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Re-Programming Photosynthetic Cells of Perennial Ryegrass (*Lolium perenne* L) for Fructan Biosynthesis through Transgenic Expression of Fructan Biosynthetic Genes under the Control of Photosynthetic Promoters

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Abstract: High molecular weight fructans are the main class of water-soluble carbohydrate used for energy storage in many temperate grass species including perennial ryegrass (*Lolium perenne* L.). As well as being important readily mobilisable energy reserves for the plant, fructans are also involved in stress tolerance. Fructans are also readily digested by grazing ruminants and hence are a valuable source of energy for sheep, beef and dairy production systems in temperate regions. This paper describes the re-programming of the expression of fructan biosynthesis genes through the transgenic manipulation of 6-glucose fructosyltransferase (6G-FFT) and sucrose:sucrose 1-fructosyl-transferase (1-SST) in perennial ryegrass. Transgenic events were developed with altered fructan accumulation patterns with increases in fructan accumulation and greatly increased accumulation of fructan in leaf blades as opposed to the traditional site of fructan accumulation in the pseudostem. This altered site of fructan accumulation has potential benefits for animal production as leaf blades form the major part of the diet of grazing ruminants. Some of the transgenic events also exhibited enhanced biomass production. This combination of high quality and enhanced yield is of great interest to forage plant breeders and whilst the expression of these phenotypes needs to be confirmed under field conditions, the identification and characterisation of the transgenic events described in this paper validate the potential for the manipulation of fructan biosynthesis in perennial ryegrass.

Keywords: fructan; ryegrass; *Lolium perenne*; transgenic; cisgenic

1. Introduction

Fructans are a class of water soluble carbohydrate whose primary function is to provide a readily accessible energy reserve for plant growth. Fructans are associated with various advantageous characters in grasses, such as cold and drought tolerance [1,2], increased tiller survival, good regrowth

after cutting or grazing, improved recovery from stress, early spring growth and increased nutritional quality. Fructans represent the major non-structural carbohydrate store in 15% of plant species [3] and play a key role in forage quality. Ruminant livestock grazing on high fructan diets shows improved animal performance including increased mass and milk production, and increases ammonia assimilation [4–6].

Fructan synthesis and metabolism in grasses and cereals are complex. Fructans consist of linear or branched fructose chains attached to sucrose. The chain length of plant fructans ranges from three up to a few hundred fructose units. Different types of fructans can be distinguished based on the linkage types present. In perennial ryegrass, three types of fructans have been identified: inulins, inulin neoseries and levan neoseries with four fructosyltransferse (FT) enzymes involved in this fructan profile. The enzyme 1-SST (sucrose: sucrose 1-fructosyltransferase) catalyses the first step in fructan biosynthesis while the remaining enzymes elongate the growing fructose chain (1-FFT: fructan: fructan 1-fructosyltransferase, 6G-FFT: 6-glucose fructosyltransferase, and 6-SFT: sucrose: fructose 6-fructosyltransferase). The enzymes 1-FEH or 6-FEH (fructoexohydrolase) reduce fructan chain length by releasing fructose molecules.

Fructans accumulate in the stems and leaf sheaths with the majority of the accumulation in the leaf sheaths or pseudostem at the base of the tillers. This has driven research and development efforts towards increasing water soluble carbohydrate levels in grasses used in improved pastures, both through molecular breeding and biotechnology. Fructan synthesis and metabolism is complex. In grasses, the level and composition of fructans can be increased in stems and leaf sheaths through the engineered expression of fructosyltransferase (FT) genes [7–9]. This, however, does not increase significantly the level of high degree of polymerization fructans (high DP fructans) in leaf blades, the tissues which are normally eaten by the large grazing animals in the field. In leaf blades, the expression of members of FT family genes involved in fructan polymerization is generally low, but can be induced by a number of abiotic stresses. Thus, accumulation of high DP fructans in leaves could provide more accessible nutrition for grazing animals.

Therefore, fructans in mature leaf blades accumulate less than in leaf sheaths and stems [10]. In order to specifically increase the level of fructans in leaf blades, a strategic approach was devised that coordinately expresses fructan biosynthesis genes in photosynthetic tissues. This involved metabolic re-programming of photosynthetic cells for enhanced sucrose and fructan production.

2. Results

2.1. Production of Transgenic Plants

The results of the biolistic transformation of embryogenic calli of perennial ryegrass are illustrated in Figure 1. The bombardment of 500 calli for each of the transformation vectors led to the recovery of between nine and 44 transgenic plants per vector that were confirmed to be positive for both the selectable marker and gene of interest (Table 1).

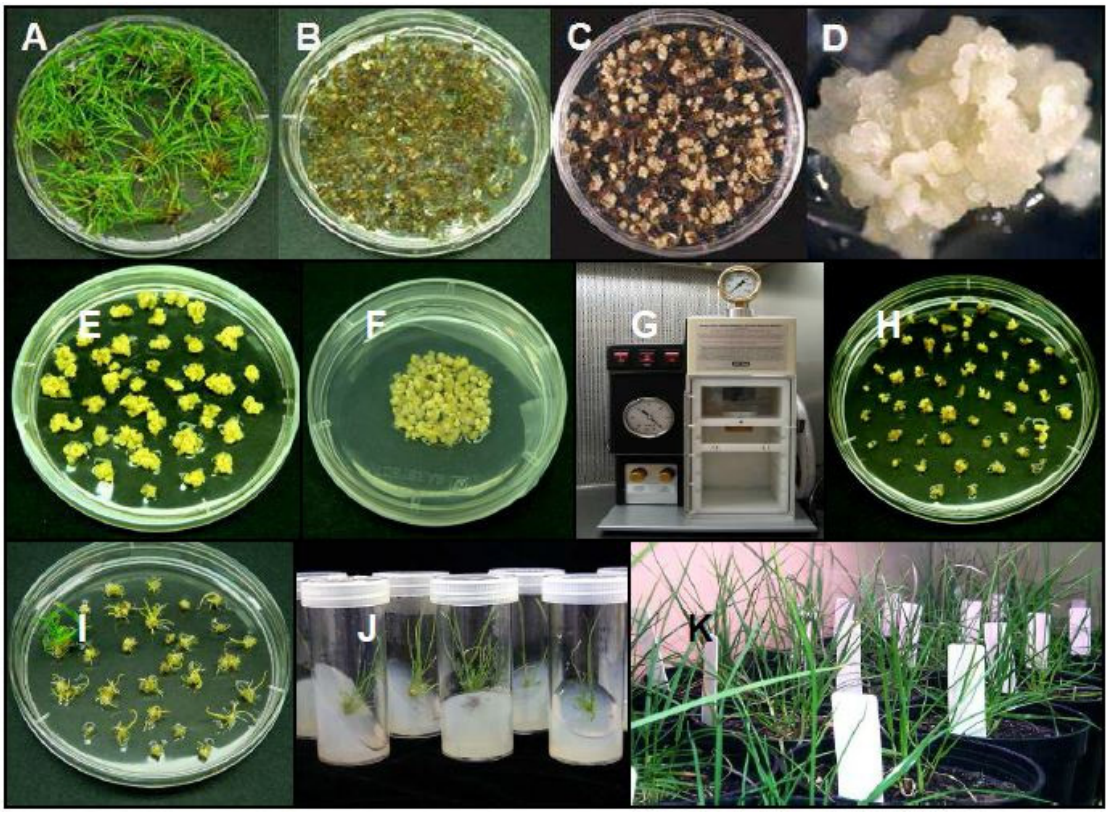

Figure 1. Production of transgenic perennial ryegrass plants from microprojectile bombardment of shoot meristem-derived calli. (**A**) Donor material for shoot meristems; high vegetative biomass, nil-to-low root development; (**B**) Distribution of basal meristematic material on callus initiation medium; (**C**) Proliferation of callus from basal meristematic regions; (**D**,**E**) Proliferation of embryogenic callus derived from basal meristems; (**F**) Distribution of calli of high osmotic medium prior to biolistic transformation; (**G**) Biolistic transformation device, PDS-1000/He; (**H**,**I**) Growth and development of hygromycin-resistant shoots, 30–75 days after bombardment; (**J**) Growth and development of hygromycin-resistant shoots in vitro; (**K**) Hygromycin-resistant plants established in soil and grown under glasshouse containment conditions.

Table 1. Summary of the transformation progress for perennial ryegrass with wheat photosynthetic-regulated expression of *Lp1-SST* and *Lp6G-FFT* and fusion open reading frames (ORFs).

Species	Genotype	Transforming DNA	No Plates Bombarded	No calli Bombarded	No +ve Transgenics	No Plates Analysed	*hph* +ve Plants	GOI +ve Plants
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST::Tarbcs +pACH1*	10	500	46	46	37	32
L. perenne	FLP 418-20	*TaRbcS::Lp6G-FFT::Tarbcs +pACH1*	10	500	50	50	48	38
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST-Lp6G-FFT::Tarbcs (1) + pACH1*	10	500	47	47	47	44
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST-Lp6G-FFT::Tarbcs (3) + pACH1*	10	500	26	26	26	23
L. perenne	FLP 418-20	*TaRbcS::GUS::Tarbcs +pACH1*	10	500	13	13	11	9

2.2. Biochemical and Morphological Characterisation of Transgenic Plants

During the regeneration of the putative transgenic perennial ryegrass lines, differences in growth phenotypes were noticed between the lines. Both the tissue culture regenerants and corresponding soil grown plants from both of the fusion-1 and fusion-3 transgenic lines showed a superior vigour phenotype compared to the transgenic plants containing either *TaRbcS*::*Lp1-SST*, *TaRbcS*::*Lp6G-FFT*, *TaRbcS*::*GUS* or control plants containing only the selectable marker, *hph* (Figure 2).

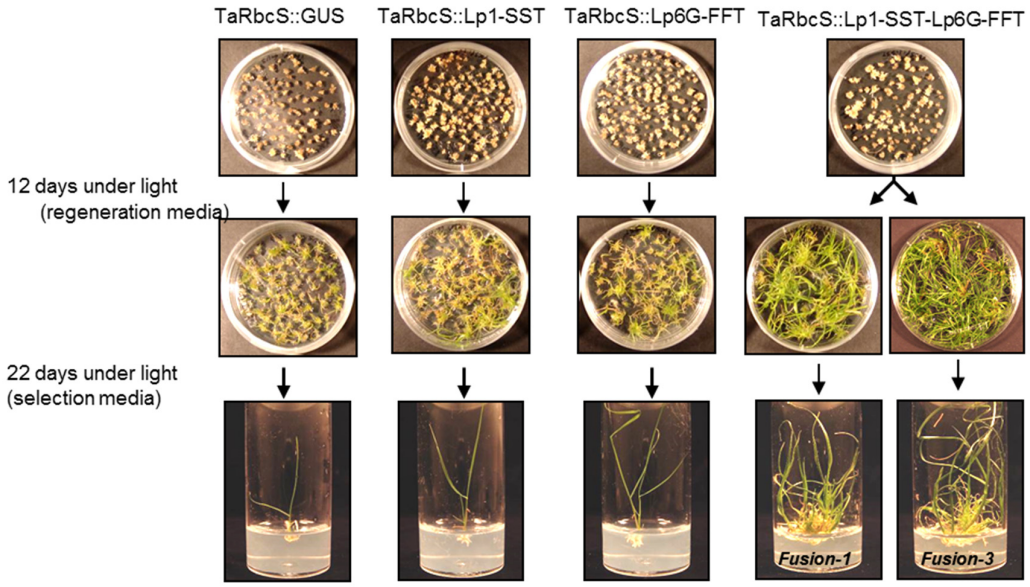

Figure 2. Plant regeneration phenotypes of transgenic perennial ryegrass (FLP-418-1) after co-transformation with the light-regulated gene constructs (Table 1) and the *pAcH*1 vector, with selection on hygromycin. The plants that contain either of the *Lp1-SST-Lp6G-FFT* fusion constructs consistently perform better in tissue culture (far right column).

The plants showing the phenotype were confirmed to contain the gene-of-interest (GOI) using real-time PCR and Southern hybridization analysis [11]. The superior growth phenotype of the transgenic fusion-1 and fusion-3 lines was first observed during the early stages of plant regeneration conducted on plates. Specifically, just 12 days after incubation under lights, the calli showed further developed green shoots. The fast growth rate of the fusion transgenic plants became more evident 22 days after transferring to rooting media. Transgenic plants containing either fusion-1 or fusion-3 constructs showed an obvious increase in tiller number. In addition, the fusion transgenics consistently showed a higher tiller density per plant compared to control lines (Figure 2).

Following transfer to soil and propagation under glasshouse conditions, more specific differences were observed between the fusion-1 and fusion-3 lines. Even though both fusion plants displayed more vigorous growth, fusion-1 lines had longer, thicker and slightly darker green leaf blades. Also, the plants were physically more robust with thicker leaf sheaths and leaf blades. Fusion-3 lines continued to grow faster than the other control plants with longer leaf blades and more vigorous tiller growth, but the leaf morphology was more similar to wild-type. An increase in root biomass was also observed in both fusion-1 and fusion-3 soil grown plants (Figure 3).

The control transgenic lines harbouring either the *Lp1-SST* or *Lp6G-FFT* as single genes did not show the increased growth rate that was observed in the fusion-1 and -3 lines. Their appearance is similar to each other, although some developed more vigorously than the transgenic plants containing either Gus or *hph* (Figure 3).

Leaf blades from individual plants were cut and hand sectioned and viewed under a microscope (Figure 4). There were apparent differences in the number of cells with chloroplasts: being more in both of the transgenic fusion lines than in the control plants. In addition, chloroplasts were present in cells located on the abaxial side (lower part of the leaf) of transgenic plants, despite both plants being grown under the same light conditions in the growth room. Sometimes it was observed that control plants produced more chloroplasts in mesophyll cells located on the adaxial side (upper side which faces the light source) than on the abaxial side, whereas the transgenic plants most often produced a near-equal number of chloroplasts on both sides. It was not possible to determine difference in cell size or overall cell numbers from hand-made sections.

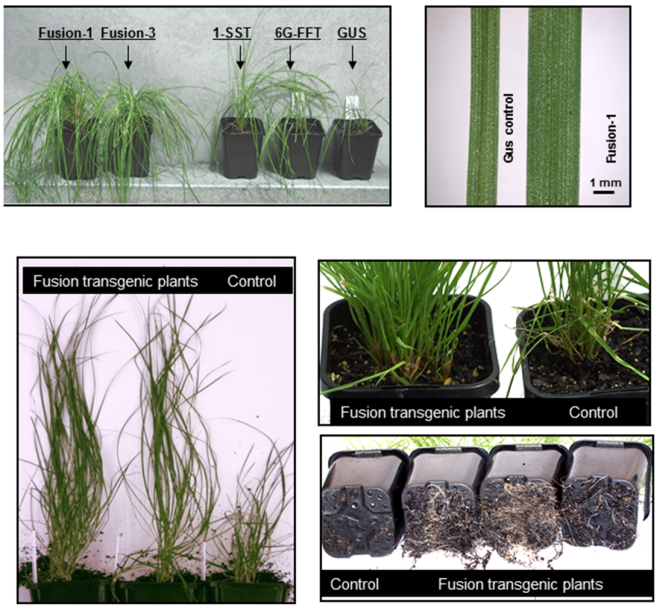

Figure 3. Mature plant phenotypes. Representative samples of transgenic plants at vegetative stage. The plants were trimmed equally three weeks earlier. Close-up micrographs of the leaf blades (right). The fusion consistently has wider blades compared to control plants.

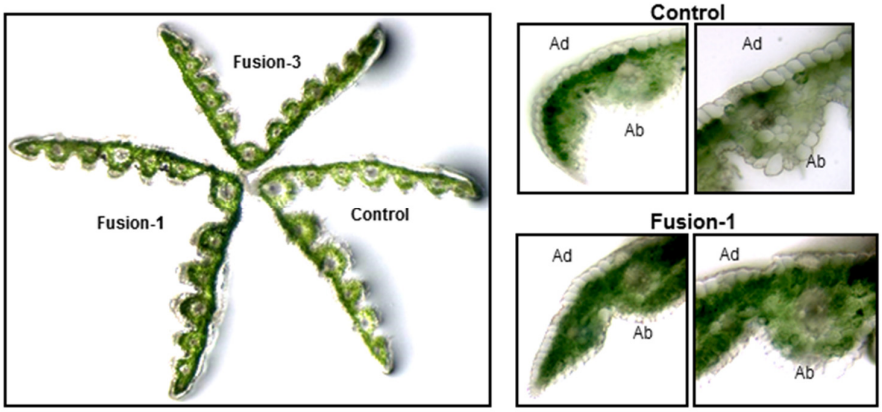

Figure 4. Leaf phenotypes. Representative samples of hand sections of leaf blades at the vegetative stage. Left shows a comparison of whole leaf sections; right shows magnified areas of leaf sections. Ad-Adaxial, Ab-abaxial.

Biochemical analysis by HPAEC of water soluble carbohydrates extracted from independent transformants harbouring the *TaRbcs::FT-Fusion-1, TaRbcs::FT-Fusion-3, TaRbcs::Lp1-SST, TaRbcS::Lp6G-FFT*, and two control lines (*hph* only) showed that the fusion-1 and fusion-3 transgenic plants contained significantly higher levels of total fructans (Figure 5), showing up to a 2.5-fold increase over the control lines (Figure 6).

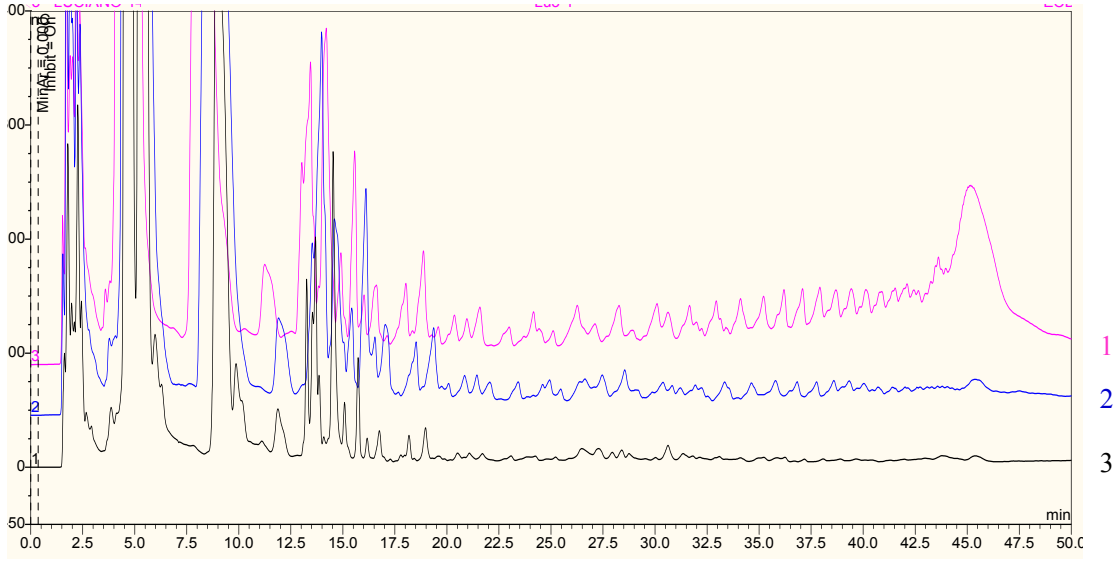

Figure 5. Biochemical analysis (HPAEC) of fructan level and composition present in transgenic TaRbcs::Fusion-3 (1), TaRbcs::Fusion-1 (2) and control plants (3) harbouring only the selectable marker (*hph* gene).

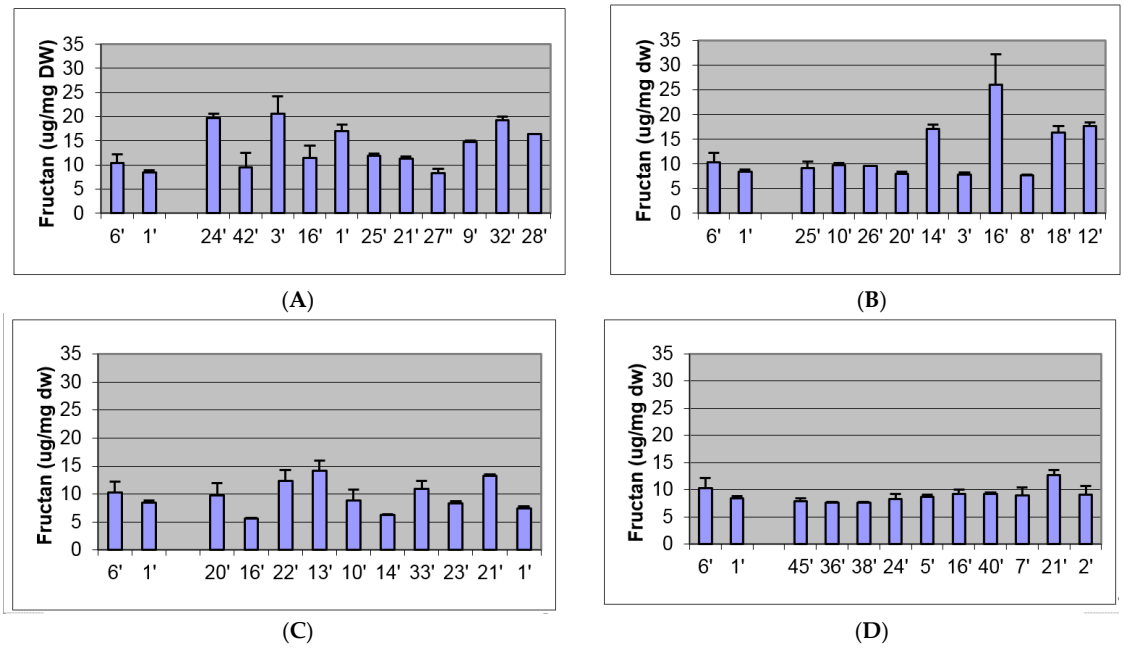

Figure 6. Biochemical analysis (HPAEC) of total fructans present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-1, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-3, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::*6G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

In addition, the levels of 1-kestose were up to four times higher in fusion-1 lines, and three times higher in fusion-3 lines compared to the *hph* controls (Figure 7). In the *TaRbcs*::*Lp1-SST* plants, 1-Kestose increased up to three-fold whereas total fructan content only increased 0.5-fold. In contrast, 1-kestose levels in the *TaRbcS*::*Lp6G-FFT* transgenic plant lines showed marginal increases in 1-kestose (up to 0.5-fold) and only one line showed a small increase in total fructans (Figure 7). Analysis of

sucrose contents of all the lines revealed that some of the high fructan lines also showed an increase in total sucrose content (Figure 8).

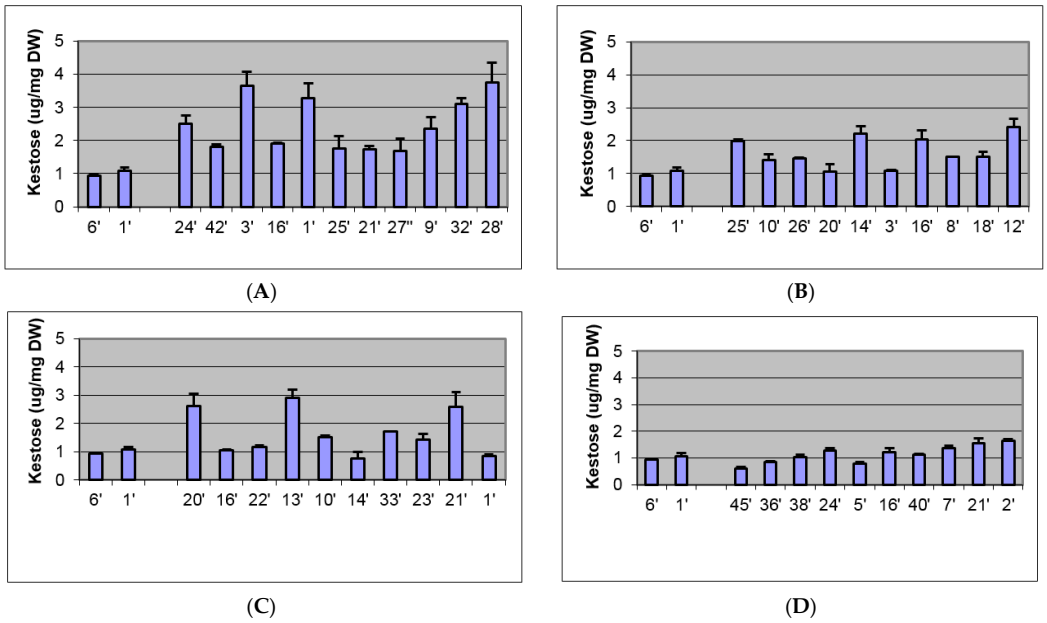

Figure 7. Biochemical analysis (HPAEC) of 1-kestose present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-1*, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-3, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::*6G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

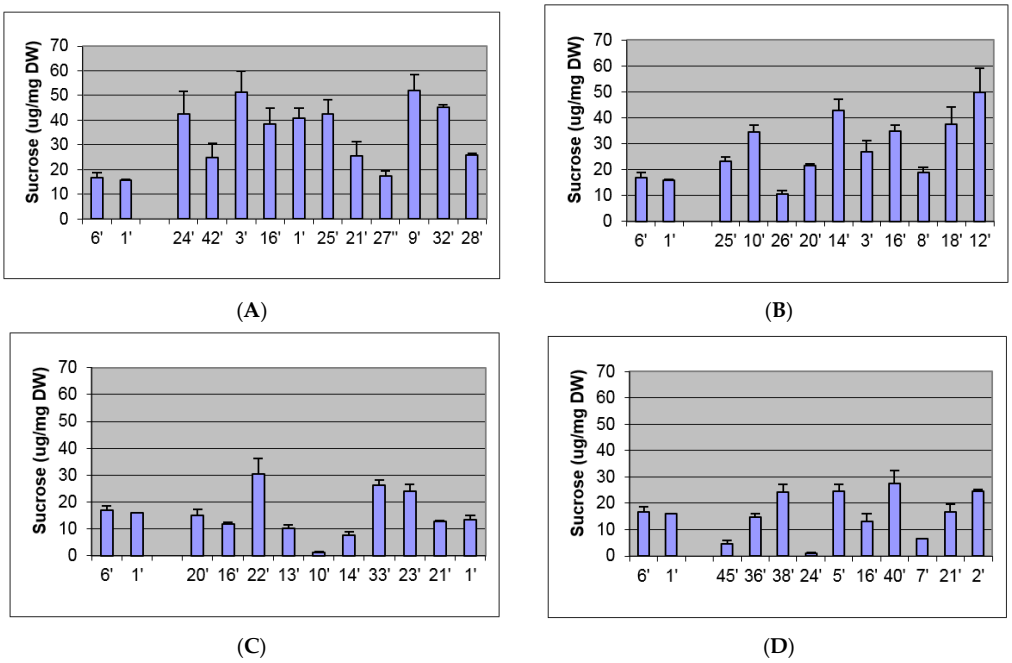

Figure 8. Biochemical analysis (HPAEC) of sucrose present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-1*, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-3*, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::*6G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

3. Discussion

The main storage carbohydrates in perennial ryegrass are high molecular weight fructans with a prevalence of β(2-6) linkages [10,12,13]. The expression of enzymes involved with fructan biosynthesis (*6G-FFT, 1-SST and 6-SFT*) [12,13] is highly tissue-specific and tends to be associated with the base of leaves and leaf sheaths/pseudostems which are also the site of fructan accumulation in perennial ryegrass which is consistent with the role of fructans as storage carbohydrates to support the regrowth of leaves [14] and their potential role in osmoregulation and membrane protection during drought and water stress [15].

In this research, we have demonstrated that through the development of transgenic plants with fructan biosynthetic genes under the control of photosynthetic promotors, not only have concentrations of fructan, kestose and sucrose been increased overall but also in the leaf blades. This is of key importance if the aim of increasing fructan concentrations is to improve the nutritive value of perennial ryegrass through increasing the concentration of water-soluble carbohydrates [16]. Increasing the concentration of water-soluble carbohydrates is important for grazing ruminants as it serves to increase both the energy concentration of the herbage but also plays a role in improving the synchrony of fermentation in the rumen and hence the efficiency of conversion of ingested grass into animal products [17]. Comprehensive bioeconomic modelling of the impact of an increase of 1 MJ/kg in dairy pasture has been shown to have the potential to increase the profitability of dairy farms both directly through the increased nutritive value of the herbage and indirectly through the reduction in the use of purchased supplementary sources of energy such as grain or pellets [18].

The mechanism of the high biomass phenotype observed in some transgenic events in this study is unknown and it is acknowledged that the phenotype was measured in primary transformants but it is clear that the combination of increased productivity combined with increased concentrations could lead to benefits over and above those described by Ludemann et al. [18].

Previous work has shown that it is important to combine high energy traits with good agronomic adaption to the target environment, otherwise the trait is not always expressed or the seasonal growth pattern may not suit the needs of the grazing enterprise [17,19]. The transgenic events described in this paper are in a tissue culture-responsive genotype from an elite perennial ryegrass breeding line and further work is planned to evaluate these events under field conditions and to further cross them into a broader range of backgrounds to investigate the effects of genotype x environment interactions on the expression of the transgene.

4. Materials and Methods

4.1. Identification and Cloning of Photosynthetic Promoters from Perennial Ryegrass

The expression of *RbcS* and Chlorophyll a/b Binding Protein (*CAB*) is well characterised by light-regulated genes in higher plants.

Both *LpRbcS* and *LpCAB* genes were chosen for promoter discovery and isolation in perennial ryegrass. Publicly available cDNA sequences (*LpRbcS*, *EC*778430 and *LpCAB*, *EC*778438) were used as query sequences in a BLAST search of the perennial ryegrass EST database in our in-house database. As both genes are members of multigene families, several contigs (each contig represents an individual gene) were identified in our perennial ryegrass EST collection. Nine contigs were identified to be homologous to the published *LpRbcS* cDNA sequence and thirteen contigs were found to be homologous to the *LpCAB* cDNA sequence. Two contigs, *LpRbcS* (*LPCL9_C*359) and *LpCAB* (*LpCL*1112_*C*12), representing the genes of the promoters to be isolated, contained (47) and (19) EST sequences, respectively. These sequences came from a variety of libraries representing a range of different tissues. This data was used for in silico expression analysis and indicated that both genes are only expressed in photosynthetic tissues.

DNA sequence alignments for each of the gene family members were performed, and gene-specific primers were designed for contigs *LpRbcS_C*359 and *LpCAB_C*12 and used to screen perennial ryegrass

BAC DNA pools by PCR. The BAC clones were identified and sequenced. Primers were designed and the *Lolium perenne*-specific promoter regulatory sequences were cloned, sequenced and the *cis*-regulatory sequences specific for photosynthetic promoters were identified by PLACE (Table 2). The sequences included the I-Box motif and the GT1 box for RbcS [19,20]. In addition, 16/19 nucleotides of the LpRbcS sequence shared homology with the monocot Rbs Consenesus sequence [21]. The I-Core box and SORLIPs *cis*-regulatory sequences were present in the CAB promoter. SORLIPs were found to be over-represented in light-induced promoters in Arabidopsis [22].

These *L. perenne*-specific promoter regulatory sequences were subsequently used in the construction of backbone-free expression cassettes with fructan biosynthesis genes.

Table 2. The position of the *cis*-regulatory sequences identified by PLACE. Common *cis*-acting regulatory sequences are listed [19,21,22,24]. Positions noted are the first nucleotide in the sequence relative to the ATG. (n.p.—not present).

cis-Acting Regulatory seq.	Accession	Position *LpRbcS*	Position *LpCAB*
I-Box Core	S000199	−184	−137
I-Box	S000124	−311	−137
GT1 consensus	S000198	−304	n.p.
RbcS monocot seq	Schaffner et al., 1991	−173 to −151	n.p.
SORLIPs	S000482	n.p.	−58, −217, −647, −695

4.2. Isolation of Fructan Biosynthesis Genes

The *Lolium perenne* cDNA clones encoding sequences for *Lp1-SST* and *Lp6G-FFT* have previously been isolated from a perennial ryegrass cDNA library [12,23]. The complete gene sequences of the isolated perennial ryegrass fructosyltransferase homologues are publicly available.

4.3. Cloning of FT Translational Fusion

It has been proposed that FT proteins may physically associate with each other to facilitate the efficient biosynthesis of fructans. Therefore, a genetic fusion was created between the open reading frames for *Lp1-SST* and *Lp6G-FFT*.

The *Lp1-SST* gene was PCR-amplified with a GATEWAY recombination site incorporated in the forward primer. A sequence that codes for three glycine residues followed by a *Hind* III site was incorporated into the reverse primer, with the stop codon removed. The *Lp6G-FFT* gene was PCR-amplified with a *Hind* III site followed by a sequence that codes for three glycine residues and the gene-specific sequence without the ATG. The reverse primer for the *Lp6G-FFT* gene was flanked by a second GATEWAY recombination site. The primer sequences are provided in Table 3. The purified fragments were digested with *Hind* III and the ligated product was cloned into the Invitrogen GATEWAY pDONR221 entry vector. When the resultant pENTRY1-*Lp1-SST-Lp6G-FFT*-2 entry clones were sequenced, one sequence (fusion-1) was confirmed to be the predicted product, with eight amino acids in the linker joining the two genes. However, another sequence (fusion-3) contained two consecutive *Hind* III sites, which would result in the addition of another two amino acids, giving a total of ten amino acids between the two FT genes upon translation.

Table 3. Primer sequences used to amplify the PCR fragments used to generate the translational fusion of the *Lp1-SST* and *Lp6G-FFT* fructosyl transferase genes (*FT Lp1-SST*::*Lp6G-FFT*). Black sequences are gene-specific, blue and red (*Hind* III RE site) sequences are nucleotides introduced to generate the linker region, and green nucleotides represent the recombination-specific sequences.

Gene	Forward Primer	Reverse Primer
Lp1-SST	GGGGACAAGTTTGTACAAAAAAGCAGG CTTCATGGAGTCCCCAAGCGCCGTC	TCTAAGCCTTTCCTCCTCCCAAGTCG TCGTTCGTG
Lp6G-FFT	ACTAAGCTTGGAGGAGGAGAGTCCAG CGCCG	GGGGACCACTTTGTACAAGAAAGCTGGG TCCTACATGTCGTCAGCCAAGAAGGCC

4.4. Generation of Vectors for Transgenic Assays

A number of vectors were constructed using Invitrogen Multisite Gateway™ technology based on recombinational cloning. This methodology relies on the generation of individual entry plasmids containing either the promoter, gene of interest (GOI), or terminator sequences flanked by recombination sites. The recombination sites facilitate the directional triple insertion of each of the entry plasmids into a gateway-enabled destination vector, by recombination. The final vector is then sequenced and used directly for plant co-transformation with a plasmid for expression of a plant selectable marker.

In order to test the function of the fusion protein, the FT fusion-1 and FT fusion-3 ORFs were cloned under the control of the enhanced cauliflower mosaic virus (CAMV)35S^2 promoter [25], using the Invitrogen Multisite Gateway™ Technology recombination into *Agrobacterium* binary vector [26].

Gateway entry vectors were constructed for the (CAMV)35S^2 promoter, the *TaRbcS* terminator sequence, as well as FT fusion-1 and FT fusion-3 ORFs. The cloned fragments were sequence-verified and used for three-way recombination cloning with the cloned GOI cDNA sequences into the *pPZP*200*ubi*:*bar-nos* R4 R3 destination vector. In addition, constructs also included the *Lp6G-FFT* and *Lp1-SST* single ORF driven by the (CAMV)35S^2 promoter as controls. The *Lp*1-*FFT* single ORF will also be cloned in the same manner. As a control, the GUS ORF was used for confirmation of expression. The following constructs were made.

- pPZP200: 35S^2::*Lp6G-FFT*::*TaRbcS*
- pPZP200: 35S^2::*Lp1-SST*::*TaRbcS*
- pPZP200: 35S^2::*Lp*1-*FFT*::*TaRbcS*
- pPZP200: 35S^2::*Lp1-SST*::6*G-FFT*::*TaRbcS*
- pPZP200: 35S^2::*GUS*::*TaRbcS*

4.5. Function of Lp1-SST, Lp6G-FFT and FT-Fusion Protein in Transient Transgenic Assays

For proof-of-function, transient expression of the 35S promoter constructs was conducted in tobacco plants, as they do not naturally store fructans. The method involved agro-infiltration of the individual constructs into *N. benthamiana* leaves [27,28] followed by biochemical analysis by anion exchange. Three days after the injection, the plant material was harvested and the water-soluble carbohydrates were extracted using a hot water extraction method. The extracts were separated using high performance anion exchange chromatography (HPAEC). The results showed production of fructans, with the increased production of both 1-kestose and 6G-kestose by the fusion protein data (not shown) so vectors were designed for the stable transformation of perennial ryegrass.

4.6. Generating Vectors for Stable Transformation

A 695 kb promoter fragment from a previously published sequence containing the TATA signal from the *TaRbcS* gene (NCBI accession number AB042069) was PCR-amplified with Gateway™ (Invitrogen) recombination sites at the primer flanks. The fragment was cloned into the Invitrogen pDONRP4-P1R entry vector using Gateway™ recombination technology. The 696 bp *TarbcS* gene termination signal sequence [29] was also PCR-amplified using primers with recombination sites and cloned into the Invitrogen pDONRP2-P3R entry vector. The cloned fragments were sequence-verified and used for three-way recombination cloning with the cloned GOI cDNA sequences into the pDEST-R4R3 destination vector: pDESTR1-R2R-*Lp1-SST*, pDESTR1-R2-*Lp6G-FFT*, and pDESTP1-P2R-*Lp1-SST-Lp6G-FFT* gene fusion expression vectors. The following constructs for photosynthetic-regulation of expression of fructosyltransferases by the *TarbcS* promoter to be used are outlined below.

- pDEST-*TaRbcS*::*Lp1-SST*::*TaRbcS*
- pDEST-*TaRbcS*::*Lp6G-FFT*::*TaRbcS*

- pDEST-*TaRbcS*::*Lp1-SST-Lp6G-FFT*::*TaRbcS* (fusion-1)
- pDEST-*TaRbcS*::*Lp1-SST-Lp6G-FFT*::*TaRbcS* (fusion-3)
- pDEST-*TaRbcS*::*GUS*::*TaRbcS*

4.7. Production of Transgenic Ryegrass Plants

Biolistic co-transformation of perennial ryegrass with the vectors containing the *TaRbcS* regulatory sequences, driving the expression of individual fructan genes or as a translational fusion, and the *pAcH*1 vector for hygromycin resistance was conducted on embryogenic calli for perennial ryegrass using the method of Spangenberg et al. [30]. The pAcH1 vector was previously constructed and has been used successfully in plant transformation experiments [30–34]. The GUS marker gene was also cloned as a positive control. Table 2 summarises the transformation and molecular analysis for the generation of these lines. Following agarose gel electrophoresis, the resulting DNA fragment was purified from the agarose gel prior to being used for plant transformation to produce DNA without vector backbone sequences. The pAcH1 vector previously constructed and used successfully in plant transformation experiments was also digested with restriction enzymes to produce a DNA fragment for the expression of the selectable marker only [30–34].

A tissue culture responsive genotype, FLp418-20, was selected for use in these experiments on the basis of observed shoot regeneration from embryogenic callus. Clonal replicates of this genotype were used to provide the material for biolistic transformation using the vectors described above and following the method of Spangenberg et al. [30] and illustrated in Figure 1.

4.8. Transgene Detection

The presence of the transgene and selectable markers were confirmed using the methods described in detail by Badenhorst [11]. In summary, following growth in selectable media during tissue culture, the presence of the transgene was confirmed using real-time PCR and Southern hybridisation. Real-time PCR results were scored in comparison to positive (plasmid DNA) and negative (non-transgenic plant DNA, no-template) control templates with the endogenous histone H3 gene (*LpHisH*3) included as a control [11]. Southern hybridization of genomic DNA with chemiluminescent detection was used to visualize the results of probes designed for the *RBcS* or *LpFT*1 promoter, the *LpFT*4 terminator and the *hph* selection cassette [11] (results not shown).

4.9. Quantification of Carbohydrates

Concentrations of monosaccharides and fructan in plant tissue samples were quantified using a high performance-anion exchange chromatography (HP-AEC) method described by Liu et al. [35].

5. Conclusions

Transgenic events were developed with altered fructan accumulation patterns with increases in fructan accumulation and greatly increased accumulation of fructan in leaf blades as opposed to the traditional site of fructan accumulation in the pseudostem. This altered site of fructan accumulation has potential benefits for animal production as leaf blades form the major part of the diet of grazing ruminants. Some of the transgenic events also exhibited enhanced biomass production. This combination of high quality and enhanced yield is of great interest to forage plant breeders and whilst the expression of these phenotypes needs to be confirmed under field conditions, the identification and characterisation of the transgenic events described in this paper validate the potential for the manipulation of fructan biosynthesis in perennial ryegrass.

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