Fructan in temperate forage grasses; Agronomy, physiology, and molecular biology

Joseph A. Gallagher¹, Andrew J. Cairns² and Lesley B. Turner²
¹Plant, Animal and Microbiology Department; ²Plant Genetics and Breeding Department, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, UK

Abstract

Temperate forage grasses are extremely important in agriculture and their reserve carbohydrate metabolism has received much attention. The bulk of this reserve carbohydrate is comprised of fructan, a polymer of fructose, which accumulates when the products of photosynthesis exceed the demands for growth. These polymers are predominantly stored in the leaf base and sheath, the region of growth and cell expansion, and can also accumulate in leaf blades and roots. The molecular makeup of fructan is species
specific and patterns of accumulation show seasonal variation. Environmental conditions have a greater influence on fructan content than genetic diversity. Fructan reserves fuel regrowth following defoliation and evidence suggests a role in stress tolerance. In addition, increased fructan content can benefit ruminant production and lead to a subsequent reduction in nitrogen release to the environment.

A number of fructan biosynthetic genes have been isolated from ryegrass and it is postulated (based on known and predicted activities) that these can account for the full range of fructan molecules observed in this species. These genes are all up-regulated in the presence of sucrose, probably through a protein kinase/phosphatase cascade, and are controlled at both the transcriptional and post-transcriptional level. The location of the activity of the encoded enzymes has yet to be determined, however, there is strong evidence contradicting existing models which place sucrose, soluble acid invertase and fructosyltransferases in the vacuole. Although much is known about the expression of the individual genes involved in fructan synthesis, the genetic control of fructan metabolism is still elusive. Quantitative Trait Loci for the high water-soluble carbohydrate trait, map to different regions throughout the genome. From mapping studies with candidate genes it appears that this trait may be controlled by regulatory genes rather than by the fructan biosynthetic genes.

1. Introduction
1.1. Forage grasslands

It is estimated that grasslands comprise approximately 20% of the vegetative cover of the earth’s surface. These areas provide forage for ruminants supporting much of the world’s milk and meat production. For example, around 75% of ruminant feed requirements are obtained from grass and other forages in the UK, varying from 60% for dairy cows up to 90% for sheep [1]. Thus forage grasses make a major contribution to the efficiency and profitability of agriculture. Consequently, they are the subject of much breeding effort in a number of countries [2]. The main cultivated grass species in temperate regions include ryegrasses, fescues and to a lesser extent timothy and cocksfoot. The nutritional quality of these grasses is correlated with non-structural carbohydrate content of which fructan is a major component [3]. Fructan comprises on average 70% of the water-soluble carbohydrate (WSC) of perennial ryegrass in field conditions [4]. Traditional plant-breeding approaches have been successful in producing high-sugar ryegrasses that have been shown to improve protein utilization by ruminants [5].

In this chapter we will discuss aspects of fructan metabolism and accumulation in temperate forage grasses which have an impact upon agricultural performance.
We will review the literature on grass fructans, their distribution within the vegetative plant, and diurnal and seasonal effects on accumulation. We will consider how fructan content affects growth, plant composition, yield and ruminant productivity. The relationship between fructan content and abiotic stress will also be reviewed. We will discuss the molecular biology of fructan synthesis in *Lolium perenne* L. (*L. perenne*), the genes necessary to synthesize the observed range of fructan molecules and consider how the regulation of these genes relates to fructan metabolism. The work on the localization of fructan synthesis within the cell will be assessed with respect to existing models. We will conclude by discussing the genetic control of fructan accumulation in forage grasses, the relationship of quantitative trait loci (QTL) with the genes responsible for fructan synthesis and to the implications for future breeding programmes.

2. Agronomy

2.1. Forage grass fructan

Fructans, branched or linear poly-fructose molecules synthesized *de novo* from sucrose, act as storage polymers of carbon, performing much the same function in temperate forage grasses (and cereals) as starch does in many plants. These polymers accumulate when the supply of carbon assimilate from current photosynthesis exceeds the demand from growth processes. They are soluble, highly accessible molecules which accumulate in both growing and storage tissues and are in a constant state of flux responding to changes in the balance between anabolic and catabolic processes, and content may vary unpredictably in uncontrolled environments. They may be stored short or long term, and can reach high concentrations without inhibiting photosynthesis [6]. Most forage grass species accumulate a characteristic range of fructan varying in molecular size and linkage type [7-9]. Cocksfoot (*Dactylis*) and timothy (*Phleum*) fructan is predominantly comprised of large polymers while ryegrasses and fescues accumulate a range of oligomeric fructan in addition to larger polymers [7,8]. Perennial ryegrass fructan consists of three linkage series including; inulin, inulin neoseries and the levan neoseries [10]. The inulin series consists of a terminal glucose and β(2,1) linked fructose residues, the inulin neoseries consists of an internal glucose residue and β(2,1) linked fructose residues, and the levan neoseries consists of an internal glucose residue and β(2,6) linked fructose residues (Figure 1). Pavis et al. [10] reported that ryegrass roots, leaf sheaths, leaf blades and elongating leaves contain similar classes of fructans, but that the size range varied in the different tissues. Starch is also present in ryegrasses [11,12] but only as a minor component of the total non-structural carbohydrate fraction of vegetative tissue.
2.2. Partitioning of fructan in forage grasses

Forage grasses are generally grazed or harvested for conservation in a vegetative stage of growth, and thus the bulk of consumed plant material is comprised of expanded leaf blades with a smaller contribution from leaf sheath tissue. It is frequently reported that high molecular weight fructan is found predominantly in the leaf sheaths and leaf bases of temperate forage grasses, where it serves as a reserve carbohydrate, and that small amounts of mainly low molecular weight fructan accumulate in expanded leaf blades [7,13,14]. This is based mainly on evidence from controlled environment studies on intact plants when overall plant carbohydrate status can be low, for example, Pavis et al. [15]. These observations of low leaf fructan content are in contrast with high WSC values reported for field-grown material [16-18]. Data from plants grown in glasshouses have also demonstrated significant amounts of polymeric fructan in leaf blades, although leaf sheath tissue was undoubtedly the main site of storage of fructan reserves in vegetative tissues of tall fescue and perennial ryegrass [5,19]. In fact, analysis of perennial ryegrass material grown in field or glasshouse environments generally shows highest fructan and/or WSC contents in the leaf sheaths (sometimes described as tiller bases or pseudostems). WSC contents of approximately 25% dry matter (DM) are not uncommon in perennial ryegrass with over 70% in the form of fructan. Leaf blade WSC values ranging from 16-20% DM (approximately 63% fructan) have been observed [4,20]. Recent findings with perennial ryegrass suggest that plants may segregate independently for tiller base, leaf and root fructan.
content. (i.e. the ratio of fructan contents between different tissue fractions can vary) (Table 1). These findings are still consistent with the conclusion that fructans only accumulate in leaf blades after the storage capacity of both leaf sheaths and growth zones has been exceeded [21], if the storage capacity thresholds for these tissues vary. Artificially reducing or removing the carbon demand of sink tissues certainly induces accumulation of high concentrations of large fructan polymers in leaves when photosynthate supply is maintained e.g., following root chilling or leaf excision [11,14,22].

Table 1. Leaf, tiller base and root fructan content of selected plants from a segregating perennial ryegrass mapping family. n = 3. Data for Aberystwyth, UK, Autumn 2004.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Fructan mg g(^{-1}) dry matter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>leaf</td>
<td>tiller base</td>
</tr>
<tr>
<td>9/9</td>
<td>288.6</td>
<td>352.6</td>
</tr>
<tr>
<td>3/5</td>
<td>236.3</td>
<td>330.3</td>
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<tr>
<td>16/4</td>
<td>274.4</td>
<td>382.8</td>
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<tr>
<td>7/3</td>
<td>301.4</td>
<td>350.0</td>
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<tr>
<td>3/6</td>
<td>318.9</td>
<td>337.3</td>
</tr>
<tr>
<td>10/5</td>
<td>259.9</td>
<td>317.1</td>
</tr>
</tbody>
</table>

2.3. Diurnal and seasonal effects on fructan accumulation

In any discussion on leaf fructan content, diurnal and seasonal effects must be considered. It is not clear if the accumulation of fructan shows a diurnal variation. In one study, the fructan content of perennial ryegrass leaves increased approximately 4-fold between early morning and just after midday on a bright sunny day in summer [23]. Subsequently fructan content fell and was low again by dusk. However, other workers have reported minimal variation in fructan content during daylight hours in studies with tall fescue [24], phalaris (*Phalaris aquatica*) [25] and Italian ryegrass [26]. Tall fescue and phalaris did show increases in sucrose content throughout the day. Differences in diurnal patterns may be related to species but could also be attributed to preceding climatic conditions and the maturity of the sward which will determine the carbon demand of sink tissues.

WSC content also varies during the course of the year, although there is some discrepancy in the seasonal patterns described in the published literature. Fulkerson et al. [27] and references therein suggest that WSC content is usually highest in winter when conditions are cool and sunny with temperatures too low to support growth but not too low to prevent photosynthesis. Photosynthesis and fructan production are relatively insensitive to low temperature in comparison to growth processes [28]. However, a number of
authors have reported highest WSC and/or fructan contents between spring/early summer and late summer and the lowest concentrations in winter [4,16,29,30]. It should be considered that most of these studies involved repeated measures on the same sward, and that seasonal patterns were often disrupted by regular cutting during the growing season. In a study where plants were sown in the field at staggered intervals, and destructively sampled at the same physiological stage throughout the year, highest fructan and total WSC contents were observed in May (Table 2), in agreement with previous studies. Patterns varied with tissue type, with leaf blade carbohydrate content decreasing between May and November in contrast with leaf sheaths, which showed no decrease in this period. Pollock and Jones [31] also monitored seasonal fructan accumulation in uncut forage and, contrary to the study described above, they observed peak contents in the winter months with some variation between species. Differences can probably be accounted for by variation in climatic conditions and indicate that environmental factors can have a profound effect on patterns of fructan accumulation.

Table 2. Fructan and total WSC content of leaf blades and leaf sheaths during the year. Data are mg g\(^{-1}\) dry matter. n = 16. Data for Aberystwyth, UK.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>February</th>
<th>May</th>
<th>August</th>
<th>November</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade fructan</td>
<td>39</td>
<td>197</td>
<td>134</td>
<td>148</td>
</tr>
<tr>
<td>Total WSC</td>
<td>167</td>
<td>294</td>
<td>164</td>
<td>223</td>
</tr>
<tr>
<td>Sheath fructan</td>
<td>96</td>
<td>322</td>
<td>295</td>
<td>338</td>
</tr>
<tr>
<td>Total WSC</td>
<td>216</td>
<td>367</td>
<td>312</td>
<td>366</td>
</tr>
</tbody>
</table>

2.4. The effect of fructan accumulation on regrowth

Forage grasses can survive depletion of the bulk of plant aerial biomass and subsequently regrow from stubble. In general terms, the carbohydrate reserves in the tiller base are used to fuel regrowth following defoliation and replenished once carbon gain from photosynthesis is greater than carbon utilization for growth. The rate of regrowth after cutting has frequently been linked to the amount of carbohydrate reserves stored in tiller bases in the stubble [29,32]. However, some reports suggest nitrogen may be more important especially in the longer term [33]. Nitrogen must undoubtedly play a role. Other reports have suggested WSC content has little effect on leaf regrowth [34] but that it does affect root growth and tiller initiation. In a study by Morvand-Bertrand et al. [35], dry matter yield following periods of approximately 4 weeks regrowth was unrelated to the WSC reserves present in
defoliated plants. The time-course of carbohydrate measurements made during regrowth and the severity of cutting may explain the varying conclusions of different workers. The initial stages, which are purely resource driven, may show a high correlation with carbohydrate reserves but once new photosynthetic leaf area is present, and current photosynthate supply available, other factors come into play and this correlation is lost. When carbon is no longer limiting, a correlation with nitrogen may be observed, as shown by experiments with elevated carbon dioxide [36]. The regrowth of perennial ryegrass has been studied in detail and three distinct phases of the process have been characterized [37,38]. The first lasts from day 0 (first day of defoliation) to day 2/3, depending on environmental conditions, when new leaf growth is entirely dependent on the mobilization of fructan reserves from the stubble. The second phase occurs between day 3 and day 6 after cutting when carbon supply from current photosynthesis is sufficient to support new growth. In phase three (after day 6), photosynthate supply is sufficient to support growth and also replenish fructan reserves to pre-defoliation levels. Patterns of regrowth in time-course studies with high and low WSC lines of ryegrass (cvs. Aurora and Perma) were consistent with this model. High WSC plants initially showed a more rapid rate of regrowth, but this trend was not maintained after day 4 [39].

Defoliation of temperate grass species will have consequences for plant carbon reserves. The effects will depend on the frequency and severity of cutting, the quantity and form of available reserves and the environmental conditions following defoliation [40]. Under most normal agricultural practices the extent of defoliation usually has little effect on plant WSC reserves in the longer term. WSC and fructan reserves are quickly replenished in the days after cutting [37-39]. However, extremely frequent defoliation may result in such severe depletion of plant reserves that regrowth becomes impossible, plant death occurs and swards lose persistence [29].

2.5. The relationship between fructan, growth and yield

The accumulation of high levels of fructan reserves in forage grasses might be anticipated to correlate with rather low inherent rates of plant growth. Indeed the growth rate of individual plants (tillers) of one high-sugar perennial ryegrass cultivar has been shown to be relatively slow [39]. This might then be expected to have further consequences on sward productivity. However, reported dry matter yields for high sugar grasses are rarely lower than for normal varieties [16,30,41]. It must be noted that one study did indicate that a high WSC trait may be linked with lower root mass [20]. One important factor may be sward structure. Mature plants of cv. Aurora, the high-sugar grass variety used in a number of studies, often consist of a large number of small
tillers. These may all grow rather slowly, but if the tiller density in the sward is sufficiently high then productivity per unit area will be maintained.

2.6. The role of fructan in abiotic stress tolerance

Fructans are frequently attributed a functional role in plants during a range of abiotic stresses. Their importance in drought and cold tolerance in particular has been repeatedly cited in research papers and reviews [6,42,43] and these are considered below.

It is now well established that growth is more sensitive than photosynthesis to water stress. Accordingly there are numerous published reports of fructan accumulation during drought; for example, fructan accumulated in the tissues that make up the tiller base and in the leaves of ryegrass during water stress [44,45]. Most studies which propose a role for fructan in stress tolerance compare fructan accumulation in stress-tolerant and stress-sensitive species, varieties, cultivars or lines [46-49]. However such studies only provide indirect evidence as other biochemical and physiological differences may have contributed to the improved drought tolerance. Transgenically-transformed plants from one genetic stock provide more experimentally robust material for testing the physiological role of fructan. Transformations of tobacco and sugar beet have demonstrated a beneficial effect on drought tolerance [50,51]. However it must be noted that these plants were transformed with a bacterial levansucrase and this resulted in the production of fructan polymers which were much larger than those normally found in plants. Other studies have failed to demonstrate that fructans have a role during drought other than as a carbon sink when required [52], and Thomas and James [53] concluded that sugar reserves did not enhance growth in drought-stressed perennial ryegrass. More convincing evidence that fructan has a role in drought tolerance comes from studies on membrane properties. Using a vesicle leakage assay, Hincha et al. [54] were able to demonstrate that chicory inulin protects membranes during dehydration and Vereyken et al. [55] showed a similar effect with levan.

In addition to drought, plant growth processes are generally more sensitive to low temperature than photosynthesis and fructan synthesis when there is sufficient light intensity [28]. This is consistent with numerous reports of fructan accumulation in plants exposed to low temperatures [e.g., 46,56-58]. There are many parallels with the work on drought stress in the approaches and conclusions of workers investigating the role of fructan in cold tolerance. In common with work on drought stress, evidence for a functional role of fructan in cold tolerance comes from comparisons of species, varieties, cultivars or lines with contrasting hardiness [46,59-61]. Suzuki and Nass [59], observed an apparent relationship between high degree of polymerization (DP) fructan and freezing tolerance. More indirect evidence comes from work by Puebla et al.
where they show that a cold tolerant species of *Bromus* synthesized fructan constitutively while a species adapted to warmer conditions only accumulated fructan under cold stress. Transgenic plants transformed for fructan synthesis have been studied under cold stress treatments, and a protective effect of fructan claimed [62,63]. However Pollock et al. [64] contrasted the effect of carbohydrate accumulation on cold tolerance in a hardy and less hardy cultivar of perennial ryegrass, and only demonstrated a correlation with the hardy variety. They also showed that fructan did not increase cold tolerance by a depression of freezing point. Whilst fructan may have a role in low temperature stress tolerance, other factors such as membrane properties are undoubtedly involved. It may be possible to demonstrate a cryoprotective role for fructan using similar procedures to those described by Hincha et al. [54] in the study of drought tolerance.

### 2.7. The effect of fructan on plant composition and ruminant production

Increased carbon flux to the WSC pool may also be considered to invariably reduce carbon partitioning to other pools. This is inevitable when total DM yield is not affected. It is a general characteristic of plants that high WSC content is correlated with reduced protein content and some comparisons of the composition of high and low WSC perennial ryegrass cultivars support this [3]. However, this trend is not consistent and some studies have reported no significant differences in crude protein content [20,65]. While protein content does not show a clear relationship with WSC, many studies with perennial ryegrass do show reduced neutral detergent fibre contents in high WSC cultivars [3,20,65]. The development of perennial ryegrass cultivars with high WSC content has led to numerous trials to investigate the effects of increased energy supply on ruminant production. Since high WSC content in perennial ryegrasses is largely comprised of fructan, results from these studies may be considered to show the effect of fructan on the ruminant diet. Miller et al. [3] carried out a feeding trial with late-lactation dairy cows and found that high WSC content increased dry matter intake and milk yields. Further trials by Moorby et al. [65] with early lactation cows confirmed these findings. Grazing trials with lambs have also demonstrated a beneficial effect on sheep production [66]. Live weight gains were significantly higher when animals were allowed to graze a high WSC cultivar sward compared with an intermediate cultivar. While these studies demonstrate increased production in response to a high WSC diet, perhaps of greater consequence are the effects on dietary nitrogen. This is especially relevant in the current climate with the increased emphasis in agriculture on sustainable farming practice. Earlier studies have demonstrated a relationship between WSC content in ruminant diets and protein utilization [67]. More recent trials have shown clear effects of
high WSC on nitrogen metabolism in ruminants. Trials with dairy cows showed increased partitioning of nitrogen into milk and decreased urinary N excretion [3,65]. In vitro rumen fermentation experiments demonstrated that increasing WSC decreased ammonia concentration and increased microbial protein synthesis, showing improved utilization of dietary protein [68]. These studies clearly demonstrate that inclusion of high WSC in ruminant diets can potentially reduce nitrogen excretion with consequent benefits to the environment.

Although there are clear benefits in feeding high WSC cultivars to ruminants, expression of the high WSC phenotype is inconsistent and is very dependent on environmental conditions. Consequently, there is a clear need to understand both the molecular biology of this process as well as physiological constraints in order to breed for more robust phenotypes.

3. The molecular biology and physiology of fructan synthesis in perennial ryegrass

There is considerable information available in the literature concerning the molecular biology and physiology of fructan metabolism in grasses, however, for the purposes of this chapter we will direct our attention to fructan biosynthesis in perennial ryegrass (L. perenne) and Lolium temulentum L. (L. temulentum).

3.1. Genes encoding for the enzyme activities necessary for fructan synthesis

Polymeric fructan is synthesized by the concerted action of a number of fructosyltransferase (FT) enzymes that donate the fructosyl group from either donor sucrose or donor fructan molecule to acceptor sucrose or acceptor fructan molecule, resulting in an increase in chain length. In the forage grasses, as previously stated, fructan molecules consist of the inulin series, the inulin neoseries and the levan neoseries (Figure 1). To produce the range of molecules observed in Lolium plants, FT enzymes with at least 4 activities are required; a sucrose: sucrose 1-fructosyltransferase (1-SST) (EC 2.4.1.99), a fructan: fructan 1-fructosyltransferase (1-FFT) (EC 2.4.1.100), a fructan: fructan 6G-fructosyltransferase (6G-FFT) (EC 2.4.1.243) and a sucrose: fructan or fructan: fructan 6-fructosyltransferase (6-(S/F)FT) (EC 2.4.1.10) [10,15,69]. 1-SST catalyses the transfer of fructose from one sucrose molecule to another to produce the tri-saccharide 1-kestose with a β(2,1) linkage. 1-FFT increases the chain length via β(2,1) linkages. 6G-FFT catalyses the transfer of fructose from a fructan molecule to glucose (C6) of either a sucrose or a second fructan molecule. 6-(S/F)FT transfers a fructose molecule from either a
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A number of FT genes have been isolated from *L. perenne*, which can be grouped together based on sequence homology (Table 3). While these genes have been ascribed specific FT activities (1-SST, 6-(S/F)FT, 6G-FFT and 1-FFT), only the FT activity of the 1-SST (AY245431, group 1) and 6G-FFT/ 1-FFT (AF492836, group 3 – referred to in the rest of this chapter as 6G-FFT) have been demonstrated *in vitro* [70,71]. The recombinant proteins encoded by these genes were studied following expression in the yeast *Pichia pastoris*. Chalmers et al. [70] showed that following incubation with sucrose, the recombinant 1-SST protein produced primarily 1-kestose and a small amount of nystose (1,1 kestotetraose GF3). Lasseur et al. [71] observed that the recombinant 6G-FFT demonstrated both 6G-FFT and 1-FFT activities in a ratio of 2:1. These authors proposed how the different oligo and polysaccharide fructan observed in *Lolium* could be synthesised from the activities of the proteins encoded by a 1-SST, a 6-(S/F)FT and the 6G-FFT characterized in their study, eliminating the requirement for a specific gene encoding 1-FFT activity (Figure 1).

The activity encoded by the genes in group 2 and 4 have as yet to be determined. It is likely they code for either a 6-(S/F)FT and/or a 1-FFT. Sequence homology shows that the group 2 FTs are more homologous to the functionally characterized 6-SFT gene from barley [72] demonstrating 72% identity at the amino acid level as opposed to 65% for LpFT4 (group 4). However, Chalmers [42] referred to a personal communication from Toshihiko Yamada indicating that the FT gene (AB186920, group 2) displayed 1-FFT activity following expression of the recombinant protein in yeast. Details of this study had not appeared in the literature at the time this chapter was prepared. The FT LpFT4 (group 4) shows greater homology to the *Lolium* soluble acid invertases than to the *Lolium* FTs. However, this gene contains a

Table 3. *L. perenne* FT genes grouped based on <90% sequence homology.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ac. No.</th>
<th>Name</th>
<th>Author</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AM 407402</td>
<td>1-SST (genomic)</td>
<td>Gallagher et al.</td>
<td>Putative</td>
</tr>
<tr>
<td></td>
<td>AY 245431</td>
<td>1-SST mRNA</td>
<td>Chalmers et al.</td>
<td>Functionally tested</td>
</tr>
<tr>
<td>2</td>
<td>AM 407403</td>
<td>6-(S/F)FT (genomic)</td>
<td>Gallagher et al.</td>
<td>Putative</td>
</tr>
<tr>
<td></td>
<td>AB 186920</td>
<td>1-FFT(TT1) mRNA</td>
<td>Hisano et al.</td>
<td>Putative</td>
</tr>
<tr>
<td></td>
<td>AF 494041</td>
<td>6-FFT mRNA</td>
<td>Lasseur et al.</td>
<td>Putative</td>
</tr>
<tr>
<td>3</td>
<td>AM 407401</td>
<td>6G-FFT (genomic)</td>
<td>Gallagher et al.</td>
<td>Putative</td>
</tr>
<tr>
<td></td>
<td>AF 492836</td>
<td>6G-FFT mRNA</td>
<td>Lasseur et al.</td>
<td>Functionally tested</td>
</tr>
<tr>
<td></td>
<td>AB 125218 (FT2)</td>
<td>6G-FFT mRNA</td>
<td>Hisano et al.</td>
<td>Putative</td>
</tr>
<tr>
<td>4</td>
<td>DQ 073970</td>
<td>LpFT4 mRNA</td>
<td>Chalmers et al.</td>
<td></td>
</tr>
</tbody>
</table>
signal sequence similar in length to that found in FTs as well as containing the amino acids FMS prior to the "invertase motif" DPNG [73]. This invertase motif is present in invertases and FT genes. The amino acid grouping [FY][MQI][SNGASY] next to the DPNG box is a conserved feature that separates FTs from invertases [14]. Invertases contain the 3 amino acid sequences WMN, WIN or to a lesser extent WQN in this position.

3.2. Gene structure

FT genes are highly homologous to each other and to acid invertases, and are therefore considered members of the 'invertase' gene family. Phylogenetic analysis reveals that monocotyledonous (monocot) FTs show greater similarity to monocot acid invertases than to dicotyledonous (dicot) FTs while dicot FTs show greater similarity to dicot acid invertases than to monocot FTs [74]. Wei and Chatterton [74] proposed that following the divergence into monocots and dicots, the vacuolar acid invertases duplicated and diversified to form FTs. When specific regions within gene sequences are analysed and compared, FTs from monocots and dicots group together, as do vacuolar acid invertases, indicating the acid invertases and FTs have different sequence characteristics. Similar results are observed following analysis of their signal peptides [14].

Both the 1-SST (AM 407402) and the putative 6-(S/F)FT (AM 407402) genomic sequences have been identified on the same bacterial artificial chromosome (BAC) from a L. perenne genomic BAC library demonstrating that these genes are linked. These two genes and the 6G-FFT (AM 407402) have similar genomic structure, containing 3 large exons (Figure 2). 6-(S/F)FT and 6G-FFT also contain a small 9 base pair exon. This 9 base pair exon, which is also present in invertases, is the smallest found in higher plants [75]. It forms part of the invertase motif (DPNG, [73]), which is contained within the active binding site. It is believed that the differences in biochemical characteristics associated with invertases and FTs result mainly from sequence alterations in the region around this motif [76,77]. There is no intron separating this region from exon 2 in the 1-SST gene. Whether this missing intron plays a role in the regulation of 1-SST has yet to be established. Bournay et al. [78] reported alternative splicing of an acid invertase following cold stress, resulting in the deletion of the mini exon from some transcripts, leading to an inactive enzyme. The physiological role of this aberrant splicing has not yet been determined. It is conceivable that the reduced likelihood for a splicing error to occur in the 1-SST gene may be an indication of its importance in the initiation of fructan synthesis. However, inclusion of the 9 base pair region within exon 2 does not appear to be a uniform characteristic of 1-SST genes, Yoshida et al. [79] reported the presence of the 9 base pair exon in a genomic 1-SST clone isolated from wheat.
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Figure 2. Genomic clones of *L. perenne*; 1-SST (AM 407402), 6G-FFT (AM 407401) and 6-(S/F)FT (AM 407403). Exons, putative TATA box and start codons are labelled.

1-SST (AM 407402), 6G-FFT (AM 407402) and the putative 6-(S/F)FT (AM 407402) code for proteins of similar sizes (653, 645 and 623 amino acids respectively). LpFT4 (DQ 073970) also codes for a protein of similar size (653 amino acids) [42]. The primary translation products of FTs, like acid invertases, may be cleaved to give two subunits with a ratio in size of 3:1 [72,80-82]. Both the monomeric and heterodimeric forms have been observed *in vivo*; however, it has yet to be determined if this represents a form of post-transcriptional regulation. The small subunit begins with the motif EAD [72] which is present in all four *L. perenne* FT genes. The large subunit contains the domains involved in substrate binding and hydrolysis (see below). Altenbach et al. [82] showed by interchanging the different subunits from the *Festuca* 1-SST (AJ297369) and the barley 6-SFT (X83233) genes, that the large subunits of each enzyme determined both substrate and product specificity of the recombinant enzymes when expressed in yeast.

The sequences of the *L. perenne* FT genes show six to seven putative glycosylation sites and, like all plant FT genes isolated to date, each includes a pre-peptide containing a signal sequence at the 5' end, which is cleaved during transport and protein maturation [75]. Comparison of this region within the
'invertase gene' family shows that FTs of monocots and dicots are more similar to each other than to monocot invertases, possibly indicating different compartmentation within the cell. The length of these sequences are similar to those described for vacuolar acid invertases and longer than those for cell wall invertase [14,42].

Along with the invertase motif (sucrose binding box), two other regions have been identified as contributing to substrate binding and hydrolysis. These are (1) the [W/Y]ECXD box and (2) the FRDP box. Both these regions are highly conserved among the 'invertase gene' family, and mutation analysis has shown that the Glu (E) in box 1 and Asp (D) in box 2 are directly involved in substrate binding [83-85].

3.3. Regulation and expression of fructosyltransferase genes

Sucrose is not only required as a substrate for fructan synthesis, but it is also involved in the induction of the biosynthetic genes. Studies with excised leaf systems, using inhibitors of transcription, have shown that there is a requirement for both sucrose accumulation and de novo gene expression for fructan synthesis to occur [11,86-88]. All three of the FT genes coding for the enzymes necessary for fructan synthesis in L. perenne are induced in the presence of sucrose (Figure 3).

Figure 3. Expression of actin, 6-(S/F)FT, 6G-FFT and 1-SST. RNA was isolated from excised L. perenne leaves placed in water and sucrose in the dark for 24 h. Prior to excision, plants were placed in low light with a short photoperiod for 5 days to deplete soluble carbohydrates.
Studies with sugars and sugar analogues in *Lolium* (Figure 4), barley and wheat [89,90] have demonstrated that induction of FT genes is independent of hexokinase or interaction of hexoses with transporters/sensors at the cell membrane but rather occurs via an interaction of sucrose with specific transporters/sensors. This was determined using the sugars/sugar analogues; mannose, 2-deoxy-glucose (hexokinase substrates which are not further metabolized) and 3-methyl-glucose (non-metabolizable hexose analogue). Sucrose specificity, as opposed to non-specific di-saccharide signal perception, was demonstrated using the di-saccharides; trehalose (α-D-gluc. α-D-gluc), palatinose (6-o- α-D-gluc.-D-fruc.) and turanose (3-o- α-D-gluc.-D-fruc). Palatinose and turanose give no induction of transcript (data not presented), while trehalose gave only a slight induction but this was negligible compared to that observed for sucrose. The transmission of the sucrose responsive signal has been postulated to involve protein kinase/phosphatase activities [90], in particular, protein phosphatase 2A. This area of fructan gene expression has yet to be fully elucidated.

*In silico* analysis of the promoter regions of *Lolium* FT genes showed that they contain regions associated with many responses [42]. These promoter regions contain sugar inducible elements as well as elements associated with light, stress, phloem-specific gene expression and low temperatures [42]. In general these promoters contain the regulatory elements expected given the range of conditions which result in fructan accumulation. However, not all elements are present in each promoter, for example, the sucrose responsive element (SURE Box) described by Urwin and Jenkins [91] present in most *Lolium* FT gene promoters, is not present in the promoter of the putative 6-(S/F)FT (AM 407402) gene (Figure 5).

![Figure 4](image_url)

**Figure 4.** Expression of 1-SST and 6-(S/F)FT (qPCR) in excised leaf tissue placed in the dark in water supplemented with a range of sugars and sugar analogues, or placed in high light for 12 h. Prior to excision, plants were placed in a low light with a short photoperiod for 5 days to reduce carbohydrate content. TLC shows the presence of sucrose in each tissue at the start and end of the experiment.
Sucrose Responsive Element (SURE)

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Figure 5. Sucrose responsive element showing SP8 consensus sequence and alignment with similar elements in the promoter region of FTs and other sugar regulated genes.

Although the FT genes are induced by the same range of sugars, their regulation is markedly different. 1-SST is believed to be the regulatory 'pacemaker' enzyme in fructan synthesis. In studies carried out in barley, Nagaraj [92] showed that 1-SST transcript and enzyme activity appeared and disappeared rapidly when excised leaves were induced to accumulate and subsequently remobilize fructan (using light and dark treatments). The transcript level and enzyme activity of 6-SFT on the other hand were found to be more stable. The 1-SST gene transcript not only appeared prior to that of 6-SFT, but its breakdown was faster and less transcript was produced. Similarly, in L. perenne, more transcript was detected for 6-(S/F)FT (AM 407402), when excised leaves were placed in high light, than for 1-SST or 6G-FFT (Figure 6). In these tissues, there was 4-fold more 6-(S/F)FT transcript than 6G-FFT transcript and 8-fold more than 1-SST. Lasseur et al. [71] studied the relationship between 1-SST and 6G-FFT transcript and enzyme abundance as well as fructan levels in different leaf tissues of L. perenne. In heterotrophic tissue (elongating leaf) which contains the highest levels of fructan, transcript levels paralleled both enzyme activity and fructan content and they concluded that both genes were transcriptionally controlled. Transcriptional control of 1-SST activity has previously been observed in Festuca and Taraxaum officinale [93,94]. However, Lasseur et al. [71] suggested that post-transcriptional regulation was occurring in leaf sheaths of mature leaves, as transcript levels for these genes became uncoupled from enzyme activity and fructan accumulation. Furthermore, 6G-FFT expression was observed in mature leaf blades where little enzyme activity or fructan was detected, further indicating post-translational regulation.
Fructan in temperate forage grasses

Figure 6. Relative abundance of FT transcript following qPCR. Transcript numbers were determined following amplification by qPCR from a common cDNA pool and compared to standard curves generated for each gene following similar amplification. cDNA was synthesised from RNA isolated from excised *L. perenne* leaf tissue subjected to a high light treatment.

These authors observed that 6G-FFT expression mirrored the sucrose content in mature blades, sheaths and elongating leaf blades. Similar results were observed in *L. temulentum* for the 6-(S/F)FT homologue FT 2:2 (AY 082350) [14].

In *Lolium* and other forage grasses, FT gene transcripts occur along the leaf blade with the highest levels of expression and enzyme activity at the base of the leaf, the major site of fructan accumulation [10,14,37,70,71,95]. Soluble acid invertase transcripts have also been found in this tissue [13,14,96]. Figure 7 shows that these soluble acid invertase genes produce active enzyme at the site of fructan accumulation. From single cell studies in barley, soluble acid invertase and FT transcripts have been shown to be expressed in the same cell at the same time (Chungui Lu; personal communication). Thus a conundrum occurs, given that FT and soluble acid invertase enzymes are functioning in the same tissue. Furthermore soluble acid invertases and FTs are believed to reside in the vacuole [57,97-99]. The question arises, how do fructans manage to accumulate in the presence of soluble acid invertase, as both these enzyme types require the same substrate? What is of interest is that both enzyme types have signal peptides which can be distinguished from each other based on their sequence, indicating that these enzymes may be targeted to different locations within the cell [14]. To understand this we have to reinvestigate the proposed models for soluble acid invertase and FT activity and localization within the cell.
Figure 7. Soluble acid invertase enzyme activity and fructan accumulation along the developing *L. temulentum* leaf. Soluble protein from plant extracts was separated by iso-electric focusing gel electrophoresis, invertase activity was visualized following incubation with sucrose and staining with triphenyltetrazolium chloride which detects reducing sugars. Fructans were separated by TLC and visualized by staining with urea phosphoric acid.

### 3.4. Localization of soluble acid invertase and fructosyltransferase activity within cells of *Lolium* leaf tissue

Recent literature gives the impression that the mechanism of fructan biosynthesis in natural fructan accumulators (as distinct from transgenics) has been determined. However, there remain a number of problems associated with the conventional vacuolar explanation for fructan synthesis in plants.

(i) Sucrose-fructosyltransferases (1-SST, 6-SFT) are universally reported to exhibit low affinities (high apparent Km) for sucrose and many are essentially unsaturable [23,99-101]. Vacuolar sucrose concentrations in barley mesophyll cells during fructan synthesis are reported to lie in the range 80 to 120 mM (by single cell sampling: [102,103] and would greatly limit the activity of the enzyme. In a particularly severe example, the levan polymerase (6-FFT) from *Phleum pratense* L. has no activity *in vitro* at 100 mM sucrose [100]. If these enzymes are
localized in the vacuole (within the physiological range of substrate concentration), the known kinetic properties of sucrose-fructosyltransferases cannot explain physiological rates of fructan accumulation.

(ii) Similarly, fructan-fructosyltransferases (1-FFT, 6-FFT, 6G-FFT) also exhibit low affinity for their substrate 1-kestose, with an apparent Km of >100 mM [80]. From published information, vacuolar 1-kestose concentrations in tissue during fructan synthesis may be estimated at ca. 12 mM [8,57,86]. FFT would function at only ca. <5 % of maximal activity under these substrate conditions.

(iii) In addition to the problem of low substrate affinity, chain elongation by disproportionation of fructose from 1-kestose by FFT is reported to be competitively-inhibited by sucrose [80]. From the above (i and ii), during fructan synthesis, sucrose would be present in an 8-fold molar excess relative to 1-kestose in the vacuole. It is not clear how this sucrose-inhibition of FFT is overcome if, as proposed, the process is fully vacuolar.

(iv) No analogous 6-SST, 6-FFT or their cognate genes have been functionally identified for the synthesis of the β(2,6) linked fructans (levans) of the temperate grasses [42] although as mentioned above, there are possible candidates. Although polymeric levan has been synthesized by a partially purified enzyme preparation from Phleum pretense leaf tissue, this reaction requires high-sucrose and high enzyme concentration for polymerization and to achieve physiological rates of synthesis [100]. Such reaction conditions are incompatible with the vacuolar model. Furthermore, high sucrose concentration does not inhibit this levan polymerase (6-FFT) reaction but conversely, actually stimulates it. Hence, the properties of this 6-FFT differ from those described for chicory 1-FFT [80].

(v) Soluble acid invertase is also thought to be vacuolar in some fructan-accumulating tissues [6] and the activity can be sufficient to hydrolyse all tissue sucrose in a few hours [104]. In view of the relatively high sucrose-affinity of acid invertase (Km <10 mM), it is not clear how sucrose accumulates or is able to persist in the vacuole to achieve the substrate concentrations necessary for the function of S(S/F)Ts.

(vi) Direct evidence for the vacuolar localization of natural fructan synthesis is based on only a small number of studies [57,97,99,105]. The latter three studies are subject to methodological criticism because the protoplasting enzyme used in the preparation of vacuoles contains high contaminating sucrose-fructosyltransferase activity [106].

(vii) Accounts supporting the vacuolar model rarely cite the study of Kaeser [107], who proposed that fructan synthesis is vesicular.
(viii) In our view, the most convincing examples of enzymatic polymerization (where the naturally-occurring, species-specific pattern of fructans are synthesized at near-physiological rates) require high enzyme and high sucrose concentrations, features which are incompatible with localization in the vacuole (a high-volume, dilute compartment) and with the reported sucrose-inhibition of FFT [9,100,101,108]. Taken together, the inconsistencies inherent in the general hypothesis indicate that the vacuolar model is at best incomplete and this invites further critical investigation. Here we provide an overview of our recent physiological studies at the whole organ level in *Lolium*. We test two fundamental features of the current hypothesis for sugar metabolism in grass leaves; (i) the (vacuolar) co-localization of sucrose and acid invertase and (ii) the energy independence of the vacuolar model for fructan biosynthesis. Our results lead us to suspect a role for cytoplasmic vesicles in grass leaf sugar metabolism and we test this possibility by using inhibitors of endomembrane function.

3.5. Sucrose and soluble acid invertase are not co-localized in leaves of *Lolium*

In principle, it is not surprising that a metabolite (sucrose) should share the same subcellular location (the vacuole) as an enzyme responsible for its metabolism (invertase), but in practice this requires a mechanism whereby the observed high sucrose concentrations can persist in the presence of a high background level of invertase-mediated catabolism. The most widely accepted explanation for this persistence is that the products of sucrose hydrolysis are continually resynthesised into sucrose via an energy-consuming futile cycle [104]. This “turnover hypothesis” is testable by observing the fate of pre-accumulated sucrose when sucrose (re-)synthesis is inhibited. The use of the four different inhibitory agents to block sucrose resynthesis in excised *L. temulentum* leaf tissue, allowed us to demonstrate that all leaf sucrose is stable in the tissue and not subject to constant breakdown and turnover [104]. Figure 8 shows the variation in tissue sucrose during vanadate-inhibition of sucrose synthesis in the light; sucrose synthesis was abolished in the tissue, but the residual sucrose concentration did not decline.

Repeat experiments using mannose to inhibit sucrose synthesis produced an identical result. Figure 9 shows the variation in tissue sucrose during a period of anoxia in the dark (a treatment which blocks the provision of metabolic energy for sucrose resynthesis). In the absence of sucrose resynthesis, tissue sucrose concentration remained constant. Transpirationally-fed cyanide produced an identical result. In all four treatments, the extractable invertase activity remained constant. The soluble inhibitors had no direct effect on acid invertase activity in vitro. It can be concluded that soluble acid invertase and
Figure 8. Effect of transpirationally-fed vanadate on sucrose accumulation in the light (300 μmol m⁻² s⁻¹ and 20°C): The curves compare the time-course of sucrose accumulation in carbohydrate-depleted excised leaves of L. temulentum fed with 5 mM aqueous NaVO₃ from 1.5 h (open circles: arrow indicates time of transfer) with that of leaves fed with water only (control: closed circles: regression: y = 3.597x - 0.14; r² = 0.98; n = 42). All points are means of triplicates ± S.E. Data redrawn from Cairns and Gallagher [104].

Figure 9. Persistence of sucrose in darkness in the presence and absence of oxygen. After 3 h pre-illumination in the light, excised leaves of L. temulentum contained ca.13 mg g⁻¹ sucrose. The curves show the variation in sucrose concentration in leaves subsequently maintained in air (control: open circles: regression: y = 13.65 - 0.57x, r² = 0.87; n = 21) or under O₂-free-N₂ (closed circles: regression: y = 0.04x + 13.62, r² = 0.03, n = 21). All points are means of triplicates ± S.E. Data redrawn from Cairns and Gallagher [104].

sucrose are not in continuous metabolic contact and cannot be simultaneously vacuolar as proposed in the current model. This may involve differential subcellular compartmentation of sucrose and acid invertase, consistent with the report of Rojo et al. [109] who found a putatively vacuolar invertase to be located in cytoplasmic vesicles.
3.6. Metabolic energy is a prerequisite for fructan biosynthesis

The vacuolar model predicts that once initiated, the enzymes, substrate and intermediates necessary for fructan synthesis are co-localized and that the process is self-contained. The energy to drive polymerization is proposed to be derived from the energy conserved in the glucose-fructose glycosidic linkage of sucrose. Because in the model, fructan synthesis should be independent of energetic co-factors (ATP, sugar nucleotides etc., as are the FTs in vitro), leaf fructan synthesis should continue in darkness in the absence of oxygen (at least until sucrose becomes limiting). We extended our methodology originally developed for the analysis of sucrose turnover, to examine the possibility that fructan biosynthesis requires an input of metabolic energy derived from respiration. Figure 10 and Figure 11 show the effect of transfer to anoxia on fructan polymerization in excised leaves of *L. temulentum*. Transfer to anoxia caused the abrupt and complete cessation of net fructan accumulation in the tissue (Figure 10) and also inhibited further polymerization (Figure 11). Changes in tissue sucrose concentration and extractable polymerase activity could not explain the inhibition of fructan synthesis.

The results indicate that respiratory energy is a prerequisite for continued fructan synthesis and polymerization in this tissue. This is contrary to the predictions of the vacuolar model, where once initiated, fructan synthesis is self-contained, energy-independent and should have continued. An in vivo

![Figure 10](image_url)

**Figure 10.** Quantitative effects of transfer to darkness and anoxia on total fructan accumulation in excised leaves of *L. temulentum*. Leaves were pre-illuminated for 16 h to establish the synthesis and accumulation of polymeric fructan. The curves compare the effects of subsequent uninterrupted illumination (Control, •: Light + O₂), and transfer to darkness under anoxia (○: Dark + Anoxia). Individual values are means of measurements from triplicate samples of three leaves ± S.E. determined by isocratic HPLC [11]. Where no bar is visible, the error is smaller than the symbol.
requirement for metabolic energy is also contrary to observations in vitro, where FTs polymerize fructan from sucrose as sole substrate, without any requirement for ATP, sugar nucleotides, etc. This contradiction may be reconciled if the energy requirement in vivo is infrastructural. As stated above, the kinetic properties of the FTs make the vacuole an unlikely site for fructan synthesis [100] and that, as proposed by Kaeser [107], cytoplasmic vesicles might be a more conducive location for the provision of the high substrate and high enzyme concentrations necessary for high-fidelity enzymatic polymerization. Metabolic energy would be necessary for the construction, transport and cycling of cytoplasmic vesicles and could explain why metabolic energy is required for polymerization in vivo, but not in vitro.

3.7. Preliminary evidence for the involvement of the endomembrane system in fructan biosynthesis

The kinetic properties of FTs and the requirement for respiratory energy are inconsistent with the vacuolar model for fructan synthesis. Hence, in common with Kaeser [107] an alternative hypothesis is proposed that fructan synthesis is cytoplasmic, microsomal, and by analogy with the synthesis of other polysaccharides (e.g., xyloglucan: [110], possibly associated with the
Golgi apparatus. The hypothesis is testable by observing the effects on fructan biosynthesis of inhibitors of (i) vesicle motility, such as colchicine which prevents microtubule assembly [111], (ii) endomembrane processes such as tunicamycin, which inhibits glycoprotein glycosylation [112] and (iii) general Golgi function, such as monensin [113].

In a screening test, these inhibitors were transpirationally-fed to excised leaves of *L. temulentum*. Their effects on fructan accumulation were dramatic (Figure 12). Sucrose accumulated whilst fructan biosynthesis was markedly inhibited, relative to water controls by all three compounds. The results are consistent with the involvement of endomembrane processes in fructan biosynthesis. Although these compounds are biologically active and inhibitory of fructan synthesis, the results do not distinguish (i) the possibility that the endomembrane system is the site of fructan synthesis, from (ii) the possibility that inhibitors only prevented the processing and transport of the nascent FTs to a vacuolar site of synthesis. To further investigate this issue, monensin was used in an experiment in which fructan accumulation had already been induced and was occurring at a linear rate of 1.4 mg h⁻¹ g⁻¹ FW. In this condition, sucrose substrate and FTs were already present in the tissue and all the prerequisites for the vacuolar model fulfilled. As argued above, once initiated, the process should be self-contained and continue independently of perturbations of cytoplasmic endomembrane processes and

![Figure 12](image)

**Figure 12.** Qualitative screen for effects on fructan synthesis of known inhibitors of endomembrane function. Aqueous monensin, tunicamycin and colchicine were presented to excised leaves of *L. temulentum* for the first 3 h of a 24 h period of illumination (Cairns and Pollock [87], 300 μmol m⁻² s⁻¹ and 20°C). Extracted soluble sugars were separated by thin layer chromatography and sugar components visualized with the urea-phosphoric acid stain [8].
therefore monensin should have no effect on current fructan synthesis. Figure 13 shows that transpirationally-fed monensin caused a rapid inhibition of net fructan synthesis. Figure 14 shows that this compound also caused inhibition of fructan polymerization. Sucrose accumulation was identical in both the control and

**Figure 13.** Quantitative effects of monensin on current fructan accumulation in excised leaves of *L. temulentum*. Excised leaves were stood in water and pre-illuminated for 14 h to establish the synthesis and linear accumulation of polymeric fructan. The time-course of total fructan accumulation in leaves subsequently transferred to 50 μM monensin (○) is compared with a water control (●). Individual values are means of measurements from triplicate samples of three leaves ± S.E. determined by isocratic HPLC [11]. Where no bar is visible, the error is smaller than the symbol.

**Figure 14.** Qualitative effects of monensin on the synthesis of ethanol-precipitated polyfructan in excised leaves of *L. temulentum* shown by HPAEC-PAD [100]. Leaves were pre-illuminated for 16 h to establish the synthesis and linear accumulation of fructan. Traces show the populations of polymers at intervals after transfer. A: fructan polymerization in the water-only control (Control), B: fructan polymerization in leaves fed with 50 μM monensin. Timings relate to the points in Figure 13. Each trace represents the equivalent of ca. 8.5 mg FW of tissue.
monensin-treated tissue and cannot explain the observed inhibition of fructan synthesis. These results are inconsistent with the self-contained vacuolar model and imply a direct role for the Golgi apparatus in fructan biosynthesis.

Overall, our whole organ studies implicate roles for metabolic energy and the endomembrane system in fructan biosynthesis. These findings illustrate the need for further critical re-evaluation of (i) the subcellular compartmentation of sucrose and soluble acid invertase, and (ii) the microsomal and vacuolar models for fructan biosynthesis.

4. Genetic control of fructan metabolism in temperate forage grasses

Investigations into the physiology and the molecular biology of fructan biosynthesis have gone some way to explaining fructan accumulation in temperate forage grasses. However in order to understand and exploit this complex trait fully, a genetic approach is required which will help to identify the underlying factors controlling this trait. Unraveling the basis of this control will assist in breeding for more robust high sugar grass varieties.

4.1. Quantitative trait loci mapping for WSC

Quantitative trait loci (QTL) mapping offers the opportunity to genetically dissect traits of interest as well as to identify markers for breeding programmes. The limitation of QTL analysis for characterizing traits of interest in outbreeding species like the ryegrasses and fescues comes from the narrow genetic base of mapping families; any one mapping family can only describe a fraction of the variation available in the species. Different regions of the genome may be identified as controlling a given trait in different mapping families of the same species, even if, in a given cross, one QTL appears to explain a large part of the variation.

QTL for WSC have been identified by a number of groups working with different grass species and mapping families, but many reports contain insufficient information to compare QTL positions [114,115]. The first association of WSC with a molecular marker was shown by Hayward et al. [116], working with ryegrass. They showed a close linkage with the isozyme marker PGI/2, which has now been mapped to linkage group 1 of the Triticeae-aligned Lolium map [117]. The importance of this region of the Lolium genome has since been confirmed, along with regions on linkage groups 2, 5, 6 and 7 [5,118-120]. In most cases the main sugar explaining total WSC QTL is fructan [5]. Indeed, total WSC QTL often co-locate with clusters of QTL for some of the individual sugars that comprise the WSC fraction [5,119]. In one mapping family, QTL for leaf blade and tiller base carbohydrate contents did not coincide, and individual QTL explained between
8 and 59% of the total phenotypic variation in the traits [5]. Most reports suggest that several QTL are spread over the genome, the effect of each QTL is small, and that much of the available variation is still unexplained.

4.2. Candidate genes: Mapping fructan and related genes

The first attempts to identify and map candidate genes for fructan metabolism were not particularly successful. Putative genes of fructan synthesis have been mapped in *L. perenne* [13,96]. However, some confusion arises from the considerable sequence homology between FTs and acid invertases, and it is probable that most of these earlier candidates were soluble acid invertases [14]. Current information [42] suggests that Lp1-SST (AY 245431) maps to linkage group 7 (for the NA<sub>6</sub> and AU<sub>6</sub> parents based on the F<sub>1</sub>(NA<sub>6</sub>xAU<sub>6</sub>) cross), two FTs; LpFT4 (DQ 073970) and 6G-FFT (AM407401) (unpublished) map to linkage group 3 as does fructan exohydrolase (DQ 073968). Cell wall and vacuolar acid invertases map to linkage groups 6 (LpCWINV, DQ 073969 and LpVINV, AY082350) and 7 (LpFT1, AF481763). From initial mapping investigations and based on the observation that 1-SST and the putative 6-(S/F)FT (AM 407403) have been isolated from the same BAC, it would appear that 6-(S/F)FT is also on linkage group 7. To date there is little evidence for FTs or fructan exohydrolase involvement in fructan QTL [5]. It is possible that further mapping of genes for the enzymes of fructan synthesis and breakdown may show closer associations. Alternatively the QTL could result from the activity of regulatory genes. One QTL-candidate gene association has been observed. Alkaline invertase has been shown to map close to sugar QTL on linkage group 6 [5], but as its role in cell metabolism has yet to be clarified, the relevance of this remains unclear. Studies are underway to dissect further the molecular basis underlying WSC/fructan QTLs described above.

5. Conclusion

Fructan metabolism is central to the success of temperate grasses providing a readily remobilizable source of energy in cooler climates. Fructan reserves are important for regrowth and protection against drought. Studies also suggest a role in cold tolerance, but this remains to be clearly demonstrated. Increased fructan content has been shown to benefit ruminant production and the environment through reduced nitrogen release. Expression of the high WSC phenotype is dependent on environmental conditions. In *Lolium*, the molecular and physiological basis of fructan biosynthesis has been partially elucidated; however the genetic control of fructan accumulation is still not understood. There remain gaps in our knowledge of the biochemistry, molecular biology and genetic control of fructan metabolism.
While to date, temperate forage grasses and reserve carbohydrate metabolism have been considered from a traditional agricultural perspective, these plants have the potential to fill a niche in the biofuel market. To exploit these grasses further as a resource in agriculture and the biofuel industry we need to breed for more environmentally stable phenotypes.

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