al. (2003) by Rasmussen (2007, unpublished)

The presence of both inulin neoseries and levan neoseries fructans in *L. perenne* suggests the presence of separate FEHs with 1-FEH or 6-FEH activity, and/or enzymes showing both types of activity. The *Lp1-FEH* gene has been cloned and its function has been characterised in a recombinant protein assay, while 6-FEH has been purified from *L. perenne*, proving evidence for FEHs with specific activity (Marx et al., 1997b).

So far, six genes (mRNA) of fructan related metabolic enzymes have been identified in *L. perenne*. They are *Lp1-SST* (AY245431; Chalmers et al., 2003), *Lp1-FFT* (AB186920;Hisano et al., 2004b), *Lp6G-FFT* (AB125218;Hisano et al., 2004c), *LpFT1* (AF481763;Lidgett et al., 2002), *LpFT4* (DQ0739201) and *LpFEH* (DQ073968). Enzyme activities for *Lp1-SST*, *Lp6G-FFT* and *Lp1-EFH* have been confirmed by enzyme assays with recombinant proteins produced in yeast using the *Pichia pastoris* heterologous expression system. Recombinant *Lp1-SST* protein shows typical 1-SST enzyme activity with primarily 1-kestose production and moderate 1, 1-kestotetraose (nystose) production in the presence of sucrose as the substrate (Chalmers et al., 2003). Recombinant *Lp6G-FFT* protein exhibited both 6G-FFT and 1-FFT activities with a maximum 6G-FFT/1-FFT ratio of two; the enzyme consumes sucrose and 1-kestose to produce 6G-kestose, 1,1-kestotetraose and 1&6G-kestotetraose (Lasseur et al., 2006). The production of 6G-kestose illustrates 6G-FFT activity, 1,1-kestotetraose is formed by 1-FFT activity, and 1&6G-kestotetraose could either be the product of 6G-FFT or 1-FFT activity, depending on the acceptor, 1-kestose or 6G-kestose, respectively. Recombinant *Lp1-FEH* protein showed high FEH activity towards 1-kestotriose, 1,1-kestotetraose, and inulin, but low activity against 6-kestotriose and levan, and lacked invertase activitiy (no hydrolysis of sucrose; Lothier et al., 2007). The remaining three genes isolated from *L. perenne* have not been functionally characterised yet. Genomic DNA blots indicate that the *LpFT4*, *LpFET*, *LpFT1* and *Lp1-SST* are all single copy genes in *L. perenne* (Lidgett et al., 2002; Chalmers et al., 2003; 2005).

Expression studies (at the mRNA level) in plants subjected to fructan accumulation conditions showed that in sheath tissues *Lp1-SST* and *Lp6G-FFT* were predominantly controlled at the transcriptional level (Chalmers et al., 2003; Lasseur et al., 2006; Lothier et al., 2007). These expression patterns matched both 1-SST and 6G-FFT activities and fructan content in these organs, while expression of both genes declined along the leaf axis, in parallel with the spatial occurrence of fructan and fructosyltransferase activities (Chalmers et al., 2003; Lasseur et al., 2006). In blade tissues, however, *Lp6G-FFT* was highly expressed, but only very low extractable fructosyltransferase activities and fructan amounts were detected. It was also shown that *Lp1-SST* transcripts and fructan content accumulated in continuously illuminated

leaf blades, but no increase in 1-SST activity was detected (Guerrand et al., 1996; Lasseur et al., 2006), indicating that *Lp6G-FFT* and *Lp1-SST* transcript levels do not always coincide with enzyme activities and fructan content, and *Lp6G-FFT* and *Lp1-SST* may be mainly regulated post-transcriptionally (Lasseur et al., 2006). Expression pattern analysis of *Lp1-FEH* revealed high transcript levels in sheaths, but low levels in blades, where fructan content is low. It was found that both *Lp1-FEH* gene transcription and 1-FEH activity were up-regulated during the period of active fructan biosynthesis and down-regulated during the period of active fructan breakdown, suggesting a role as a ß-(2-1) trimming enzyme acting during fructan synthesis in concert with fructan synthesising enzymes (Lothier et al., 2007).

Based on amino acid sequence information, *Lp1-SST* encodes a 653 amino acid polypeptide with a molecular mass of 61 kDa (Chalmers et al., 2003); *Lp1-FFT* encodes a 623 amino acid polypeptide with a molecular mass of 62.5 kDa; *Lp6G-FFT* encodes a 645 amino acid polypeptide; and *LpFEH* encodes a 570 amino acid polypeptide with a molecular mass of 62 kDa. As FTs are localised in vacuoles, each of them has a signal peptide at the N-terminus: 104, 59, 63, 23 amino acid in length in Lp1-SST, 1-FFT, 6G-FFT and FEH (Chalmers et al., 2003), respectively. These peptides are post-translationally cleaved. Six putative N-glycosylation sites were predicted in 1-SST protein (Chalmers et al., 2003); while Lp1-FFT, Lp6G-FFT and LpFEH proteins contain seven each, suggesting that they are glycoproteins processed in the ER (Sprenger et al., 1995; Van den Ende and Van Laere 1996). The three biosynthetic enzymes: Lp1-SST, Lp1-FFT and Lp6G-FFT, share high homology to each other. Amino acid sequence identity between Lp1-SST and 1-FFT with 6G-FFT is 66% and 82% (Chalmers et al., 2003), respectively; between Lp1-FFT and 6G-FFT 65%. Amino acid sequence comparison of these enzymes has shown that sequences of the signal peptide regions are less conserved than those of the mature protein regions. However, homology between LpFEH and 1-SST, 1-FFT and 6G-FFT are much lower: 36% for 1-SST (Chalmers et al., 2003), 37% forl-FFT, and 38% for 6-G-FFT (Chalmers et al., 2005).

Most information on the fructan biosynthesis in *L. perenne* has been gained in studies with excised leaves or young seedlings exposed to continuous light in order to increase

source activity, and root and meristem cooling to reduce sink activity. Information on the genetic regulation of fructan metabolism under more natural growth conditions and in mature plants is comparatively rare. Considering the agricultural and agronomic importance of *L. perenne* as a major grassland species and the nutritional value of WSCs in this forage grass, several aspects of fructan physiology, biochemistry and enzymology under natural conditions need to be further investigated.

1.5. Summary and research objectives

Cultivars of *Lolium perenne* with high concentrations of water soluble carbohydrates offer opportunities to mitigate greenhouse gas emissions (nitrous oxides) from grazed pastures and improve meat and milk production in livestock. The major reserve carbohydrates in cool-season grasses are fructans, accumulating predominantly in pseudostems, which are a minor component of grazed diets. 'High Sugar' grass (HSG) cultivars have been bred in the UK by IGER and these cultivars are supposed to accumulate high levels of fructans in the blades, the major grazed component of pasture grasses. However, research conducted in New Zealand showed that full expression of the WSC advantage of HSG cultivars is not reliably achieved in field conditions in New Zealand. The differential in WSC levels between cultivars seen in overseas is far less in New Zealand's climate. Previous studies demonstrated that low temperatures, either low night temperatures, or previous periods of sustained cold, induced full expression of a sustained higher WSC trait in herbage of HSG cultivars (Parsons et al., 2004). It was also shown that the major differences in WSC content are due to high levels of fructans (fructose polymers). These results suggest that fructan accumulation in the blades of high WSC grasses involves a strong gene x environment interaction. However, the precise mechanisms leading to this induction are unknown. In order to understand how fructan metabolism is controlled in HSG cultivars and how the individual enzymes, 1-SST, 1-FFT, 6G-FFT and FEH in these *L. perenne* cultivars are regulated, as compared to other, low sugar cultivars, requires further study. Especially the different responses of HSG cultivars to low temperatures warrant further investigation.

The general objective of this Masters project is to reveal which particular genes/ enzymes play a key role for fructan accumulation in HSG cultivars, and at which stages they are controlled, at transcription or translation level. The specific aims of this project are to investigate:

1. The temperature effects on the expression of the high sugar trait in HSG cultivars, by comparing water soluble carbohydrate (WSC) content in four selected ryegrass cultivars.
2. The expression patterns of key genes at the transcriptional level using RT qPCR of *1-SST*, *1-FFT*, *6G-FFT* and *1-FEH*.
3. The protein accumulation patterns of key enzymes under these conditions by western blotting using polyclonal antibodies against 1-SST, 1-FFT and 1-FEH, generated in this project.

CHAPTER TWO

Materials and Methods

A variety of techniques were used in this study to analyse effects of temperature and tissue in different ryegrass cultivars on the accumulation of sugars, and on gene and protein expression profiles relevant to fructan biosynthesis. This chapter describes materials and methods employed in this project.

2.1. Plant materials

Four cultivars of *L. perenne* were used in this study, three of which were designated as "High Sugar" grasses, they are named by breeders as AberDart (A), Aber Magic (H), PG1113 (P), and one control F (Fennema), which is a UK conventional commercial cultivar.

Seeds were germinated on floats in the greenhouse and seedlings were transferred into pots filled with potting mix (Midland, NZ). Plants were grown in the greenhouse for 40 days (January/ February 2006, Palmerston North, NZ), and subsequently each plant was divided into three plants (clonal replicates); each set consisted of 4 cultivars with 8 genotypes each (treatment replicates). The plants were cut back to 6 cm above ground and each set transferred into three controlled environment chambers (HortResearch, Palmerston North NZ) with day (14 h light; light intensity 620 μmol m^{-2} s^{-1}) and night (8 h dark) temperatures of 20/20°C, 20/10°C and 10/10°C, respectively. Plants received daily water and later during the experiment a modified Hoagland nutrient solution. Plants grown at 20/20°C, 20/10°C were cut back to 6 cm above ground weekly, but fortnightly in the 10/10°C chamber to account for differences in re-growth.

After 10-weeks, plants were harvested within a period of less than 3h (1 pm to 4 pm) to minimise effects of diurnal variation on sugar metabolism. Plants were divided into two parts: blades of mature leaves together with the emerged part of elongating leaves, and stubble consisting of both sheaths and young, elongating leaves. Dead sheath material was removed and the harvested material immediately frozen in liquid nitrogen and subsequently ground to a fine powder under liquid nitrogen in a baked mortar and pestle (to prevent RNA degradation by RNases). Part of the plant powder was freeze-dried and stored at -20°C until water soluble carbohydrate extraction was carried out. The remaining frozen plant powder was stored at -80°C until RNA and protein were extracted

2.2. Analysis of water soluble carbohydrates

Water soluble carbohydrates (WSCs) were extracted and quantified using the method described by Rasmussen et al. (2007). Freeze-dried plant material (25 mg) was extracted with 2 × 1 ml of 80% (v/v) ethanol in a 2 ml screw cap tube and incubated in an Eppendorf Thermo Mixer (12,000 rpm, 65°C, 30 min). After each extraction the homogenate was centrifuged (13,000 rpm, 15min) and the supernatants combined. This fraction contains fructose, glucose, sucrose and fructans of low degree of polymerisation (DP; referred to as low molecular fraction (LMW) fraction). Fructans of high DP (referred to as high molecular weight (HMW) fraction) were extracted from the remaining residue with 1 ml water (Millipore) twice under the same condition as above.

WSCs in the LMW and HMW fractions were analysed by the anthrone method (Jermyn et al., 1956) adapted as follows. The assay used a standard 96-well microtitre plate with Spectramax plate reader and Softmax Pro 3.0 software (Molecular Devices, Sunnyvale, CA). Aliquots of 3 µl (LMW fraction) or 10 µl (HMW fraction) of extracts were diluted to 50 µl with 50 mM KH_2PO_4/ K_2HPO_4 (pH: 7.0) and reacted with 250 µl 1.25% (w/v) anthrone in a mixture of H_2SO_4 and EtOH at a ratio of 6:4 (v:v). The mixture was agitated on the plate reader by using its mixing function and then incubated at 65°C for 25 min. The absorbance of the blue-green colour product formed

by the reaction of fructose with anthrone was read at 620 nm. LMW carbohydrates were calibrated against a series of known concentration of sucrose and HMW carbohydrates were calibrated against inulin standards. All samples and standards were determined as a mean value of three replicate assays.

2.3. General DNA, RNA and protein methods

2.3.1. Polymerase Chain Reaction (PCR)

Standard PCR reactions were carried out on an iCycler (Bio-RAD) in a total volume of 20 µl in 0.2 ml strip tubes (Axygen). The reaction consisted of 1 X *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 100 µM dNTPs, 200 nM of each primer, and 0.2 U of *Taq* DNA polymerase (Invitrogen). A quantity of approximately 5 ng of DNA or 1 µl cDNA was used as the template in the amplification. The thermal-cycle conditions were an initial reaction at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 50-64°C for 30 sec (depending on the Tm of the primers), and at 72°C for 1-2 min (1 min/ kb), respectively, and one final reaction at 72°C for 10 min. To amplify *L. perenne* 1-SST, 1-FFT and 1-FEH genes for recombinant protein expression, the TripleMaster PCR System (Eppendorf) with High Fidelity PCR Buffer (Eppendorf) were used following the manufacturer's instructions.

2.3.2. TA cloning

PCR products were cloned into TOPO 2.1 vectors (Invitrogen) and transformed into OneShot® *E. coli* cells by chemical transformation following the manufacturer's instructions. For 4 µl of PCR product, 1 µl TOPO 2.1 vector and 1 µl salt solution were added. The mixture was incubated at room temperature for 30 min to ligate PCR fragments into the vector. The reaction mix (2 µl) was added into one vial of OneShot Chemically Competent *E. coli* cells and incubated on ice for 30 minutes. After a 30 second heat-shock at 42°C, 250 µl of S.O.C (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM

glucose) medium was added and incubated for 1 h in a 37°C shaking incubator at 200 rpm. Bacteria were subsequently plated in three dilutions (10 µl, 50 µl and 100 µl) onto pre-warmed Luria-Bertani (LB) (Invitrogen) agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, and 1.5% (w/v) agar) containing100 µg ml^{-1} ampicillin or 50 µg ml^{-1} kanamycin and 40 µl of 40 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Invitrogen). Plates were incubated at 37°C overnight.

Positive colonies were selected using white-blue selection in combination with antibiotic selection. For each primer pair, 8 individual white or light blue colonies were picked and inoculated into 6 ml LB-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl) containing 100 µg ml^{-1} ampicillin or 50 µg ml^{-1} kanamycin and incubated overnight in at 37°C in a shaking incubator at 200 rpm.

2.3.3. Plasmid DNA isolation

To screen selected colonies for the presence of correct inserts, plasmid DNA was isolated in a first instance using the alkaline lysis method described by Sambrook et al. (1989) from 1 ml LB-broth cultures, followed by *EcoRI* enzyme digestion. Overnight *E. coli* cell broth (1 ml) was centrifuged at 13,000 rpm for 1 min and the pellet re-suspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-HCl and 10 mM Na_2EDTA; pH 8.0). To the suspension, 200 µl of freshly prepared solution II (0.2M NaOH and 1% (w/v) SDS) was added and mixed thoroughly by inversion. An aliquot of 150 µl of solution III (29.44% (w/v) potassium acetate; 11.5% (v/v) glacial acetic acid) was added to the solution, the tubes were inverted several times to mix thoroughly. The mixture was incubated on ice for 5 min, and then centrifuged at 13,000 rpm for 5 min. The supernatant was transferred into a new tube and DNA in the supernatant was precipitated by the addition of two volumes of 95% (v/v) ethanol, and centrifuged for 5 min. The DNA pellet was washed with 70% (v/v) ethanol, then air dried and resuspended in 50 µl of sterilised water containing 200 ng ml^{-1} RNases.

Plasmid DNA was subsequently digested by *EcoRI* enzyme (New England Biolabs) (see 3.3.4), fragments separated on an agarose gel, and visualised by ethidium bromide staining.

2.3.4. Restriction Enzyme Digestion

Restriction enzyme digestion was used for checking inserts and for subcloning. The reaction was carried out in a total volume of 20 µl reaction mixture containing 0.5 - 2 µg plasmid DNA, 1 X buffer matched to the restriction enzyme being used, and 5 – 30 U restriction enzyme. After incubating at 37°C for 1 - 2 h, the reaction mixture was loaded on an agarose gel and the DNA fragments generated by the restriction enzyme digestion separated by electrophoresis (section 2.3.5). The amount of plasmid DNA, restriction enzyme and incubation conditions used depended on the enzyme used and the purpose of the digestion, i.e. for checking inserts or for subcloning.

2.3.5. Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used to separate and quantify DNA fragments generated by restriction enzyme digestion or PCR. According to resolution requirements the concentration of agarose used varied from 0.8% to 2.5% (w/v) in 1 X TAE buffer (40 mM Tris acetate pH: 8.5, 2 mM EDTA) with 1 ug ml^{-1} ethidium bromide (Invitrogen). DNA samples in DNA loading dye solutions (6 X stock solution containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose in water) were loaded onto the gel, alongside a 1kb Plus DNA ladder (Invitrogen), and then electrophoresed in 1 X TAE buffer at 100V for 30 min. DNA fragments were visualised in a UV-transilluminator and the image captured using the Gel Doc 2000 (Bio-RAD) and Quantity One software.

2.3.6. Purification of DNA fragments from agarose gels

PCR products or DNA fragments generated by restriction enzyme digestion were separated by agarose gel electrophoresis and bands of interest were cut out of the gel using a sterile scalpel blade under UV light. DNA was subsequently extracted from the agarose gel slice using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instruction. To the excised gel slices, three volumes of QG Buffer was added and incubated in a 50°C water bath for 10 min to dissolve the gel. After the gel slice was completely dissolved, the gel solution was transferred into a QIAquick column, and centrifuged at 13,000 rpm for 1 min. The column was washed with 0.75

ml of PE Wash Buffer and DNA was eluted in 50 µl of EB Elution Buffer (10 mM Tris-HCl, pH 8.5).

2.3.7. Ligation of DNA fragments

Ligation of DNA fragments into digested vectors was performed in a reaction mixture of 20 µl containing 30-60 ng DNA fragments, 20 ng vector DNA, 1 X ligation buffer and 10 units of T4 DNA ligase (New England Biolabs), and incubated at 4°C overnight.

2.3.8. Plasmid DNA isolation using the Qiagen Miniprep Kit

Bacterial cultures which tested positive for correct plasmid DNA (as identified by restriction enzyme digestion) were extracted and purified from LB-broth culture using the Qiagen Prep Plasmid Miniprep Kit (Qiagen) following the manufacturer's instruction. Overnight cultures (5 ml) were centrifuged at 13,000 rpm for 5 min and pellets re-suspended in 200 µl of cell re-suspension solution. To the suspension, 250 µl of cell lysis solution was added, and the solution was inverted several times. An aliquot of 250 µl of neutralisation solution was added. The solution was mixed and then centrifuged at 13,000 rpm for 10 min to remove cell debris. The supernatant was transferred into a spin column which was inserted into a 2 ml Eppendorf tube, and centrifuged at 13,000 rpm for 1 min. The column was washed twice with 500 µl wash solution and DNA was eluted by the addition of 50 µl elution buffer to the column and centrifuged for 1 min at 13,000 rpm.

2.3.9. DNA Sequencing

Isolated plasmid DNA was sequenced to confirm that the cloned products had the correct sequence. The plasmid DNA was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977), using Big-Dye (Version 3.1) chemistry (Applied Biosystems). Either M13 forward and reverse primers or specific gene primers were used.

Cycle sequencing was carried out in a reaction volume of 20 µl. For each reaction the following solutions were added: 2 µl Big Dye, 3.5 µl 5 X Sequencing Buffer, 1.6 µl 2 µM M13 Forward or Reverse primer, ~250 ng DNA and the appropriate amount of water. The tubes were placed in an iCycler (Bio-RAD) and the reaction was carried out in 40 cycles of 20 seconds at 95°C, 5 seconds at 50°C, and 1 minute at 60°C. After the amplification, 3 µl 3 M NaOAc (pH 5.2), 62.5 ul 100% ethanol and 14.5 µl sterile water were added into each tube to precipitate the PCR products. Following mixing and incubation at room temperature for 15 min, the reaction mixture was centrifuged at 13,000 rpm for 20 min. The supernatant was removed immediately after centrifugation and 200 µl of 70% (v/v) ethanol were added and centrifuged again for 15 min. The DNA was air-dried and re-dissolved in 10 µl ABI HiDi Formamid (Applied Biosystem).

The products were separated on an ABI Prism 3100 Genetic Analyser (Applied Biosystems) by the Allan Wilson Centre Genome Service, Massey University, Palmerston North. Sequence data were compared with the sequence information published in GenBank (NCBI) using Alignix (Invitrogen).

2.3.10. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE)

SDS-PAGE is a denaturing gel electrophoresis system used to separate proteins based on their molecular weight. The concentration of the gels used in this work varied from 7.5% to 12.5% (w/v) of acrylamide depending on the size range of proteins to be separated. A "Mini PROTEIN® 3 Electrophoresis Module" (Bio-RAD) was used for gel electrophoresis. To prepare SDS–PAGE gels, 2.5 ml buffer (4 X concentrated) consisting of 0.4% (w/v) SDS, 1.5 M Tris-HCl (pH 8.0) and sufficient acrylamide stock (40% (w/v), Sigma), according to the concentration of the gel required, were mixed and the mixture was brought to a final volume of 4 ml with distilled water to get the desired percent acrylamide monomer. Polymerisation was initiated by adding freshly prepared 40 µl 10% (w/v) ammonium persulfate (AP) to the mix followed by 4 µl N,N,N',N'-tetramethylethylenediamine (TEMED). After the solution was quickly pipetted into the space to approximately 5 mm from the top of the gel mould, immediately 1 ml isopropanol was then overlaid to form an even layer on top of the separating gel. When the gel had set (approximately 30 min) the isopropanol was

discarded. The stacking gel was prepared by mixing stacking gel buffer stock (4 X concentrated) consisting of 0.5 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS, with 0.5 ml 40% (w/v) acrylamide and 1.25 ml of distilled water. After degassing by incubating the solution in hot water for 30 sec, 40 µl 10% (w/v) AP and 4 µl TEMED were added. Subsequently, the solution was mixed and poured onto the top of the gel. The gel comb was then inserted to form the wells, and the gel was allowed to stand until set. Protein samples were mixed with 4 X SDS-PAGE sample buffer (2% (w/v) SDS, 20% (v/v) glycerol, 20 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, and a small amount of bromophenol blue as a tracking dye) and boiled for 5 min to denature the proteins. The required volume of sample was loaded into the well, alongside the Benchmark™ Pre-stained protein ladder (Invitrogen), and electrophoresed in protein running buffer (25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS) at 90V for 20 min followed by 145 V for 60 min. The run was stopped when the dye front nearly reached the bottom of the gel. The gel was then either stained in Coomassie Blue dye solution to visualise the separated proteins (2.3.11) or transferred to a membrane for western blotting (2.3.12).

2.3.11. Coomassie Blue staining

Once the proteins were separated by gel electrophoresis, the gel was stained in Coomassie Blue dye solution to visualise the separated proteins. The gel was stained in 0.1% (w/v) Coomassie Blue dye in 50% (v/v) methanol, 10% (v/v) glacial acetic acid for 1 h, with agitation, followed by destaining in a solution of 10% (v/v) acetic acid and 25% (v/v) methanol to remove background staining from the gel.

2.3.12. Western blotting

Western blotting was used to examine the size, and/or amount of the protein of interest in a given sample using an antibody specific to the protein. After gel electrophoresis, the separated proteins were transferred from the SDS-PAGE gel to a PVDF (polyvinylidene difluoride) membrane using the Xcell IIATM blotting apparatus (Invitrogen) following the manufacturer's instructions. A piece of gel sized PVDF membrane (Sequiblot™, Bio-RAD) was wetted in methanol for 1 min. After washing twice with H_2O, the membrane was soaked in transfer buffer (25 Tris base, 192 mM glycine and 20% (v/v) methanol) for 20 min. The membrane sandwich was assembled

as follows: cathode plate, two pieces of blot sponge, followed by three pieces of gel sized 3 M filter paper, gel, membrane, three pieces of 3 M filter paper and two pieces of blot sponge, finally the anode lid. The whole assembled transfer chamber was placed into the transfer tank and filled up with transfer buffer and proteins were transferred at a constant voltage of 30 V for 2 h.

After transfer, the membrane was blocked in blocking buffer (0.05 M Tris-HCl, 100 mM NaCl (pH: 7.4), 0.1% (v/v) Tween 20, and 5% (w/v) non-fat milk powder) for 1 h. The membrane was then incubated in 10 ml 1 X TBST buffer (0.05 M Tris-HCl, 100 mM NaCl (pH: 7.4), 0.1% (v/v) Tween 20) containing the primary antibody (1: 500) for 1 h with gentle shaking at room temperature, followed by three washes with 1 X TBST buffer (10 min each) to remove unbound primary antibody. After rinsing, the membrane was incubated in 10 ml of TBST buffer containing 2 µl secondary antibody (anti-rabbit/ mouse IgG conjugated with horseradish peroxidase (Amersham) for 1 h followed by three washes with TBST (10 min each). The chemiluminescence detection was performed using ECL Plus Western Blotting Detection Reagents (GE Healthcare). Detection solution A and B were mixed in a ratio of 40:1 and 5 ml of the mixture was added to the membrane. After 5 min incubation, the membrane was sandwiched between two pieces of acetate foil. In a dark room a sheet of Bio MaxMR film (Kodak) was placed on top of the sandwich in a film cassette and exposed for an appropriate period of time. After exposure, the film was developed using an automatic X-ray film processor (All-Pro Imaging).

For re-probing, membranes were stripped by incubating in stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.7)) for 30 min with occasional agitation. The membrane was then washed for 2 X 10 min in TBST buffer. After the membrane was re-blocked in blocking buffer, it was probed with a different antibody.

2.4. Gene expression profiling

2.4.1. cDNA synthesis

2.4.1.1. Isolation of total RNA with TRIzol Reagent

Total RNA was isolated from frozen tissue powder using TRIzol Reagent (Invitrogen) according to the manufacturers' protocol. Frozen tissue powder (100 mg) was mixed with 1 ml of TRIzol Reagent. After incubation at room temperature for 5 min, the mixture was centrifuged at 13,000 rpm for 15 min. The supernatant was transferred into a new tube, vigorously mixed with 0.2 ml of chloroform, followed by centrifugation at 13,000 rpm for 15 min. The upper aqueous phase was transferred into a new tube, and RNA was precipitated by adding 0.25 ml isopropanol and 0.25 ml of 1.2 M NaCl and 0.8 M sodium citrate salt solution, followed by centrifugation at 13,000 rpm for 15 min. The supernatant was discarded and the RNA pellet was washed with 1 ml 75% (v/v) ethanol, air-dried and resuspended in 100 µl DEPC (Diethylpyrocarbonate) –treated water.

2.4.1.2. Removal of genomic DNA from total RNA

Isolated total RNA (100 µg) was treated with 20 U RNase free-DNase I (Roche) for 1 h at 37°C in a 100 µl reaction mixture containing 5 mM $MgSO_4$, 0.1mM NaOAc, followed by deactivation at 85°C for 10 min.. The DNase treated RNA was subsequently cleaned using the RNeasy Mini spin kit (Qiagen) to remove enzyme, salts and degraded DNA fragments. To 100 µl treated RNA, 350 µl RLT Lysis Buffer was added to denature the enzyme. Following 250 µl ethanol addition, the mixture was transferred to an RNeasy Mini spin column and centrifuged for 15 sec at 10,000 rpm. The column was washed with 500 µl RPE Wash Buffer twice, and RNA was eluted with 50 µl DEPC water.

The absence of genomic DNA was confirmed by RT qPCR amplification using a pair of primers designed for *L. perenne* 18S rRNA at the same conditions used for gene expression profiling RT qPCR reactions (2.4.4). One µl DNase treated RNA was used as template, while 1 µl of the untreated RNA was used as positive control.

2.4.1.3. Quantification of RNA

RNA quality was checked by gel electrophoresis. RNA (500 ng) was loaded onto a 1.25% (w/v) TAE agarose gel (2.3.5). RNA samples with sharp rRNA bands were used subsequently for cDNA synthesis.

The concentration and purity of the RNA samples was measured by the ratio of absorbance at 260 and 280 nm using a NanoDrop ND-100 Spectrophotometer, with good quality RNA samples having a ratio of A260/A280 around 2.0. Each sample concentration was determined as a mean value of three replicates, and the mean concentration (with an error less than 2 ng/ µl for each measurement) was used to adjust the final RNA concentration to 100 ng/ µl by DEPC treated H_2O for subsequent cDNA synthesis.

2.4.1.4. Reverse transcription of RNA into cDNA

Total RNA (1 µg) was reverse transcribed into cDNA using random hexamer primers and the ThermoScript RT-PCR kit (Invitrogen) following manufacture's instructions. An aliquot of 1 µl of random hexamer primers (Invitrogen) was mixed with 10 µl of 100 ng/ µl DNase treated RNA. After 5 min incubation at 65°C, the mixture was immediately chilled on ice to minimise secondary structure formation in the RNA sample. A reaction master mix was prepared containing 4 µl 5 X RT buffer, 1 µl 0.1 M DTT, 2 µl 10 mM dNTPs, 1 µl RNase OUT (40 U/µl) and 1 µl ThermoScript Reverse Transcriptase (15 U/ µl). The reaction master mix (9 µl) was added to the RNA to bring the total reaction volume to 20 µl. RT reactions were performed in an iCycler (Bio-RAD) for 10 min at 25°C, 50 min at 50°C, and finally deactivation of the enzymes at 85°C for 5 min, followed by adding 1 µl RNase H and incubation at 37°C for 20 min to remove RNA templates.

The cDNAs were then diluted 50-fold with sterilised water and stored at -80°C until used.

2.4.2. Primers designed for RT qPCR

Individual transcript analysis was carried out using gene-specific primers. Primers were designed based on the sequence of *Lp1-SST* (AY245431), Lp*1-FFT*(AB188920), Lp*6G-FFT* (AB125218), Lp*1-FEH* (DQ073968) and *LpActin* (EF460315), published in GenBank (NCBI), respectively, using Primer Express 3.0 software (Applied

Biosystems) and criteria required for qPCR primer design. These included predicted melting temperatures of 60±1°C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) content of 50-60%, no repeats of G's or C's longer than 3 bases, no 3' complementarities in the sequence of forward and reverse primers and primers targeted to the outside of the target sequence secondary structure. A BLASTN search was then performed against GenBank to ensure that all primers were unique to the gene of interest.

The primers were synthesised by Sigma Genosys (Australia). Each primer was resuspended in sterilised water to a final concentration of 100 µM and stored at -20°C. The stock primer solutions were diluted to a working concentration of 10 µM for PCR reactions and 3.2 µM for sequencing reactions.

Table 2.1. Primer sequences used in this study for RT qPCR analysis.

FW- forward primer; RV – reverse primer

		Sequence (5'-3')	Length (bp)[a]	Accession [b] No
Lp1-SST	FW	AAGTCCTCCGGTGCCTACTC	196	AY245431
	RV	GCGATGTTGCCCCAGCT		
Lp1-FFT	FW	GTTCTCGCTCACCCACAG	186	AB188920
	RV	GACACGCTCGAAGCTAAGG		
Lp6G-FFT	FW	AGGACGGCGAGGTGTCT	93	AB125218
	RV	CGCCGTCGGCGGGCGACT		
Lp1-FEH	FW	AAGGTGCCAAACATGTCCTC	239	DQ073968
	RV	TGCGACGTCATCTGAAGAAC		
LpActin	FW	TGGATTCTGGTGATGGTGTC	189	EF460315
	RV	GCTTCTCCTTGATGTCCCTTAC		

[a] Amplicon length in base pairs.

[b] Genbank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/

To ensure sensitivity and specificity of the primers for qPCR, several different primer pairs were initially designed for each gene of interest and tested for efficiency and specificity. Preliminary RT qPCR was performed with each primer pair using a cDNA sample derived from pooled cDNAs (30 µl of each cDNA sample), this pooled cDNA was also used as a sample control in later experiments, under the same conditions used for gene expression analysis (2.4.4). The primer pair that generated the lowest Ct for its specific product without any nonspecific products and primer dimers was chosen for the RT qPCR experiments. To confirm this primer pair was specific to the gene of interest without amplification of other related genes, the PCR product generated from the selected primers was cloned into TOPO 2.1 vectors (2.3.2) and sequenced (2.3.9), the sequence data were compared with the sequence information published in GenBank (NCBI) using Alignix (Invitrogen). Selected primers are listed in Table 2.1.

2.4.3. Plasmid DNA standards for qPCR

After sequencing, purified plasmid DNA containing the sequence of interest was quantified using NanoDrop ND-100 Spectrophotometer. A serial dilution was performed to give final concentrations between 2×10^5 to 2×10 copies as standards to calibrate the RT qPCR reactions.

2.4.4. qPCR analysis

qPCR analysis was performed using the MyiQ^{Tm} single-color Real-time PCR detection system (Bio-RAD) interfaced to the MyiQ^{Tm} optical system software version 1 (Bio-RAD). Each qPCR run (in 96-well plates) included triplicates of 24 experimental samples, five standards derived from a series dilution of plasmid DNA containing the amplicon of interest, ranging from 2×10 to 2×10^5 copies, one non-template reaction as negative control and two pooled cDNA samples as external sample controls. A master mix containing 2 X SYBR Green Master Mix (Applied Biosystems) with 400 nM forward and reverse primers was prepared and thoroughly mixed. The master mix (10 µl) was pipetted into thin-well PCR tubes and 10 µl of the diluted cDNA or plasmid DNA was added to one of the wells containing the mastermix to a final volume of 20 µl. The DNA and the master mix were mixed by vortexing 3×1 second.

After briefly spinning down by centrifugation to collect all reagents in the bottom of the wells, the tubes were placed into the MyiQ^{Tm} single-color Real-time PCR detection system. The thermal cycling program consisted of 95°C for 5 min to activate the polymerase, followed by 40 cycles of 95°C for 20 s, 60°C for 30 s; 72°C for 30 s. Fluorescence was monitored during the 60°C annealing step. Dissociation analysis was conducted after the final amplification cycle to verify that no primer dimers and extraneous, unintentional amplicons were formed. Samples were also analysed by agarose gel electrophoresis to verify amplification fidelity.

In order to reduce plate to plate variation, thresholds were held constant for all runs. The threshold was placed at 110, this value was in the exponential part of the amplification plot for all of the products being generated.

General recommendations were followed to prevent a carryover of RNA and cDNA during the whole experiment, such as regularly changing gloves, maintenance of separated, clean areas; negative controls (which were included at the beginning of the extraction procedures), as well as using sterile filter tips.

2.4.5. Analysis of results

The qPCR results were represented as copy numbers calibrated against the standard plasmid DNA. Data were then normalised against values obtained from amplification of the *LpActin* gene.

2.5. Antibody generation

2.5.1. Expression of recombinant proteins in a bacterial expression system

2.5.1.1. Cloning of *Lp1-SST*, *Lp1-FFT* and *Lp1-FEH* into pET 22b(+) (Novagen) vector

The 1-SST, 1-FFT and 1-FEH cDNAs of *L. perenne* were expressed in *E. coli* for recombinant protein production and subsequent antibody generation. The *Lp1-SST*,

Lp1-FFT and *Lp1-FEH* genes were amplified by PCR from *L. perenne* cDNA using the following primers with adapters, respectively:

aa**CCATGG**AGTCCC CAAGCGC (1-SST-F),

aa**CTCGAG**CAAGTCGTCGTTCGTGAAG AT (1-SST-R);

aa**CCATGG**AGTCCCGGGCCAT (1-FFT-F),

aa**CTCGAG**TGAGTCCTTAACCATGACGGTCT (1-FFT-R);

aa**CCATGG***GG*ACTAACGAGTCAGATAGTTCTTCAG (FEH-F),

aa**CTCGAG**ACCAAAACTCTCCACGGC (FEH-R).

In the 5' forward primers a *NcoI* restriction site was added in frame to flank the translation start codon ATG; and *XhoI* was added after the last amino acid codon and before the STOP codon in the 3' reverse primers to fuse the translated proteins to a 6-His tag at the C-terminus. The restriction sites of *NcoI* and *XhoI* are indicated in pink color and in bold form, and the additional bases added for primer protection (in lowercase form) or in frame reading (in underline form) are highlighted with yellow color. The PCR reaction was conducted using the TripleMaster system for high fidelity following the manufacturer's instruction (2.3.1). The PCR products were loaded onto a 1 % (w/v) agarose gel. After electrophoresis, the fragments of interest were cut out and purified from the gel slice using the Qiagen® gel purification kit (Qiagen) (2.3.6). The eluted PCR product was incubated with 1 X PCR buffer, 1.5 mM $MgCl_2$, 5 pmol dNTPs and 0.2U *Taq* for 10 min at 72°C and then cloned into TOPOpCR2.1 TA-cloning vector (Invitrogen) following the manufacturer's instructions (2.3.2). The Plasmid DNA was isolated using a Qiagen Miniprep Kit (Qiagen) (2.3.8) and the putative positive clones were selected by digesting the plasmid DNA with *EcoRI* (2.3.4) and verified by sequencing (2.3.9).

After confirming the correct sequence of the inserts, both the TOPOpCR2.1 plasmid DNA containing the fragment to be translated and the pET 22(b+) vector (Novagen) were digested with *NcoI* and *XhoI* (New England Biolabs) (2.3.4). The DNA fragments were separated by gel electrophoresis; the band of interest was cut out of the gel and further purified using the Qiagen® gel purification kit (Qiagen) (2.3.6). After ligation (2.3.7) of the pET 22(b+) vector and the fragment to be translated, the ligated

product was transformed into DH5α competent cells, and transformants were selected on plates containing the appropriate antibiotic. Single colonies were transferred to 4 ml of LB broth containing the appropriate antibiotic and grown at 37°C, overnight with shaking (200 rpm). Plasmid DNA was isolated and positive colonies were screened by digestion of the plasmid DNA with *NcoI* and *XhoI* and confirmed by sequencing the junctions of the cloning enzyme sites.

2.5.1.2. Induction of pET 22b(+) vector

The constructs of pET 22b(+) vector containing *Lp1-SST*, *Lp1-FFT* or *Lp1-FEH* fragments, respectively, were transformed into BL21 (DE3) *E. coli* competent cells (Novagen) by a chemical transformation method and plated onto LB plates containing ampicillin (100 μg ml^{-1}). A 50 ml LB broth containing ampicillin (100 μg/ ml) in a 250 ml flask was inoculated with a single colony and incubated at 37°C with shaking (200 rpm). Expression was induced by addition of IPTG (isopropyl-β-thiogalactopyranoside) to a final concentration of 1 mM when the A_{600} of the broth had reached approximately 0.6. After IPTG induction, 1 ml of the broth was harvested at 0, 1, 3 h and overnight, respectively. Cells were collected by centrifugation at 13,000 rpm for 5 min and resuspended in 100 μl of 1 X protein loading dye. Samples (5 μl) were loaded onto SDS-PAGE gels for analysis.

2.5.1.3. Cell disruption and extraction of inclusion bodies

Induced cells (5 ml) were collected by centrifugation at 5,000*g* for 10 min and then resuspended in 5 ml lysis buffer (25 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH8.0), 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$, 1 mg ml^{-1} lysozyme, 0.01 mg ml^{-1} RNase and 0.05 mg ml^{-1} DNase) and incubated at 37°C for one hour with occasional agitation. After centrifugation at 15,000g for 20 min to precipitate inclusion bodies, the soluble protein fraction was collected and stored a -20°C until further usage. The pellet containing inclusion bodies was resuspended in 5 ml washing buffer (50 mM Tris-HCl (pH 7.7), 1 mM EDTA (pH 8.0), 0.1% (v/v) Triton X-100 and 0.3 mM NaCl) and then re-pelleted, this was repeated two more times. The soluble and the inclusion body proteins were subsequently analysed by SDS-PAGE.

2.5.2. Purification of recombinant proteins

2.5.2.1. Purification of recombinant proteins using Ni-NTA (nickel-nitrilotriacetic acid) affinity column

The 6-His tag fused to the recombinant proteins at the C-terminus allows protein purification using Ni-NTA affinity columns (Invitrogen). The inclusion body fraction containing 1-SST, 1-FFT or 1-FEH fusion proteins extracted from 500 ml induced culture were resuspended in 20 ml binding buffer (20 mM Tris (pH 7.9), 500 mM NaCl, 5 mM imidazole, 6 M urea, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF)) and incubated on ice overnight. After 30 min centrifugation at 38,000*g* to remove cell debris, the supernatant was collected and loaded on a Ni-NTA affinity column, washed with ten column volumes of binding buffer and subsequently 15 column volumes of washing buffer (20 mM Tris (pH 7.9), 500 mM NaCl, 20 mM imidazole, 6 M urea). Elution was performed with elution buffer (20 mM Tris (pH 7.9), 500 mM NaCl, 250 mM imidazole, 6 M urea) and 0.5 ml fractions were collected. The protein concentration of each fraction was determined using Bradford reagent (Bio-RAD) and the fractions containing protein were pooled. An aliquot of total supernatant, binding buffer, washing buffer and elution (purified protein) were analysed by SDS-PAGE.

2.5.2.2. SDS-PAGE whole gel elution of Ni-NTA affinity column purified proteins

The purified proteins were dialysed, using dialysis tubing with a cut-off of 10,000 Daltons (Bio-RAD), against 0.2 X PBS buffer (200 mM NaH_2PO_4, 150 mM NaCl (pH 7.2)) in a cold room (4°C) with three buffer changes, 2 h each. Subsequently, the dialysed proteins were freeze-dried, and then resuspended in one fifth of the original volume of MQ H_2O to a final concentration of 1 X PBS. The protein was loaded on a SDS-PAGE gel along with a pre-stained molecular weight protein ladder (Invitrogen). After the proteins were separated by electrophoresis, the gel was removed and soaked in elution buffer (50 mM Tris, 25 mM boric acid (pH 8.7)) for 30 min. The whole gel elution was performed using Whole Gel Eluter (Bio-RAD) following the manufacturer's instructions, at a constant current of 80 mA for 20 min, and then 20 sec

with reversed current. The fractions with the predicted size were collected and analyzed by SDS-PAGE followed by coomassie brilliant blue staining to confirm the purity of the protein. A small piece of the gel containing the protein band of interest was analysed by mass spectrometry (AgResearch Proteomics Facility). After confirmation of the correct sequence, the protein concentration of the eluted fraction was determined using Bradford reagent, followed by dialysis in PBS buffer. The dialysed protein was freeze-dried and resuspended in MQ H_2O to a final concentration of 0.5 μg μl^{-1} in 1 X PBS.

2.5.3. Production of polyclonal anti- Lp1-SST, LpI-FFT and LpI-FEH antisera in rabbits

Production of polyclonal antisera was carried out at the Small Animal Production Unit, Massey University, Palmerston North. A total of 300 μg purified recombinant *Lp*1-SST, 1-FFT or 1-FEH protein was injected into a rabbit in a series of three injections: (i) on day 0, New Zealand White rabbits were immunized subcutaneously with a 400 μl emulsion comprising 100 μg of the purified protein in 200 μl of 1 X PBS and 200 μl of Freund's complete adjuvant; (ii) on day 21, each of those rabbits was injected subcutaneously with 400 μl emulsion of 200 μl of 1 X PBS containing 100 μg of the purified protein and the same volume of Freund's incomplete adjuvant; (iii) on day 42, each rabbit was boosted again with the same emulsion used for the second injection. Blood was collected 10 days after the third injection and sera were collected by centrifugation at 1,500*g* for 20 min at 4°C. Merthiolate and phenol was added to the sera in a final concentration of 0.01% (w/v) and 0.25% (v/v), respectively, and the titre was assayed by immunoblotting of 100 ng antigen. The antibodies were stored at 4°C for short term or at -80°C for long term storage.

2.5.4. Determination of antibody quality

The quality of the antibodies was determined by antibody titre tests. An aliquot of 2.3 μg of purified antigen protein was loaded on a preparative SDS-PAGE gel containing a single well for the protein sample, and a reference well for protein ladders. After electrophoresis, the protein was transferred onto a PVDF membrane. The membrane

was blotted in blocking buffer, and then cut into strips approximately 3 mm wide with the upper right corner cut. Subsequently, each strip was incubated in TBST buffer (with 1 h shaking) containing different concentrations of antisera at a ratio of 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1: 6,400, 1:12,800 and 1:25,600. The ratio for the pre-bleed sera was 1:200. After three times washing with TBST buffer, the strips were incubated in TBST buffer containing the secondary antibody at a 1:5,000 dilution for 1 h, followed by three washes. Chemiluminol solution (1 ml) was added to each strip, which were arranged in the antisera dilution order, and exposed for 10 sec, 30 sec, 1 min, 2 min and 5 min on X-ray film in a dark room. After exposure, the film was developed using an automatic X-ray film processor (All-Pro Imaging).

2.6. Plant protein expression profiling

2.6.1. Protein extraction

Total soluble protein was extracted from 150 mg frozen plant tissue powder. The powder was homogenised in 300 µl of 25 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 0.04% (v/v) 2-mercaptoethanol and incubated for 10 min at 4°C with 1000 rpm agitation in an Eppendorf Thermo Mixer. The homogenates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was transferred into a fresh tube and kept at 4°C. The protein concentration in the supernatant was determined using the Quant-iT™ Protein Assay Kit (Invitrogen) following the manufacturers' instruction. In a 0.5 ml tube, 2 µl of the protein extracts were added to 198 µl Quanti-iT working solution. After 15 min incubation, fluorescence was determined in a Qubit ™ fluorometer and protein concentrations determined using a calibration curve based on a series of protein standards (from 0- 20 µg/ ml) supplied with the kit.

2.6.2. Protein separation and Western blotting

Total proteins were separated on 7.5% (w/v) polyacrylamide SDS gels (2.3.10). Each gel contained a protein molecular weight marker, six experimental samples (one cultivar with two genotypes grown at three temperature regimes), and one external

sample control, which was selected randomly from experimental samples and employed for every gel to monitor the uniform loading between gels. To test for equal loading, two gels loaded with the same samples were run in parallel. After electrophoresis, one gel was stained with Coomassie Blue dye (2.3.11) and the intensity of the ribulose 1,5-bisphosphate carboxylase (Rubisco) protein bands (55 kDa) were compared. Ensuring equal sample loadings by this method, the second gel was then used for western blotting (2.3.12).

The target proteins were semi-quantified by comparing the band intensity on the films and normalized to the external sample control, to allow comparison of band intensities between different films.

2.7. Statistical analysis

In this study, the experiment was analyzed as a full three-way ANOVA with 4 cultivars (A, H, P and F), 2 tissues (blade and sheath) and 3 temperature regimes (20/20 °C, 20/10 °C and 10/10 °C). Each of the $4 \times 2 \times 3 = 24$ treatments was replicated 8 times (genotypes). Statistical analysis was conducted by Professor Jonathan Newman (University of Guelph, Canada) using JMP statistical software version 5.1. The data were Box-Cox transformed to homogenise the error variances, and the untransformed means and standard error of the means are reported as a measure of data dispersion.

CHAPTER THREE

Experimental Results

This chapter reports the experimental results from a climate chamber experiment using four ryegrass cultivars (A, P, H and F) grown at three temperature regimes (14 h day/ 10 h night: 20/20°C, 20/10°C and 10/10°C). These four cultivars were a subset of 8 cultivars examined in a previous experiment, and were selected according to their WSC content with significantly higher WSC content in A, P and H compared to the control cultivar F. We report here significant main effects of cultivar and tissue, and cultivar by tissue and tissue by temperature interactions on WSC levels and gene expression profiles.

3.1. Water soluble carbohydrate (WSC) concentrations

3.1.1. Cultivar effects

Concentrations of high molecular weight (HMW) WSC's (fructans) were significantly higher in cultivar P (P<0.0001), but levels in A were only marginally higher than in the control cultivar F; and levels in H were not different compared to F (Fig. 3.1A). Low molecular weight (LMW) WSC's (glucose, fructose, sucrose, and low degree of polymerisation (DP) fructans) concentrations were not significantly different in the four cultivars (Fig. 3.1B). None of the interactions were significant.

3.1.2. Tissue effects

Concentrations of HMW WSCs were significantly higher in sheath tissue (P<0.0001; Fig. 3.2A), but LMW WSC concentrations were only marginally higher compared to blades (P=0.0224; Fig. 3.2B). None of the interactions were significant.

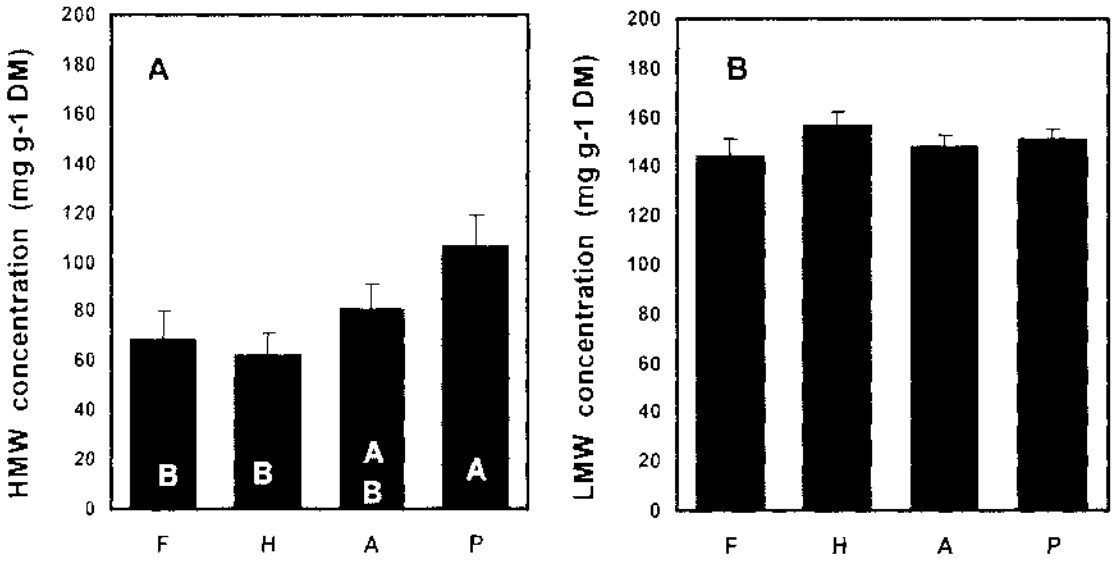


Fig 3.1 Main effect of cultivar on the concentration of (A) – HMW WSCs and (B) – LMW WSC. Different letters denote significant differences as determined by Tukey's Honestly Significant Difference (HSD) test.

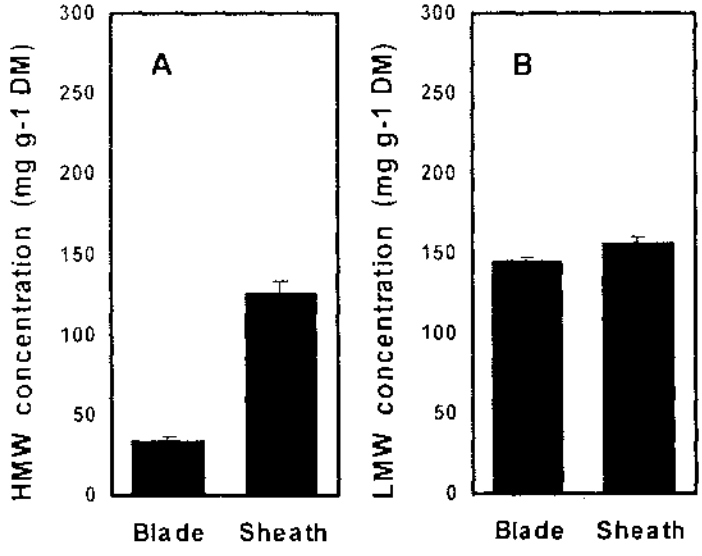


Fig 3.2 Main effect of tissue on the concentration of (A) – HMW WSCs and (B) – LMW WSC.

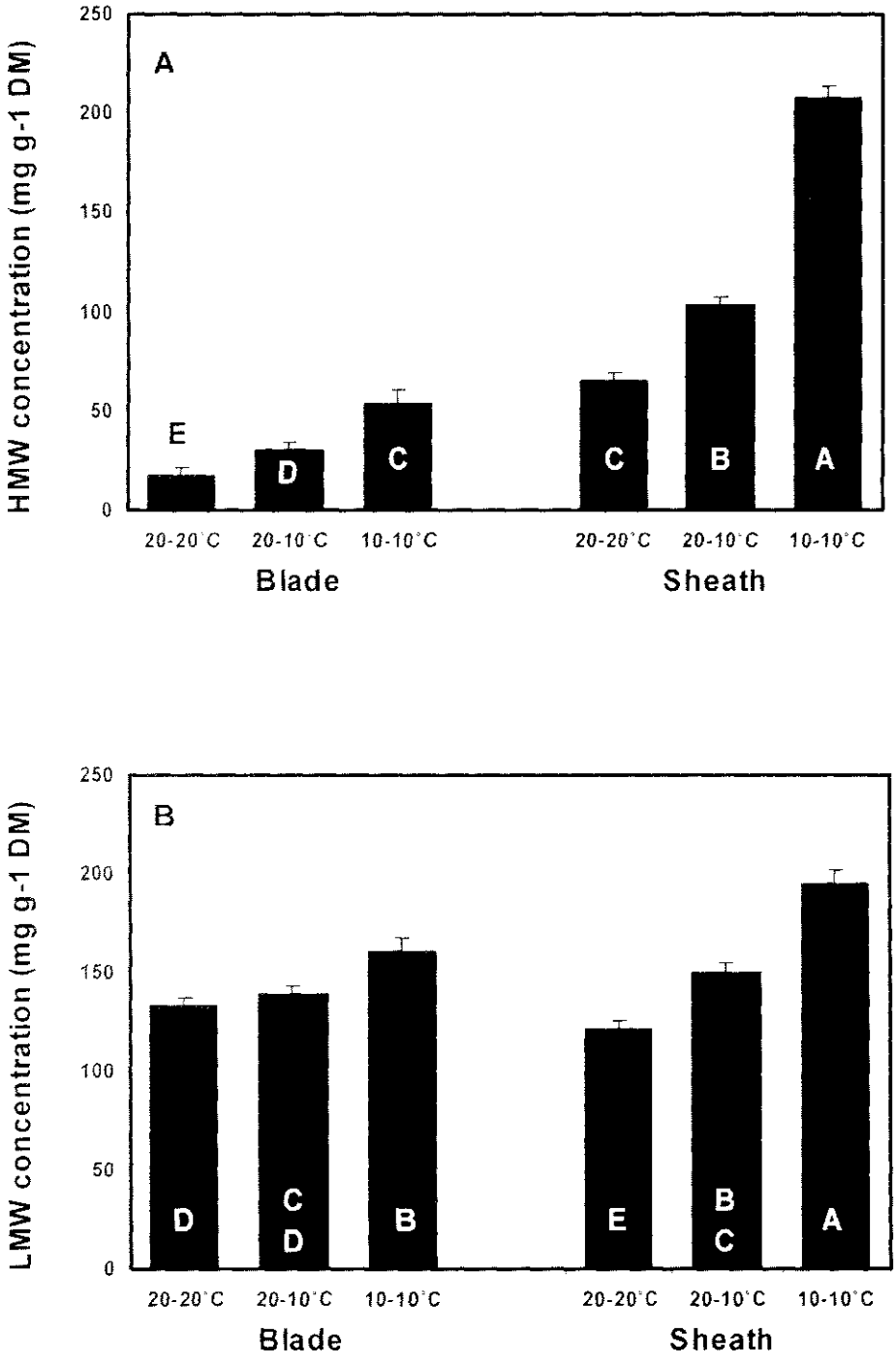


Fig 3.3 Tissue by temperature interactions on the concentration of (A) – HMW WSCs and (B) – LMW WSC. Different letters denote significant differences as determined by Tukey's Honestly Significant Difference (HSD) test.

3.1.3 Tissue by temperature interactions

There were significant tissue by temperature interactions on the concentrations of HMW and LMW WSCs (P=0.0068, P<0.0001, respectively). Plants grown at 10°C/10°C compared to plants grown at 20°C/20°C accumulated significantly higher levels of HMW (Fig. 3.3A) and LMW (Fig. 3.3B) WSCs, both in blade and sheath tissues. Plants grown at 20°C/10°C accumulated intermediate levels of WSCs. HMW WSC levels were mostly significantly lower in blade tissue compared to sheath tissue, but blades grown at 10°C/10°C accumulated the same amounts as sheath tissue grown at 20°C/20°C. LMW WSC concentrations in plants grown at 20°C/20°C were higher in blades compared to sheaths; but they were lower in blades compared to sheaths in plants grown at 10°C/10°C.

3.2. Gene expression profiling

The expression levels of 1-SST 1-FFT 6G-FFT and 1-FEH mRNAs were determined by RT-qPCR in both plant blade and sheath tissues. The transcript amounts are presented as the ratio of copy numbers of the target gene and the "housekeeping" gene *Lp.Actin*.

3.2.1 Methodological tests for RT- qPCR quantification

3.2.1.1. RNA quality

RNA free from genomic DNA contamination is prerequisite for accurate RT-qPCR results. In this experiment, the RNA samples were treated with RNase free DNase I to remove trace amounts of genomic DNA. The absence of genomic DNA after DNase treatment was verified by performing qPCR using a pair of primers designed for *L. perenne18SrRNA* (because of its abundance) with the DNase treated RNA as template, whilst the untreated RNA was used as a positive control. After 40 cycles of qPCR, no detectable amplification was observed from the DNase treated RNA samples whereas the untreated RNA sample showed a clear band (data not shown). The DNase, salt and degraded DNA can affect downstream cDNA synthesis, RNA samples were therefore purified using RNeasy Mini spin columns (Qiagen) before cDNA synthesis. The

integrity of RNA after DNase treatment and purification was documented on 1.25% (w/v) agarose gels and all samples showed sharp ribosomal bands on ethidium bromide stained gels (Fig. 3.4).

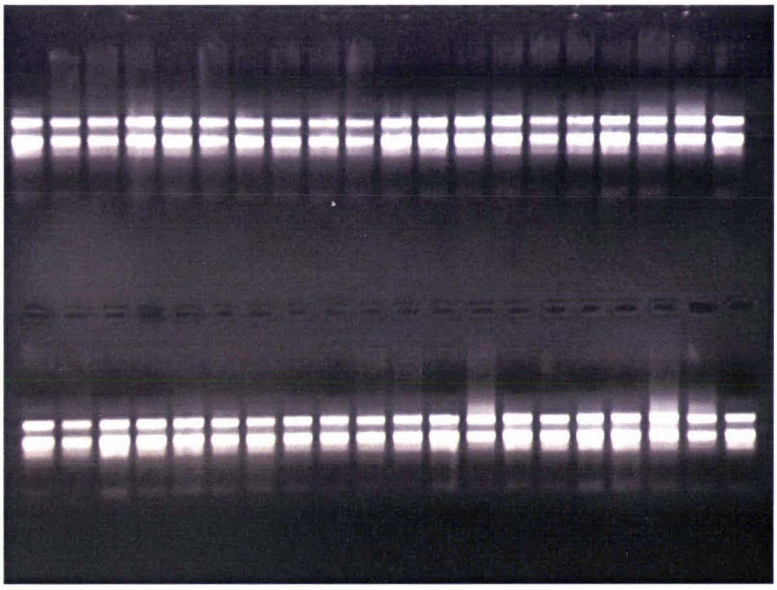
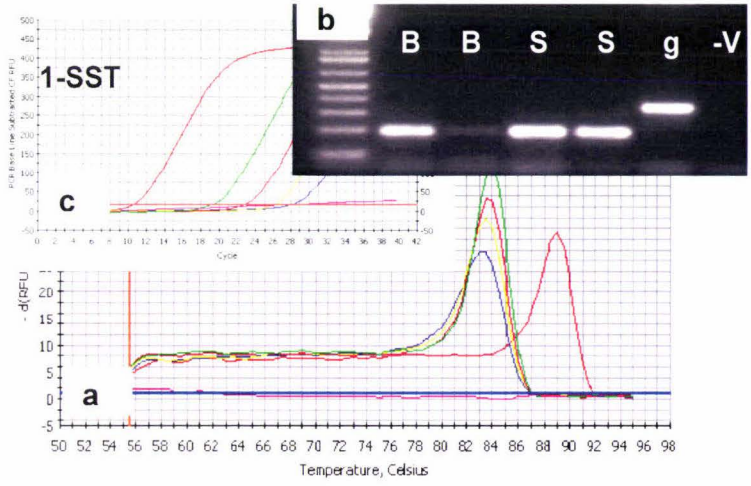
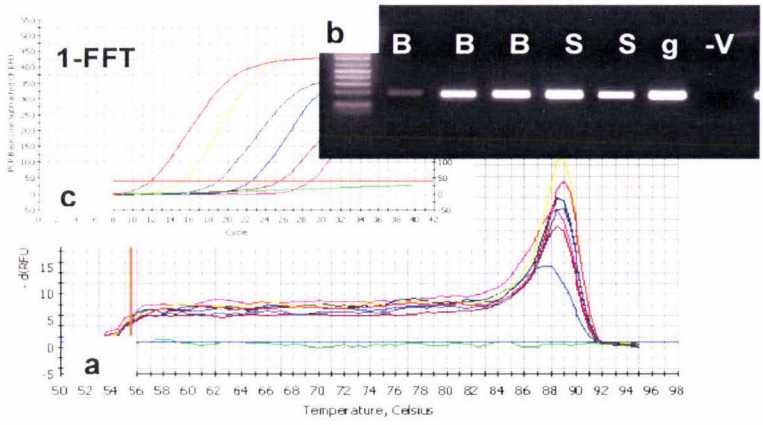


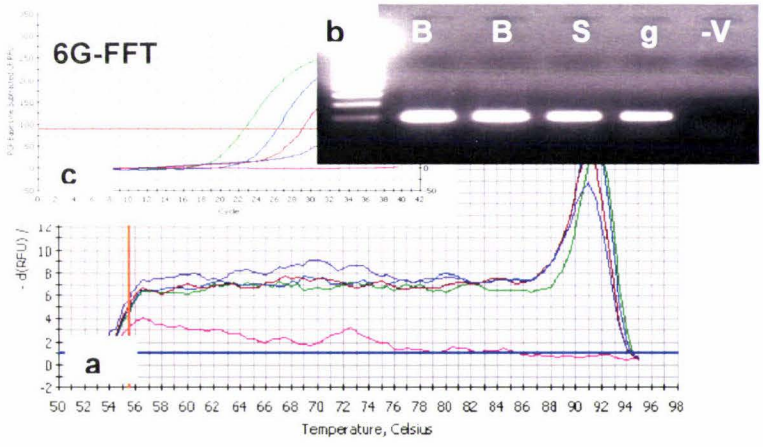
Fig. 3.4 Agarose gel electrophoresis analysis of RNA integrity after DNase treatment and purification. Each lane represents one RNA sample. 30ng RNA were loaded per lane.

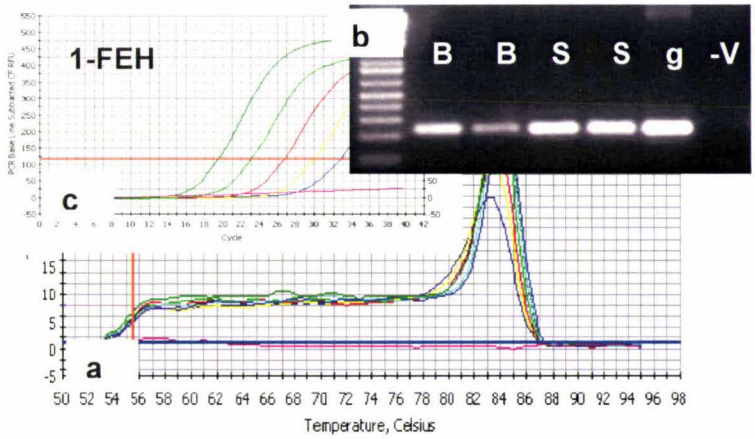
3.2.1.2. Primer design and primer specificity

PCR primers are often designed to span an exon-exon boundary to control for genomic DNA contamination. However, this strategy could not be used in this study as fructosyltransferase genes share a high degree of sequence homology; the homology between *Lp1-SST* and *Lp6G-FFT* is 81%; and 60% between *Lp1-FFT* and *Lp1-SST* or *Lp6G-FFT*. Furthermore, intron–exon boundary information was not available for these genes. Therefore, primers were designed to the sequence region which is unique to the target gene using criteria required for qPCR primers, such as the size of the amplicons, high sensitivity and specificity.


1-SST
b
B B S S g -V
c
- d(RFU)
a
50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98
Temperature, Celsius


1-FFT
b
B B B S S g -V
c
- d(RFU)
a
50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98
Temperature, Celsius


6G-FFT
b
B B S g -V
c
- d(RFU) /
a
50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98
Temperature, Celsius


1-FEH
b
B B S S g -V
c
a
50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98
Temperature, Celsius

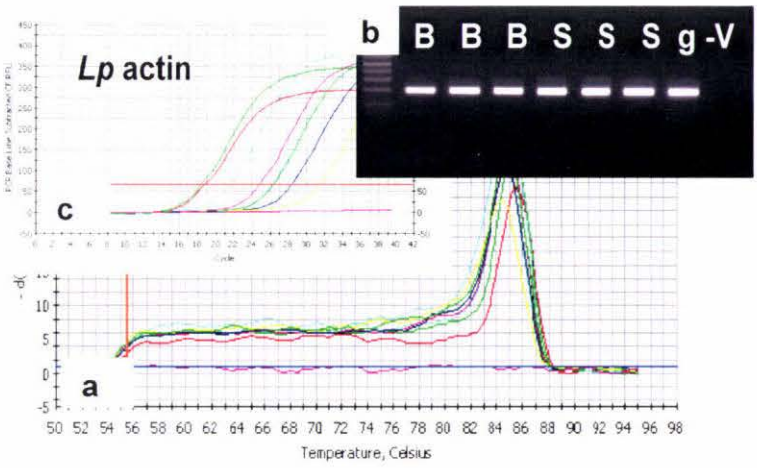


Fig. 3.5 (a) Representative melting curve analysis of the qPCR amplicons generated from cDNA of blades and sheaths. (b) The amplicons were separated by electrophoresis on a 2.5% (w/v) agarose gel and visualised with ethidium bromide staining. Lane assignments are as follows: B: blade cDNA; S: sheath cDNA; g: genomic DNA; -V: non-template control. (c) Amplification profile of the selected primers.

Because of the high sequence homology between fructosyltransferase genes, especially between *Lp1-SST* and *Lp6G-FFT*, primer specificity for the target gene is very important. Three primer pairs were designed for each gene based on comparison of the published sequences (NCBI data base), then tested by qPCR with cDNA from blades and sheaths. One pair was selected for each gene according to its specificity and efficiency. Chosen primer pairs showed lowest Ct's and melting curve analyses showed single products and the absence of extraneous amplicons and primer dimers (Fig. 3.5). Specificity of the oligonucleotide primers was further demonstrated on 2.5% (w/v) agarose gels showing single bands of the expected length (*Lp1-SST*, 196; *Lp1-FFT*, 186; *Lp6G-FFT*, 93; *Lp1-FEH*, 239; and LpActin, 189bp). In addition, a more stringent test of the specificity of the primers was performed by sequencing the qPCR products; the sequences of the amplification products showed almost 100 percent identity to the sequences published in GenBank (NCBI).

3.2.1.3. qPCR amplification efficiencies and linearity

The standard curves constructed from plasmid DNA containing the target sequence of *Lp1-SST*, *Lp1-FFT*, *Lp6G-FFT*, *Lp1-FEH*, and *LpActin*, respectively, displayed high qPCR efficiency rates, from 94 to 102% (Table 3.1), with strong linear relationships between C_t and initial template copy number of the series of dilutions of standard plasmids (from 20 - $2 \cdot 10^5$ copies).

Table 3.1 qPCR amplification efficiencies and linearity

Target gene	r^2	Slope	Amplification efficiency (%)
Lp1-SST	0.992	-3.34	101
Lp1-FFT	0.997	-3.38	96
Lp6G-FFT	0.990	-3.36	98
Lp1-FEH	0.994	3.45	95
Lp actin	0.995	3.33	94

3.2.2. Gene expression profiling

3.2.2.1. Tissue effects

Significantly higher transcript levels of the genes tested were found in sheath compared to blade tissues (*Lp1-SST*, *Lp6G-FFT*, and *Lp1-FEH*: P<0.0001; *Lp1-FFT*: P=0.0012; Fig. 3.6). This corresponded with higher concentrations of HMW WSCs in sheath tissues (see Fig. 3.1A). The expression level of *Lp1-FFT* was much higher in blades compared to *Lp1-SST* and *Lp6G-FFT*, and almost comparable to expression levels in sheaths, suggesting that *Lp1-FFT* may have a prominent role in fructan biosynthesis in blades.

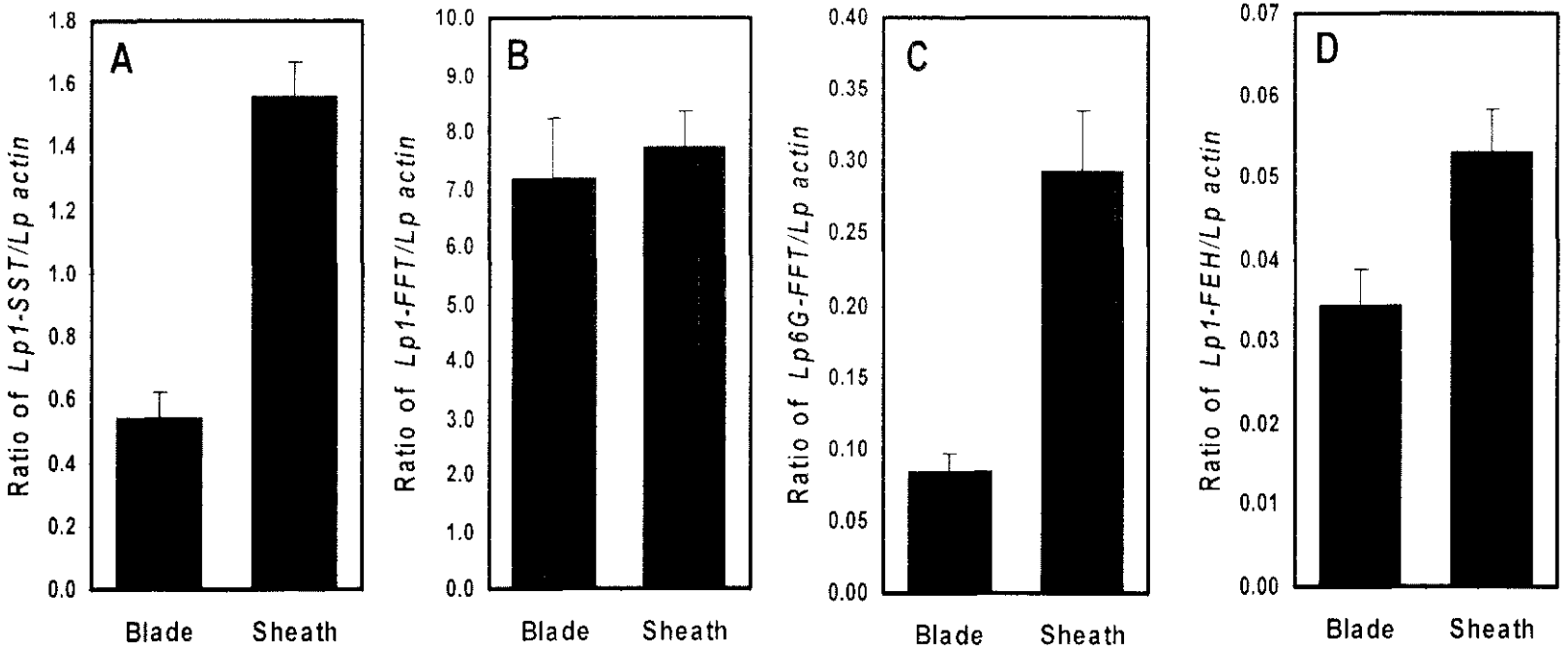


Fig. 3.6 Main tissue effects on the expression of *Lp1-SST* (A), *Lp1-FFT* (B), *Lp6G-FFT* (C) and *Lp1-FEH* (D) gene expressions.

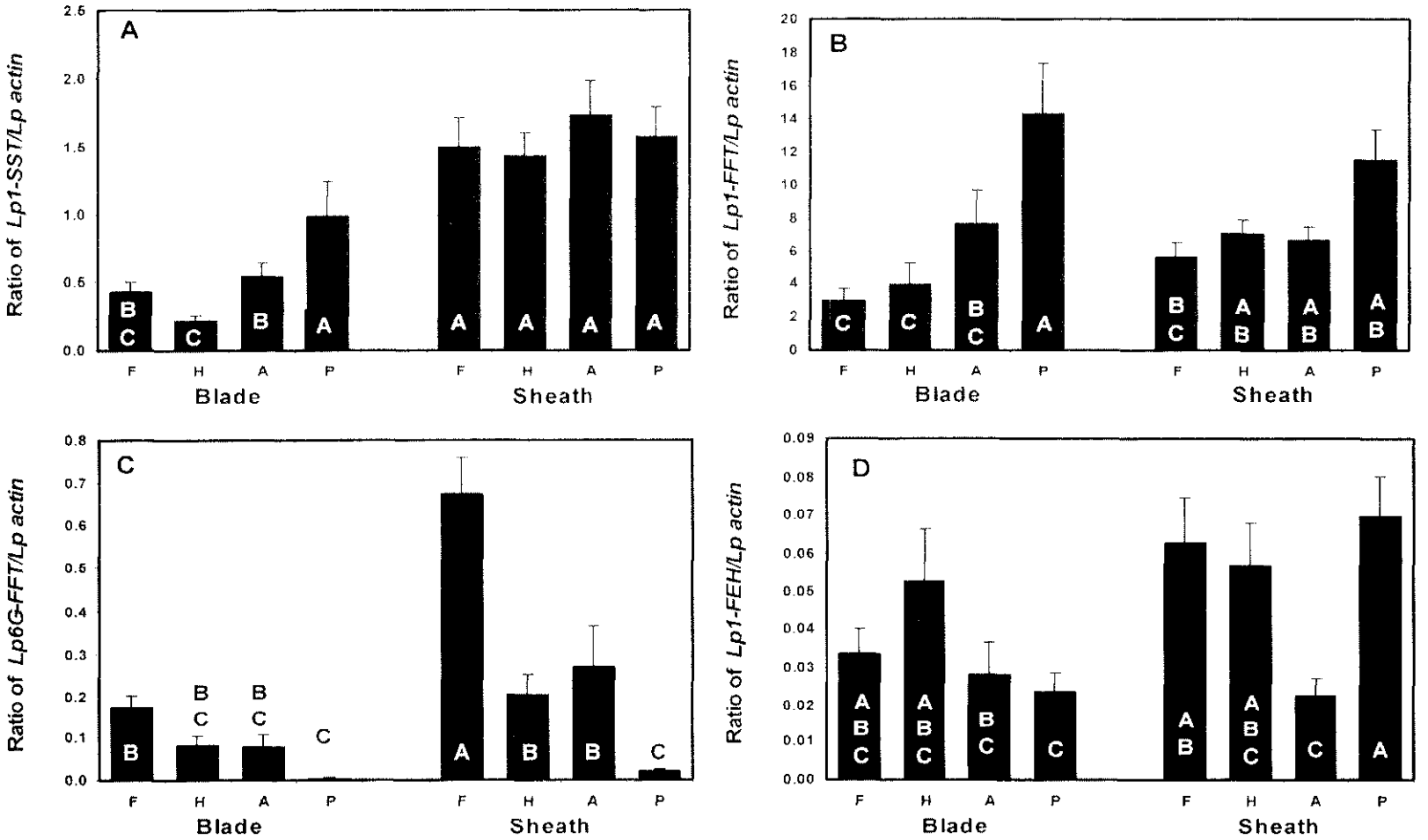


Fig. 3.7 Interactions of cultivar and tissue on the expression of *Lp1-SST* (A), *Lp1-FFT* (B), *Lp6G-FFT* (C) and *Lp1-FEH* (D). Different letters denote significant differences as determined by Tukey's Honestly Significant Different (HSD) test.

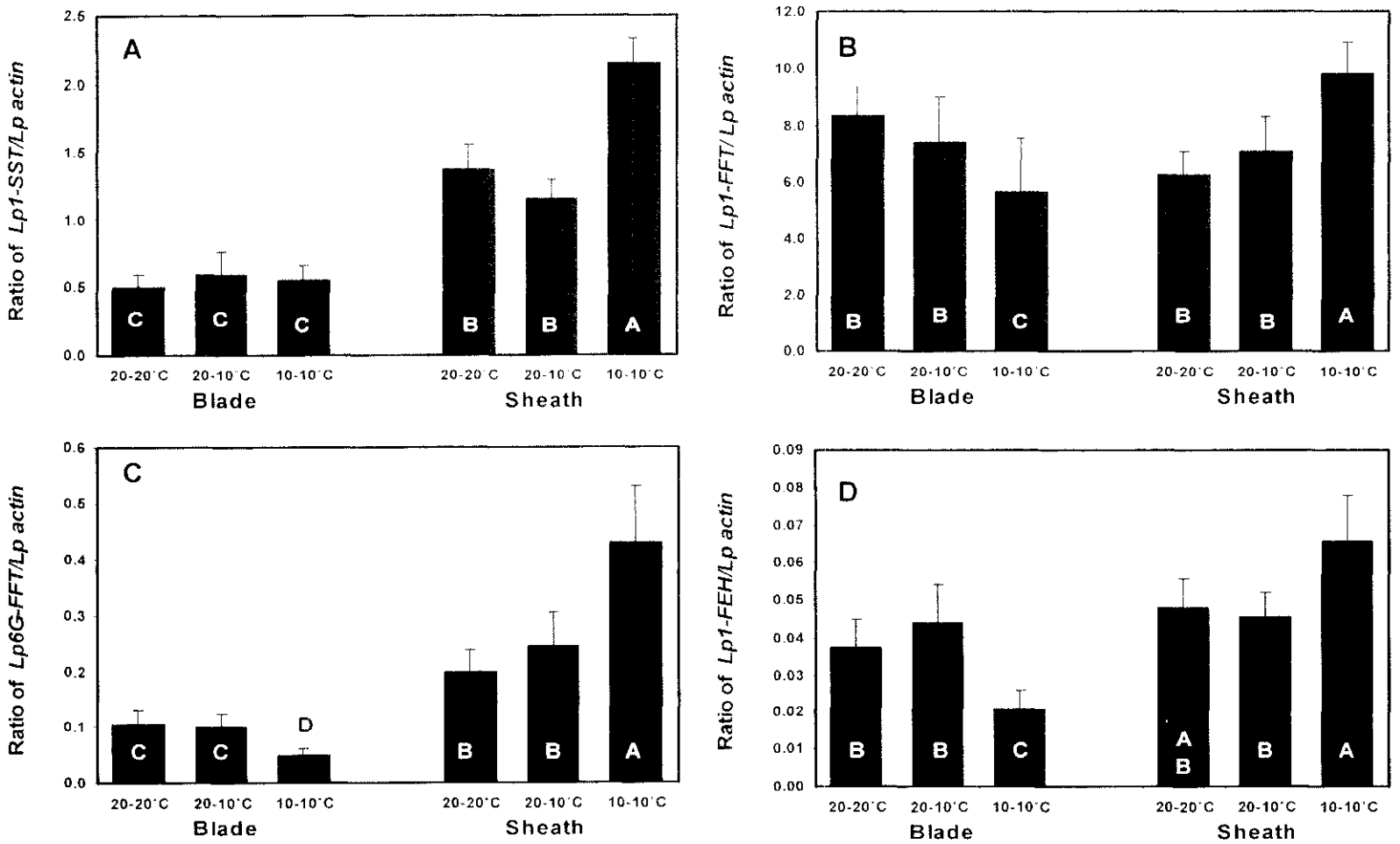


Fig. 3.8 Temperature by tissue interactions on the expression of *Lp1-SST* (A), *Lp1-FFT* (B), *Lp6G-FFT* (C), and *Lp1-FEH* (D). Different letters denote significant differences as determined by Tukey's Honestly Significant Different (HSD) test.

3.2.2.2. Cultivar by tissue interactions

Expression levels of *Lp1-SST* were significantly (P<0.0001) higher in sheaths compared to blades. There was no significant difference between the cultivars in sheath tissue (Fig. 3.7A); but in blades, *Lp1-SST* (P<0.0001) and *Lp1-FFT* (P=0.0212; Fig. 3.7B) transcript levels were significantly higher in cultivar P compared to the other cultivars, consistent with higher concentrations of HMW WSCs in this cultivar (see Fig. 3.1A).

In sheath tissue, expression of 6G-FFT was significantly (P=0.0009) higher in the control cultivar F and lowest in P (Fig. 3.7C). Expression was much lower in F blades compared to F sheaths, but there was no significant difference between the tissues of the other three cultivars.

1-FEH was significantly (P=0.0187) more expressed in the sheaths of cultivar P compared to blades, but the other cultivars did not show a tissue specific difference (Fig. 3.7D). In general there was no significant difference of 1-FEH expression between the cultivars, except that it was significantly less expressed in sheath tissue of A compared to sheath tissue of the other cultivars.

3.2.2.3. Tissue by temperature interactions

Gene expression levels were significantly affected by tissue x temperature interactions. Expression of *Lp1-SST*, *Lp1-FFT*, *Lp6G-FFT* and *Lp1-FEH* was significantly (P<0.0001) higher at 10°C/10°C, but only in sheaths (Figs. 3.8A to D). Expression of *Lp1-SST* in the blades was not affected by temperature; and it was decreased at 10°C/10°C in the case of *Lp1-FFT*, *Lp6G-FFT*, and *Lp1-FEH*.

3.3. Protein expression profiling

The protein expression levels of the fructan pathway specific enzymes 1-SST (and/or 6G-FFT), 1-FFT and 1-FEH were determined by Western blot analysis using anti1-SST (and/ or 6G-FFT), anti1-FFT and anti1-FEH polyclonal antibodies, respectively.

3.3.1. Antibody generation

3.3.1.1. Expression of recombinant proteins

The prokaryotic vector pET 22(b) was used for the expression of *L. perenne* proteins involved in the fructan biosynthetic pathway. Full length *Lp1-SST*, *Lp1-FFT*, and partial *Lp1-FEH* cDNAs were amplified with primers that introduced *NcoI* restriction sites at the 5'end and *XhoI* at the 3' end, respectively. Use of these primers in PCR yielded a product of the predicted size for each gene, about 2 kb for both *Lp1-SST* and *Lp1-FFT* and 600bp for *Lp1-FEH*. These fragments were cloned in-frame with the 6×His-tag sequence into the expression vector pET 22(b), which was transformed into *E. coli BL21(DE3)* cells. To confirm the identity of the cloned genes and to verify that no mutation was introduced by the PCR step, the plasmids were digested with *NcoI* and *XhoI* restriction endonucleases and the fragments sequenced. Sequence alignments showed that the cloned *Lp1-SST* and *Lp1-FEH* had 100% identity to the sequences published in the NCBI data base (Accession#: AY245431 and DQ073968). Unexpectedly, the cloned *Lp1-FFT* showed a three base difference to the NCBI sequence; however, its deducted amino acid (AA) sequence was the same as the AA sequence in the NCBI data base (Accession#: BAD35132.1).

For the expression of recombinant proteins, cultures were induced with 0.5 mM IPTG for different time periods (1–18 h) at 37 °C. Among tested recombinant proteins, 1-FFT was successfully expressed in *E. coli BL21(DE3)* cells, and high protein levels were already present after 1 h induction by IPTG in LB cultures. The 1-FFT fusion protein was located entirely in insoluble inclusion bodies (Fig. 3.9). The molecular mass of the expressed protein in the bacterial lysis extract (Fig. 3.9 A, lane 1 h) was approximately 68 kDa, which corresponds to the expected size.

The expression of full-length 1-SST and partial 1-FEH in *E. coli BL21(DE3)* cells was not as successful as 1-FFT, although various attempts had been conducted to improve the level of expression, such as changing IPTG concentrations, modifying the medium and culture temperatures (25, 30, 37 °C) and lengthening the incubation time. As a consequence, the 5'end sequences of 1-SST and 1-FEH were removed, as the high GC content in their 5' end nucleotide sequence might have reduced expression in *E. coli*. The truncated proteins of the above genes were then expressed under the same conditions that were chosen for 1-FFT. The expression of both truncated 1-SST and 1-FEH recombinant proteins was strongly induced by IPTG in overnight cultures; proteins were also targeted to the insoluble inclusion bodies (Fig. 3.9 BandC).

Truncated 1-SST was about 52 kDa, and 1-FEH 22 kDa, corresponding to the expected sizes.

3.3.1.2. Purification of the recombinant proteins

The insoluble inclusion bodies of 6× His-tagged proteins were solubilised with 6 M urea and incubated with 2 ml Ni–NTA affinity resin under denaturing condition, which efficiently retained the recombinant protein. After washing of the column, the recombinant proteins were eluted with 250 mM imidazole. For each recombinant protein, the eluted samples presented a main single band in the SDS–PAGE, corresponding to the purified recombinant proteins (Fig. 3.10 A to C).

Because the purity of the antigen is critical to generate high quality antibodies, the Ni^{2+} purified proteins were further purified by whole gel elution. The analysis of the fractions containing the target protein on SDS-PAGE revealed a distinct single band for each recombinant protein (Fig. 3.10A, B&C lane wge), showing that the target proteins were of high purity. These purification procedures were highly efficient in producing large amounts of pure recombinant proteins and about 11 mg of 1-SST, 8 mg of 1-FFT and 15 mg of 1-FEH recombinant proteins were obtained from 1 L of culture, respectively.

The comparison of the peptide mass fingerprint of trypsin-digested purified recombinant proteins by MALDI–TOF-MS with protein sequences deposited at the NCBI database demonstrated that the top match of recombinant protein of 1-SST was sucrose: sucrose 1-fructosyltransferase from *L. perenne* (Accession#: AAO86693) with a 'match-score' of 245 and 'expect' of 6.4e-19, and matched peptides within the protein with a coverage of 54% (Fig. 3.11.A); 1-FFT had highest homology with a putative fructosyltransferase1 from *L. perenne* (Accession#: BAD35132) with a 'match score' of 238 and 'expect' 3.2e-18, and the matched peptides within the protein with a coverage of 54% (Fig. 3.11.B); and 1-FEH had highest homology with a putative fructan 1-exohydrolase (Accession#: AAU14219) with a 'match score' of 114 and 'expect' 7.7 e-07, and matched peptides within the protein with a coverage of 54% (Fig. 3.11.C). The results obtained were in very good agreement with the proteins expected to be expressed and confirmed the identity of these three recombinant fructan biosynthesizing proteins expressed in *E. coli*.

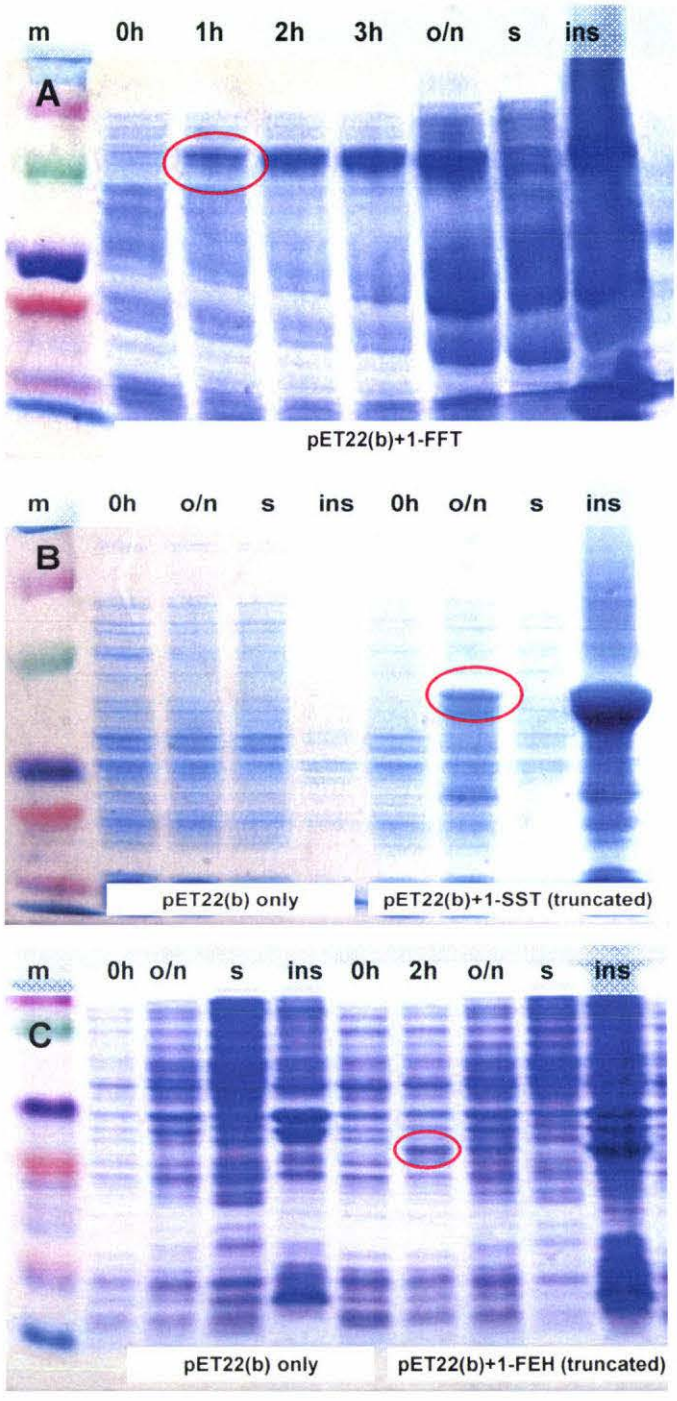


Fig. 3.9 SDS–PAGE analysis of recombinant proteins of *L. perenne* 1-SST, 1-FFT, and 1-FEH proteins in *E. coli*. After addition of IPTG, cells were grown at 37 °C overnight. m, molecular marker (Bio-RAD); pET 22(b) only: pET 22(b) expression vector only (control); pET 22(b)+1-SST, or 1-FFT, or 1-FEH: recombinant expression vector pET 22(b) with target genes; 0h: before IPTG induction; 1 h - o/n: after IPTG induction from 1 h to overnight; s: soluble fraction; ins: insoluble fraction.

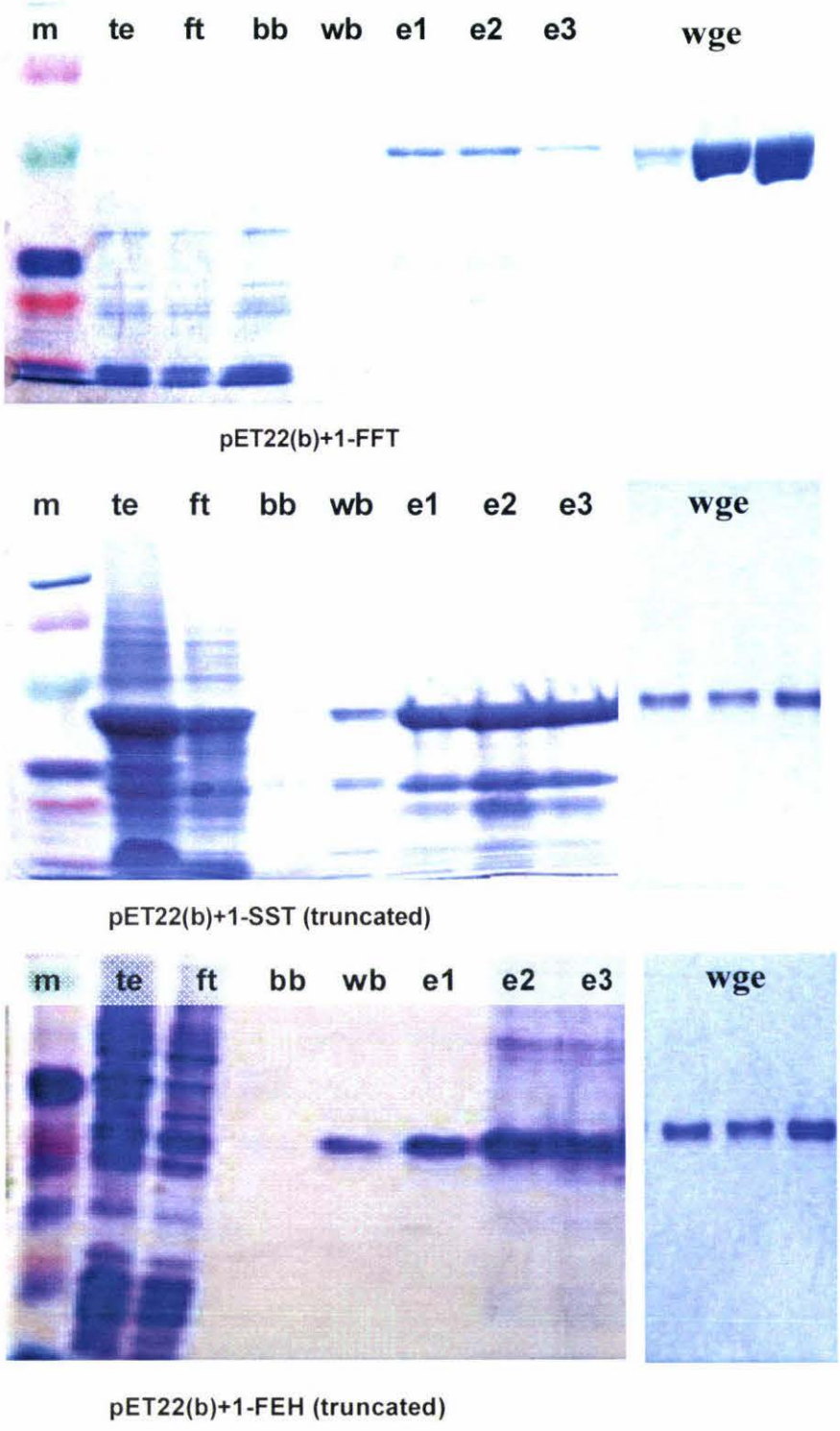
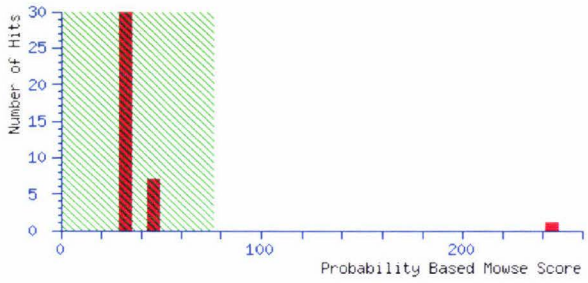


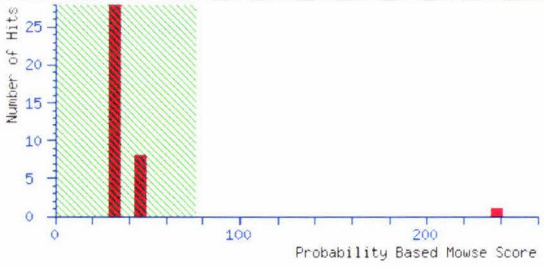
Fig. 3.10 SDS–PAGE analysis of purified recombinant proteins from *E.coli.* m, molecular marker (Bio-RAD); te: total *E. coli* cytoplasmic extract; ft: the column flow-through sample; bb: the column binding buffer sample; wb: the column wash buffer sample; e1–e3: the first three elution aliquots from the Ni–NTA column; wge: the samples after whole gel elution.

A



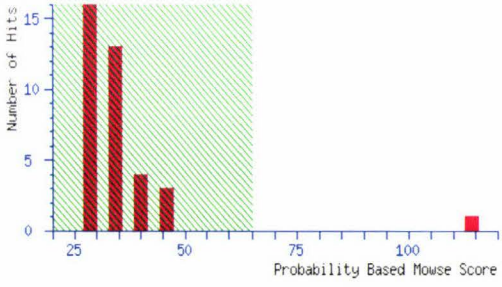
1SSTCUT(54%)MVPDQWYDSNGVLTGSITVLPDGQVILLYTGNTDTLAQVQCLATPADPSDPLLREWVK**HPANPILYPPPGIGLKD**
FRDPLTAWFDHSDHTWRTVIGSKDDDGHAGIILSYKTKDFVNYELMPGNMHRGPDGTGMYECIDLYPVGGNSSEMLGGDDSPGVLFV
LKESSDDER**HDYYALGRFDAVANVWTPIDRELDLGIGLR**YDWGKYYASK**SFYDQK**KNR**RIVWAYIGETDSEQADITKGWANLMTIPR**
TVELDRKTR**TNLIQWPVEEVDTLRR**NSTDLGR**ITVNAGSVIR**LPLHQGAQLDIEASFQLNSSDVDAINEADVGYNCSTSGAAVR**GAL**
GPFGLLVLANGRTEQTAVYFYVSKGVDGGLQTHFCHDESRSTRAKDVVNR**MIGSIVPVLDGETFSVRVLVDHSIVQSFAMGGR**ITAT
SR**AYPTEAIYAAAGVYLFNNATGATVTAER**LVVHEMASADNHIFTNDDLL

B



1FFT(54%)MESR**AIPSAAYAPLLPSAADDVALAKQDRPGVGWR**GFLTVLAACGVVVLLVGATLLAGSRMGQAGDGEGNTDEDGAGG
FPWSNEMLQWQR**AGFHYQPEGHFMSDPNGPVYYRGYYHLFFQYNR**RGVAWDDYIEWGHVVSQDLVHWRPLPLAMRPDHWYDKKGVLS
GTITVLHNGTLVLLYTGVTEDPMAESQCIAVPTDPNDPLLRHWTK**HPANPVLAHPQGVQGMDFRDPTSAWWDKSDSTWRILIGSKDD**
DNGSHAGIAFIFKTKDFLSFERVPGIVHRVEGTGMWECIDFYPVGGGHNSSSEELYVIK**ASMDDERHDYYSLGRYDAAANTWTPLDA**
ELDLGIGLRYDWGK**LYASTSFYDPLK**QRR**IMLGYVGEVDSAR**ADVAK**GWASLQSIPR**TVALDEKTR**TNLLLWPVEEVEALR**YNSTDL
SGITVENGSIFHLPLHQATQLDIEASFRLDASDVAAINEADVGYNCSSSGGAAAR**GALGPFGLLVHAAGDLRGEQTAVYFYVSRALD**
GSLRTSFCNDETRSSRARDVTKR**VVGSTVPVLDGEALSMRVLVDHSIVQSFAMGGRV**TATSR**VYPTEAIYARAGVYLFNNATGASVT**
AERLIVHEMASAVYDETVMVKDS

C



NHGTNESDSSSDDVAKGWAGIYGIPR**TIWLDRDGKQLLQWPVEEIESLRRNEINYQGLELEKGDLYEIK**GVDTLQADVEIDFELTSI
DDAESFDPSWLLDPQKHCREAGASVHGGIGPFGLVILASSDMEEHTVVHFRVYKSQKEYMVLMCSDIRRSSL**RQGLYTPAYGGFFEF**
DLEKERKISLRTLIDRSAVE

Fig. 3.11 Mass spectrometry analysis of recombinant 1-SST, 1-FFT and 1-FEH proteins. Probability based Mowse score and the peptides detected by the peptide fragment mass fingerprint. Matched peptides shown in Bold Red. A: recombinant 1-SST; B: recombinant 1-FFT; C: recombinant 1-FEH.

3.3.2. Determination of antibody quality

Rabbits were immunised with purified 1-SST, 1-FFT and 1-FEH recombinant proteins, respectively. Bloods were collected 10 days after the third immunisation and analysed for titre and sensitivity by immunoblotting.

The titre test was carried out by immunoblotting using certain amounts of antigens with different concentrations of antisera. Serial dilutions of antisera (1:200, 1:400, 1:800, 1:1,600, 1:3,200, and 1:6,400) in TBST buffer were incubated with 100 ng antigen.

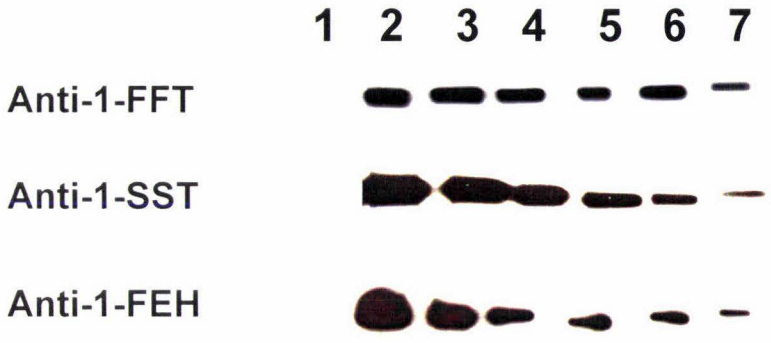


Fig. 3.12 Determination of titres of the three antibodies by detecting 100 ng antigen with different concentrations of antisera. Lane assignments are: 1 - 1:200 dilution pr-bleed; 2 - 1:200 dilution antisera; 3 - 1:400 dilution antisera; 4 - 1:800 dilution antisera; 5 - 1:1,600 dilution antisera; 6 - 1:3,200 dilution antisera; 7 - 1:6,400 dilution antisera.

The immunoblot results showed that both anti-1-FFT and anti-1-FEH serum strongly reacted with their corresponding antigens and could be diluted down to a titre of 1:6,400, each giving a single prominent band. In comparison, the prebleed sera did not result in any bands even at a titre of 1:200 (Fig. 3.12), clearly demonstrating that the bands observed with the antisera were due to the specific interaction of the antibody with 1-FFT and 1-FEH proteins. The anti-1-SST serum obtained was of poor quality and only detected 100 ng antigen at a dilution of

1:800 (data not shown). Hence, a second rabbit was immunised with purified 1-SST protein. The titre test results revealed that, like anti-1-FFT and anti-1-FEH serum, anti-1-SST detected 100 ng antigen at a dilution of 1:6,400, while its prebleed serum did not detect any antigen even at a titre of 1:200 (Fig.3.12).

The sensitivity of these three antibodies was tested by using1:1,000 dilution antisera with different amounts of antigens. Serial dilutions of antigens, from 100 ng, 50, 25, 12.5 and 6.25 ng were electrophoresed by SDS-PAGE, followed by immunoblotting with 1:1,000 diluted antisera to detect minimal detection amounts. Both anti-1-SST and anti-1-FEH were able to detect 12.5 ng of their respective antigens, whereas anti-1-FFT was able to detect 6.25 ng of 1-FFT antigen, indicating that these three antibodies are highly sensitive to their corresponding antigen (Fig. 3.13).

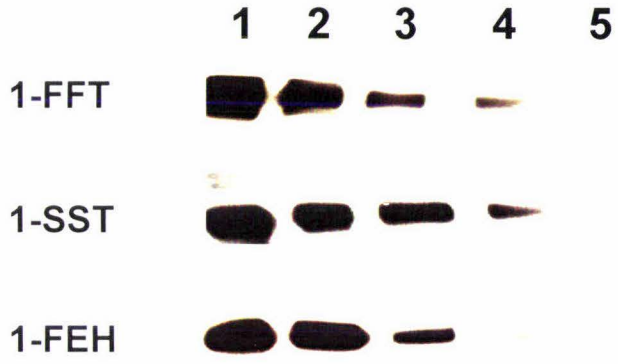


Fig. 3.13 Sensitivity determination of the three antibodies with 1:1,000 diluted antisera to different amounts of antigens. Lane assignments are as follows: 1: 100ng antigen; 2: 50ng antigen; 3: 25ng antigen; 4: 12.5ng antigen; 6: 6.25ng antigen.

3.3.3 Analysis of 1-SST, 1-FFT and 1-FEH protein levels

Western blot analysis was used to semi-quantify the amounts of 1-SST, 1-FFT, and 1-FEH proteins in crude plant extracts using anti-1-SST, anti-1-FFT and anti-1-FEH polyclonal antibodies generated in this study. Based on amino acid sequence information, *Lp1-SST* encodes a 653 amino acid polypeptide and has a molecular mass

of 61 kDa (Chalmers et al., 2003). *Lp1-FFT* encodes a 623 amino acid polypeptide with a molecular mass of 62.5 kDa (Chalmers et al., 2003); and *LpFEH* encodes a 570 amino acid polypeptide with a molecular mass of 62 kDa (Chalmers et al., 2003).

The molecular mass of the protein detected by anti-1-SST antibody in the blades and sheaths (Fig.3.14) was approximately 65 kDa, which corresponds to the expected size of this protein. However, Lp1-SST and Lp6G-FFT proteins share 81% amino acid sequence identity, making it highly likely that the polyclonal antibodies used in this study cross-react with both proteins.

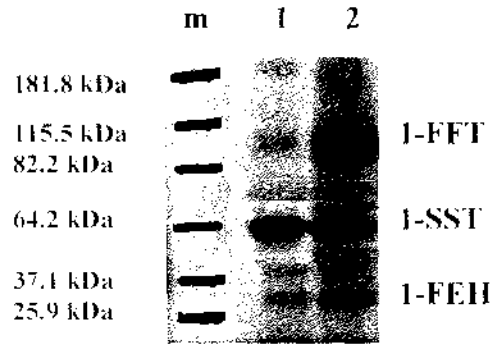


Fig. 3.14 Western blot analysis of Lp1-SST, 1-FFT and 1-FEH using polyclonal antibodies. m: molecular marker (Invitrogen). 1: Sheaths. 2: Blades.

The protein detected by anti-1-FFT antibody was approximately 110 kDa (Fig.3.14), indicating dimerisation of the native protein under the conditions used in this study. Attempts to purify the protein reacting with anti-1-FFT antibodies using immunoprecipitation were unsuccessful and further studies are needed to confirm the identity of the detected protein with 1-FFT.

It has been reported that monocotyledon 1-FEH is a homodimer, and has a molecular mass of about 33 kDa under denaturing conditions (Henson and Livingston 1996). Thus, the two bands detected by 1-FEH antibody may be: (i) two different isoforms of

1-FEH, (ii) the upper band might be a glycosylated form 1-FEH (Chalmers et al., 2003).

1-SST protein seemed to accumulate more in sheaths compared to blades, whereas 1-FFT protein was more abundant in blades. 1-FEH was slightly more abundant in blades, but in general its levels were much lower compared to 1-SST and 1-FFT (Fig.3.14).

Figures 3.15 and 3.16 show a complete data set on WSC concentrations, gene transcript levels and protein levels in one representative genotype of each of the four cultivars studied here at the three different temperature regimes in blades (Fig. 3.15) and sheaths (Fig. 3.16). As can be seen, 1-SST protein levels were increased at low temperatures, both in blades and sheaths. In blades this effect was strongest in the HSG cultivars A and P, whereas in sheaths all four cultivars responded to low temperatures in a comparable manner. Protein levels of 1-FFT were mostly unaffected by low temperatures, and the HSG cultivar P had the lowest levels of 1-FFT protein compared to the other cultivars.

In the control cultivar F both protein bands for 1-FEH were approximately of the same intensity, whereas cultivars H and A accumulated more of the smaller protein. The HSG cultivar P, on the other hand, accumulated more of the larger sized protein, especially in sheaths. 1-FEH protein levels only responded weakly to low temperatures, especially in sheaths of A and P.

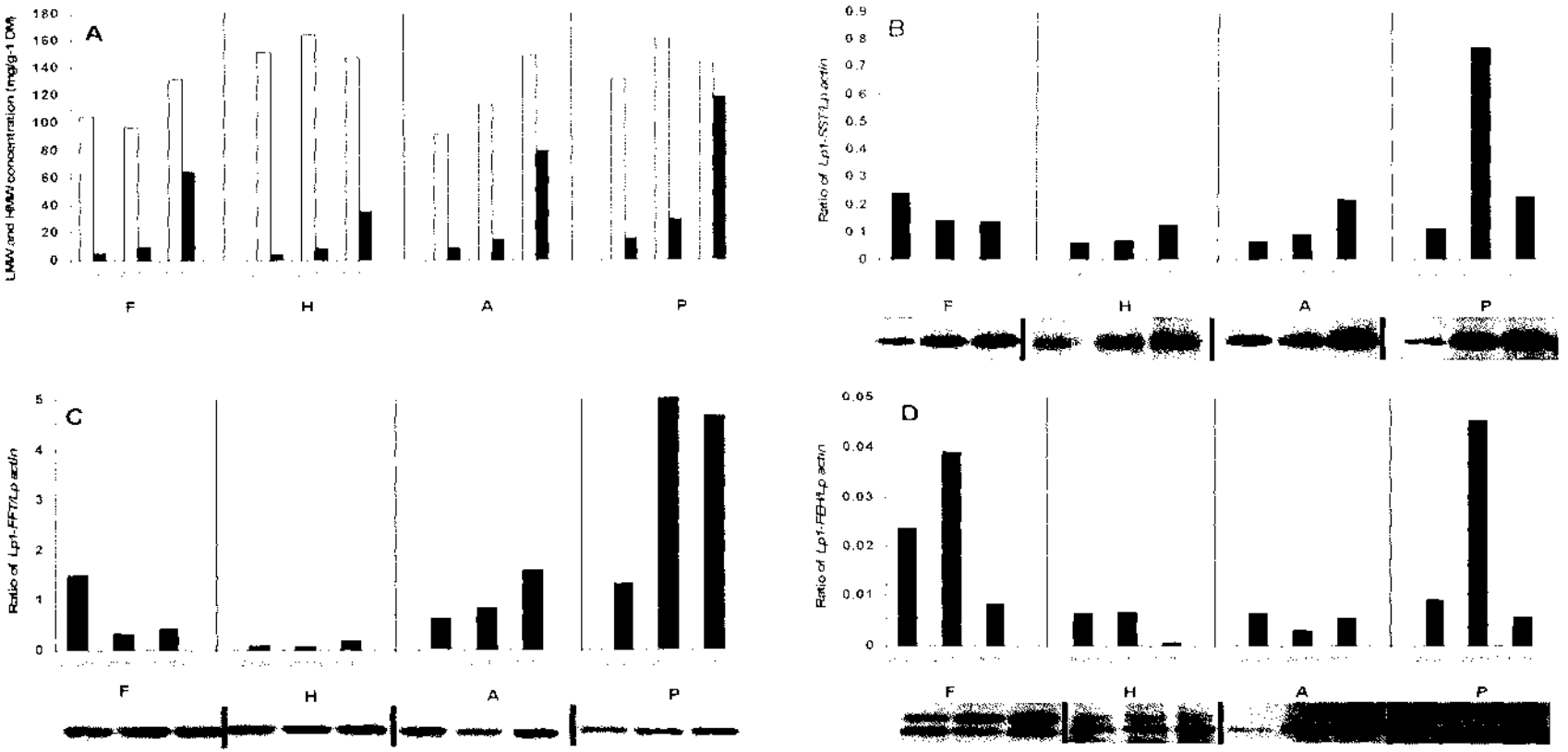


Fig. 3.15 WSC levels, gene transcript levels (qRT-PCR), and Western blot analysis of Lp1-SST, 1-FFT and 1-FEH proteins in blades using polyclonal anti-1-SST, anti-1-FFT, and anti-1-FEH antibodies, respectively. A: LMW (white bars) and HMW (black bars) concentrations in blades. B: Transcript profiles and protein levels of 1-SST. C: Transcript profiles and protein levels of 1-FFT. D: Transcript profiles and protein levels of 1-FEH.

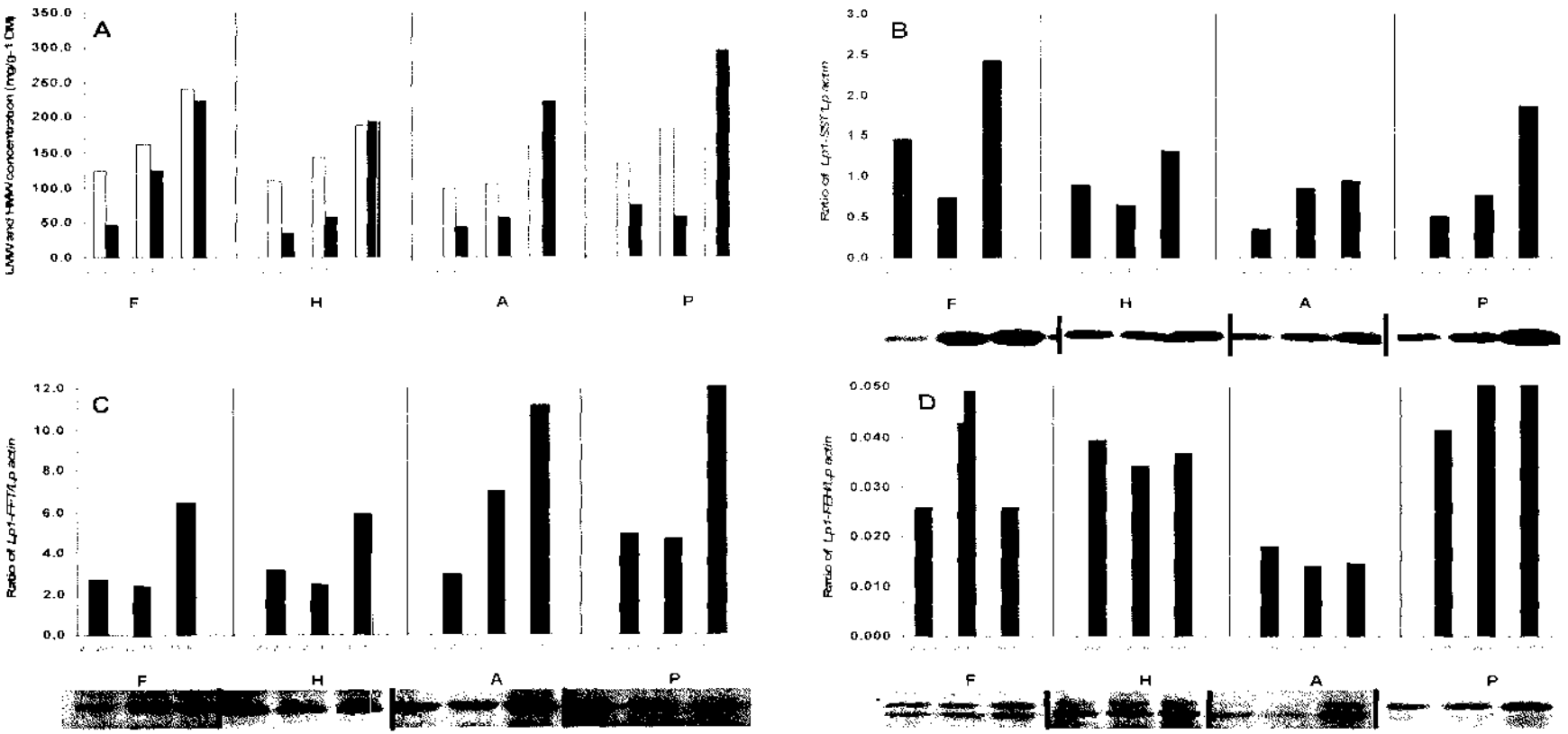


Fig. 3.16 WSC levels, gene transcript levels (qRT-PCR), and Western blot analysis of Lp1-SST, 1-FFT and 1-FEH proteins in sheaths using polyclonal anti-1-SST, anti-1-FFT, and anti-1-FEH antibodies, respectively. A: LMW (white bars) and HMW (black bars) concentrations in blades. B: Transcript profiles and protein levels of 1-SST. C: Transcript profiles and protein levels of 1-FFT. D: Transcript profiles and protein levels of 1-FEH.

CHAPTER FOUR

Discussion and Conclusion

There is considerable and growing interest in the development of perennial ryegrass cultivars accumulating high levels of water soluble carbohydrates (WSC's) in the blades, the grazed component of forage grasses (Humphreys 1989 a, b, c; Turner et al., 2006). High WSC levels in forages have been associated with increased animal performance and environmental benefits due to reduced N losses from ruminants (Lee et al., 2001; Miller et al., 2001b).

WSC's can be separated into two major components - a low molecular weight (LMW) fraction, consisting mainly of glucose, fructose and sucrose, and a high molecular weight (HMW) fraction, consisting of fructans with variable chain lengths. The LMW fraction undergoes mainly diurnal changes with increasing levels during the day, but is in general relatively resilient to long-term changes in the environment. The HMW fraction (fructans) on the other hand is very strongly affected by environmental and other growth conditions (Pollock and Jones, 1979; Pollock, 1986). The difference in WSC levels between high sugar grass and other cultivars was found to be mainly due to the difference in fructans in the blades, rendering this trait especially prone to environmental and developmental regulation (Parsons et al., 2004; Edwards et al., 2007a, b).

Detailed studies on differences in sugar accumulation and their relation to gene and protein expression of fructan biosynthetic enzymes in high sugar grass cultivars are still lacking and this study attempts to provide further insights into the regulation of this important trait in perennial ryegrass.

4.1 Tissue effects

The plant material analysed in this study was separated into blades (above the ligule) and sheaths (below the ligule). It should be noted here that "sheath" actually comprises

two different major tissues - true sheath material and elongating, immature blades located in the inside of the sheaths in a ratio of approx. 5: 1. Due to experimental constraints, mainly to keep the harvest period as short as possible to avoid large diurnal variations of sugar content and gene expression, we did not separate these two tissues. Because of the much higher abundance of true sheaths in this mixture, we assume that observed sugar, transcript, and protein levels are mainly representative for processes in the sheaths.

As expected, sheaths contained significantly higher concentrations of HMW WSCs (fructans) than did leaf blades (Fig. 3.1A and B). Sheaths have been described metabolically as a reservoir of non-structural carbohydrates in grasses, where fructans accumulate when photosynthetic carbon supply exceeds demand for growth and development (Housley and Volenec, 1988; Pavis et al., 2000a). Mature blades, on the other hand, usually contain relatively low levels of fructans, which only accumulate in this tissue when storage capacities of sheaths and growth zones have been exhausted (Guerrand et al., 1996). Fructans in mature blades, the photosynthetically active tissue, may also function as a control mechanism to keep sucrose concentrations low and thereby preventing sugar-induced feed-back inhibition of photosynthesis (Wagner et al., 1983; Pollock, 1986; Hendry and Wallace, 1993). Fructans in blades can also serve as a short-term carbohydrate storage (Allard and Nelson, 1991). In blades, LMW WSC concentrations were much higher compared to HMW WSCs (Fig. 3.2), consistent with their functioning as photosynthetically active organs.

All four genes analysed for transcript levels (*Lp1-SST*, *Lp1-FFT*, *Lp6G-FFT*, and *Lp1-FEH*) were highly expressed in sheaths (Fig. 3.6), consistent with high levels of fructan accumulation in this tissue. This is in agreement with previous studies on *L. perenne* (Chalmers et al., 2003; Lasseur et al., 2006; Lothier et al., 2007) and a variety of other plant species including *Taraxacum officinale* (Van den Ende et al., 2000), *Hordeum vulgare* (Nagaraj et al., 2004), *Allium cepa* (Vijn et al., 1997; 1998), *Festuca arundinacea* (Lüscher et al., 2000), *Agropyron cristatum* (Wei and Chatterton, 2001), *Poa secunda* (Wei et al., 2002), and *Triticum aestivum* (Kawakami and Yoshida, 2002; Kawakami et al., 2005). The higher transcript levels of *Lp1-FEH*, an enzyme generally

described as being involved in fructan degradation, in sheaths compared to blades, indicates that 1-FEH might also be involved in fructan biosynthesis together with other fructan biosynthetic gene products. The co-expression of fructan biosynthetic and hydrolysing genes reported here is in agreement with recent studies in wheat (Van den Ende et al., 2003a) and *L. perenne* (Lothier et al., 2007). These authors reported that 1-FEH transcript levels and activity followed the same pattern as 1-SST transcript levels and activity under conditions of fructan accumulation. It was therefore suggested that, besides their main roles during fructan mobilisation, FEHs might also be involved in ß-(2,1) trimming during fructan biosynthesis in concert with fructan synthesising enzymes and possibly play an important role in determining the *in vivo* fructan patterns (Bancal *et al.*, 1992; Van den Ende et al., 2003a; Lothier et al., 2007).

Western blot analysis revealed that only 1-SST protein accumulated to high levels in sheaths, whereas protein levels of 1-FFT and 1-FEH were higher in blades. It must be noted here that 1-SST and 6G-FFT share a high degree of homology on the amino acid level (81 %), and both proteins are approx. of the same size; it is therefore possible that the antibodies raised against 1-SST also cross-react with 6G-FFT protein. We can not explain the low levels of 1-FFT and 1-FEH proteins in sheaths, compared to blades, as this is not consistent with the observed fructan accumulation and transcript levels. As transcript and protein levels are not necessarily an indication of biosynthetic activity, future studies should include the determination of enzyme activities as well.

4.2 Cultivar by tissue interactions

Fructan levels were significantly higher in the HSG cultivar "P", but there was no significant difference in "A" and "H" compared to the control grass. This result is a further indication that currently existing "High Sugar" cultivars do not express the trait of high fructan accumulation consistently, as was previously shown (Parsons et al., 2004; Edwards et al., 2007a, b). Levels of LMW WSCs did not differ between the cultivars, indicating that these cultivars have similar rates of sugar synthesis, but different carbohydrate storage capacities.

Expression profiling revealed that *Lp1-SST* was more highly expressed in sheaths compared to blades, but its expression level did not differ between the cultivars in any of the tissues. In contrast, *Lp1-FFT* was significantly more expressed in two of the HSG cultivars ("P" and "A", Fig. 3.7B), but only in blades. 1-FFT has been proposed to be an elongating enzyme leading to the production of long chain fructans of the inulin series and inulin neoseries fructans in ryegrass and other plants (Edelman and Jefford, 1968; Koops and Jonker, 1996; Lüscher et al., 1996; Van den Ende and Van Laere, 1996) It must be noted here that the annotation of the published sequence of 1-FFT is based on sequence homology only; no functional characterisation studies have been published so far. Recently, the role of 1-FFT in *L. perenne* was questioned based on a study with recombinant proteins, which demonstrated that *L. perenne* 6G-FFT was bi-functional and could also display 1-FFT activity (Lasseur et al., 2006). It was suggested, that the putative 1-FFT is actually an allelic variant of 6G-FFT in different *L. perenne* varieties. However, sequence homology between *Lp1-FFT* and *Lp6G-FFT* is much lower (60%) compared to e.g. *Lp6G-FFT* and *Lp1-SST*, which share 81% nucleotide sequence identity. Protein sequence identity between *Lp1-SST* and *Lp6G-FFT* is 82%, but only 65% between *Lp1-FFT* and *Lp6G-FFT*. Functional characterisation of both, *Lp1-SST* and *Lp6G-FFT*, has shown that they are unique genes with different catalytic activities (Chalmers et al., 2003; Lasseur et al., 2006). Thus, it is doubtful that the less homologous gene *Lp1-FFT* is an allelic variant of *Lp6G-FFT* in the *L. perenne* genome as was proposed.

On the other hand, the highest sequence identity of the putative *Lp1-FFT* gene was found to the functionally characterised fructosyltransferase 6-SFT from wheat (NCBI accession No. AB029887; Kawakami and Yoshida, 2002). Kawakami and Yoshida (2002) reported that levels of 6-SFT transcript in winter wheat during cold hardening increased concomitantly with fructan content. Additionally, transgenic perennial ryegrass plants that overexpress Ta*6-SFT* under the control of CaMV 35S promoter accumulated higher levels of fructans in plants, which also displayed increased freezing tolerance compared to non-transgenic plants (Hisano et al., 2004a). In *Lolium* species, the existence of 6-SFT is debated, as bifurcose, the first product of 6-SFT activity, is absent (Pavis et al., 2001b). It was therefore proposed that, in the absence of 6-SFT, 6-SST- or 6-FFT-like enzymes might be responsible for the (ß2-6) linkages in

the branched levan neoseries, the predominant HMW fructans in *Lolium* species (Guerrand et al., 1996; Pavis et al., 2001a). However, so far these kinds of enzymes have never been identified or characterised in *Lolium* species. The higher expression level of *L.p1-FFT* compared to other fructosyltransferase genes in the blades of the HSG cultivars "P" and "A" suggests that this *Lp1-FFT* (AB188920) may have a prominent role in fructan biosyntheses in blades. Therefore, identification and characterisation of *Lp1-FFT* is required for future research.

Pavis et al. (2001b) reported that levan neoseries fructans are the major type of HMW fructans, comprising up to 76% of total high molecular weight fructans (DP > 8) in *L. perenne*. 6G-FFT catalyses the initiation of the 6G-linked inulin and levan neoseries fructans using 1-kestose as substrate, and it is thought to be the key enzyme in grass fructan biosynthesis (Lasseur et al., 2006). Unexpectedly, 6G-FFT transcript levels were the highest in the control cultivar F, especially in sheaths, and the lowest in cultivar P, which accumulated the highest levels of fructans (Fig. 3.7C). This might indicate that either 6G-FFT is not critical for fructan accumulation in high sugar cultivars, or the presence of a second allele of 6G-FFT, which is highly expressed in HSG cultivars, but was not amplified with primers used in this study. Full-length sequencing of 6G-FFT and any allelic variants of it will be critical to resolve this question.

4.3 Temperature by tissue interactions

It has been reported previously that low temperatures lead to strong elevation of WSC concentrations in blades and sheaths of temperate grasses (Bancal and Gaudillère 1989; Chatterton et al., 1989; Pollock, 1984). In the present study, both blades and sheaths also showed increased levels of HMW and LMW WSCs in plants grown at lower temperatures (Fig. 3.3A and B). The increased HMW and LMW WSC concentrations in blades and sheaths can be explained by the fact that photosynthesis and fructan synthesis are less sensitive to low temperatures than growth rate and carbohydrate utilisation (Levitt, 1980; Wagner et al., 1983; Thorsteinsson and Tillberg, 1990).

In sheaths, transcript levels of *Lp1-SST*, *Lp1-FFT*, and *Lp6G-FFT* increased with increases in fructans as a response to low temperatures. However, in blades the cold treatment had no effect on the expression of *Lp1-SST* and, surprisingly, down-regulated *Lp1-FFT*, *Lp6G-FFT* and *Lp1-FEH* transcripts (Fig. 3.8), which is inconsistent with increased levels of WSCs in this tissue. This result might indicate that fructan biosynthesis and accumulation in blade tissue is not regulated on the transcript level, but rather on the protein and/ or enzyme activity level. A study on gene expression and enzyme activity in perennial ryegrass subjected to root and leaf base cooling and simultaneous exposure of blades to continuous light revealed that *Lp6G-FFT* transcript was highly expressed in blades under these conditions, but only very low extractable fructosyltransferase activities and fructan amounts were detected (Lasseur et al., 2006). Similarly, *Lp1-SST* transcript accumulated in continuously illuminated leaf blades, but 1-SST activity remained at a low level (Guerrand et al., 1996; Lasseur et al., 2006). It was also shown in barley, that although high levels of WSCs accumulated in cold-treated blades, expression of *Hv6-SFT* was down-regulated (Wei and Chatterton, 2001).

Protein levels as analysed by western blotting were not consistently affected by any of the treatments. In sheaths, 1-SST protein levels were generally increased in plants grown at low temperatures, consistent with fructan and *Lp1-SST* transcript profiles. In blades, this increase was only seen in the HSG cultivars P and A, which also had the highest level of fructans. However, 1-FFT protein was not affected by low temperatures in blades, and levels were lowest in P, inconsistent with transcript and fructan levels in this cultivar. In sheaths, 1-FFT protein was also largely unaffected by low temperatures and no consistent difference could be observed between the different cultivars. Analysis of 1-FEH protein levels is further complicated by the presence of two protein bands cross-reacting with anti-1-FEH antibodies. In F, the control cultivar, both bands were of similar intensity and neither showed a response to low temperatures. In H and A, the smaller protein band seemed to accumulate more than the larger band, and a slight increase of this protein was observed at low temperatures. In contrast, the larger protein was more highly expressed in P, and it showed a slight increase at low temperatures. We are not aware of any previous studies in *L. perenne* or other temperate grasses using antibodies against fructan biosynthetic proteins and

our results suggest that further studies of post-transcriptional and post-translational modifications of enzymes involved in fructan biosynthesis and accumulation in blades under various temperature regimes are needed.

4.4 Conclusions

In this study, we tested water soluble carbohydrates concentrations, the expression of the *L. perenne* fructan biosynthetic pathway key genes and proteins, 1-SST, 1-FFT, 6G-FFT and 1-FEH, in the blades and sheaths of three selected high sugar cultivars (P, A and H) and a common cultivar (F) grown at three different temperature regimes. Comparison of the fructan concentrations with gene and protein expression profiles strongly suggests that: (i) 1-FFT is very likely to play an important role for fructan accumulation in the blades of HSG cultivars, therefore, identification and characterisation of *Lp1-FFT* is required for future research; (ii) 1-SST is significantly regulated at transcriptional and translational levels by temperature effects; (iii) unexpectedly, 6G-FFT was more significantly expressed in the control cultivar F, but not in the high sugar cultivar P, further studies are needed to resolve this; (iv) 1-EFH might be involved in ß-(2,1) trimming during fructan biosynthesis in concert with fructan synthesising enzymes in addition to its main role during fructan mobilisation.

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