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Seasonal Carbohydrate Dynamics and Climatic Regulation of Senescence in the Perennial Grass, Miscanthus

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Genetic Differentiation in Response to Selection for Water-Soluble Carbohydrate Content in Perennial Ryegrass (*Lolium perenne* L.)

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Abstract Plant carbohydrates are of increasing interest as renewable feedstocks to replace petrochemicals in the generation of fuels and production of high-value chemicals. Greater understanding of the genetic control of diversity in fructan synthesis and accumulation would facilitate more directed channelling of feedstock to process in a ryegrass biorefinery. Divergent populations produced by phenotypic selection for water-soluble carbohydrate content have been used to investigate relationships between traits, and to identify patterns of genetic differentiation which indicate genomic regions under high and low selection pressure. Selection for high water-soluble carbohydrate content was associated with increased synthesis of large fructan polymers and increased accumulation of above-ground plant biomass, particularly during spring. Three rounds of selection and two rounds of recombination resulted in widespread genetic differentiation across the whole genome, causing reduced allelic richness and increasing homozygosity at some loci. A smaller number of loci were shown to be subject to high selection pressure. Breeding material subjected to many years of selection for water-soluble carbohydrate also showed allelic differences which may reflect the consequences of high selection pressure at some of these same loci. However, some of the loci unaffected in the divergent selection experiment showed similar effects. This might arise from differences in linkage disequilibrium in these two sets of plant materials, but more likely from the

different genetic background of the germplasm. This illustrates the complex nature of the water-soluble carbohydrate trait in perennial ryegrass.

Keywords Allelic richness · Biorefining · Dry matter yield · Fructan polymers · SSR markers · Sugars

Abbreviations

ANOVA	Analysis of variance
C2 populations	Second-generation selected populations after two rounds of recombination (C2 ^s with random selection, C2 ^{s-} with negative selection for WSC content, C2 ^{s+} with positive selection for WSC content)
CP	Crude protein
DM	Dry matter
DP	Degree of polymerisation
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High-performance liquid chromatography
LTS	<i>Lolium</i> test set
QTL	Quantitative trait locus/loci
SNP	Single-nucleotide polymorphism
SSR	Simple sequence repeat
WSC	Water-soluble carbohydrate

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Introduction

Plant carbohydrates are of increasing interest as renewable feedstocks to replace petrochemicals in the generation of fuels and production of high-value chemicals. In many temperate regions of the world, a large proportion of land area is occupied by grassland. Historically, this has been used

for livestock production, and forage grasses, particularly ryegrasses, have been improved to provide increased nutritional value. One approach for achieving this has been by increasing water-soluble carbohydrate (WSC) content. Considerable genetic variation for WSC is available in perennial ryegrass and has been characterised to some extent [1]. It is this variation that has enabled the recurrent selection breeding programme at Aberystwyth to realise significant gain in WSC content over the past 30 or so years [2, 3]. Consequently, high-sugar grasses offer the potential to produce high sugar yield for biorenewable applications without major change to the landscape.

Ryegrasses store their carbohydrate reserves in the form of polymers of fructose, water-soluble sugars called fructans, rather than as starch. Perennial ryegrass produces a diverse range of fructan molecules with different bond types ($\beta(2,1)$ and $\beta(2,6)$) which may be branched and/or linear and vary in length from 3–90 fructose units [4]. Potentially, this leads to the presence of a complex range of molecules available for conversion in a biorefinery. Different molecules may be particularly appropriate for specific applications. For example, some polymers may be suitable for the production of biosurfactants; carbohydrate surfactants are of interest because they are biodegradable and non-toxic to the environment [5, 6].

Greater understanding of the genetic control of diversity in fructan synthesis and accumulation would facilitate more directed channelling of feedstock to process. As part of the EU GRASP project (<http://www.grasp-euv.dk/>), trait-specific selections were carried out over two generations using populations derived from an initial set of 20 test genotypes (the *Lolium* test set (LTS)) in order to examine changes in allele frequencies associated with divergent selection as a means of validating single-nucleotide polymorphism (SNP) markers in candidate genes. One set of divergent populations was developed from test genotypes which included two members of the ryegrass WSC F2 mapping family in which WSC quantitative trait loci (QTL) have been characterised [1]. Significant changes in total WSC content were demonstrated in the GRASP divergent selections [7]. In addition, Farrar et al. [7] reported the possible co-selection of biomass accumulation during the experiment. Such straightforward co-selection would be extremely valuable if it could be demonstrated to be stable and consistent. The source of high WSC in this plant material came predominantly from the WSC mapping family LTS parents [8]; it would be expected mainly from Aurora. Aurora is a very early-heading cultivar and it might be that the biomass data published arise from early spring growth linked to flowering time [9]. The data of Farrar et al. [7] were for early spring only, and it is therefore unknown whether the positive effect observed on spring biomass accumulation is maintained over the growing season or whether a trade-off exists between spring growth and biomass accumulation later

in the growth season. Comparison of the high WSC and low WSC selections revealed differences in allele frequency for only one of the six candidate genes studied (soluble acid invertase 1:4). Simple sequence repeat (SSR) markers were included in this GRASP experiment as putative neutral markers. However, SSRs linked to QTL regions will not necessarily prove to be neutral. For a complex trait, like WSC content, under multi-locus control, significant numbers of SSRs may be located next to QTL regions and would show selection effects. Indeed, Turner et al. [8] showed preliminary evidence of such effects and, in fact, selection effects on these markers may provide further insight into the genetic control of the trait being studied.

These divergent populations from the GRASP project provide excellent material for further study of the genetic control and physiology of the high-sugar trait. The aims of the work described here were threefold: first, to examine the co-selection of traits (sugar/fructan content and biomass production) in the GRASP experiment in more detail to confirm the associations of these traits; second, to extend the set of SSR markers to cover more of the genome and to study patterns of genetic differentiation in the second-generation populations (after two rounds of recombination (C2)) to confirm the widespread nature of the selection effects; and third, to examine certain genomic regions (identified by the GRASP experiment as being under high and low selection pressure during selection for WSC content) in early- and advanced-generation high-sugar ryegrass cultivars to investigate the generality of their role in the genetic control of WSC content.

Materials and Methods

Plant Material

The structure of the GRASP selection experiment, along with basic maintenance and characterisation of plant material in the glasshouse, was previously described by Farrar et al. [7]. Briefly, a synthetic C0 of 600 plants was first created by pair-crossing four plants (LTS01, LTS05, LTS09 and LTS18) with contrasting WSC phenotypes from the GRASP LTS of 20 plants. The 3-year mean total WSC content (by anthrone colour reaction) of samples taken from potted plants of these founder plants in the glasshouse during spring and autumn ranged from 16.4 to 22.3 % dry matter (DM). The range was 16.9 to 25.9 % DM in 2007, the year samples were taken from the C2-selected populations in the GRASP selection experiment. Further information on these four LTS founder plants was reported by Posselt et al. [10]. The C0 population was randomly divided into two. One half underwent three rounds of divergent selection for WSC content at a selection pressure of 10 % and two rounds of recombination whilst the other half was subject to random selection under the same conditions.

The phenotypic scores used for selection were from anthrone measurements of total WSC content of all herbage above a cut at a stubble height of approximately 4 cm. This above-ground biomass was predominantly leaf but included some sheath/tiller base material. For each generation, these procedures produced full populations of 300 unique individual plants (C, C⁻ and C⁺) from which selected populations of 30 plants (C^s, C^{s-} and C^{s+}) were identified for polycrossing to produce the following generation. The populations analysed here are those from the second generation and all members of the populations are genetically unique individual plants. They have been renewed each year from a sub-set of a few tillers and are currently still maintained in 130-cm pots in a frost-free glasshouse.

Additionally, two ryegrass cultivars were grown for genetic analysis. Pre-basic seed of AberElan (not actively selected for WSC) and AberMagic (a recent high-sugar grass cultivar selected for WSC and yield) was sown in March 2011 in an unheated, unlit glasshouse. Fresh young leaf material was sampled in August 2011 from 96 individual plants of each cultivar for DNA extraction and marker analysis. Mean herbage WSC content of the same plants was measured in the glasshouse in March 2012 and was 16.4±1.45 % DM for AberElan and 22.1±2.26 % DM for AberMagic.

Field Experiments

Tillers were removed from the 900 C2 generation plants (300 C2 random, 300 C2⁻ negative and 300 C2⁺ positive populations) soon after germination in August 2006 (the year the seed was produced) and used to establish a field trial of spaced plants for assessing heading date the following spring. This trial was close to the institute on an open site close to sea level and exposed to westerly winds. The soil was a well-drained, permeable, medium loam. Single copies of each individual plant were planted out in October 2006. The plants were randomised within, but not between populations. Rows of 10 plants at a spacing of 30 cm were surrounded by a border row of the cultivar Parcour. Fertiliser (60 kg/ha N:P:K, 5:24:24) and a spray to control broad-leaved weeds were applied in March 2007. Heading date was scored on individual plants as the first date, in days after April 1, when three heads had emerged from the flag leaf. No variation in heading date was noticed in the border plants, suggesting few positional effects occurred within the scale of the spaced plant field trial.

Also, in August 2006, and as part of a larger trial of various test populations and controls, small 2-m² plots of the three C2 populations were sown with further seed from the original stocks. The conditions at the field site were the same as for the spaced plant trial. A seed rate of 3 g/m² was used in a fully randomised design with four blocks. The plots were cut back and broad-leaved weeds controlled with a single spray at the

end of September 2006. Before the winter, a further cut was carried out in October 2006. The plots received 100 kg/ha nitrogen in the sowing year. In all subsequent years (2007, 2008, 2009), they received 400 kg/ha nitrogen divided into eight applications; two in early spring and one after each cut. The plots were harvested for analysis at 5–6-week intervals from April to October in 2007, 2008 and 2009 with a Haldrup plot harvester set at a cutting height of approximately 6 cm. The fresh weight of the full sample was measured in the field. A sub-sample of between 300 and 400 g fresh matter was dried overnight in an oven set at 80 °C and DM yield calculated from DM percentage of the sub-sample. In year 1, around half of the dry sample was milled to pass through a 2-mm screen, and this was further sub-sampled for chemical analysis of nutritional value by near infra-red spectroscopy (NIR), calibrated against a suitable validation set. Crude protein (CP) was calculated from nitrogen content with a multiplication factor of 6.25. The yields of the individual harvests were summed to give annual yields.

Glasshouse Experiments

Some of the samples from the experiment previously described by Farrar et al. [7] were used for part of this study. Briefly, single copies of the 900 C2 generation plants (300 C2 random, 300 C2⁻ negative and 300 C2⁺ positive populations) were grown in 90-cm pots, stood 24 to a flat tray, in a frost-free glasshouse. The individual plants were randomised within but not between the populations. The 30 selected C^{s-} and C^{s+} came from across the full populations so it is unlikely that any significant positional effects on phenotype occurred. Extracts were produced by Farrar et al. [7] in March 2007 and assayed for the total WSC content of each individual plant from the C2 full populations of 300 plants. Here, the extracts from the three selected populations of 30 plants were further analysed by high-performance liquid chromatography (HPLC) to identify the different sugars present. Sugars were separated and quantified by isocratic HPLC on a Dionex (Camberley, UK) instrument comprising an ASI-100 automated sample injector, P580 pump and Chromeleon instrument interface, on a 300×7.8-mm column of Aminex HPX87-C (Bio-Rad, Hemel Hempstead, UK) at 85 °C, protected by an in-line 0.5-µm filter and a Carbo-C guard column. The mobile phase was degassed water at a flow rate of 0.6 ml/min. This column completely resolved fructan molecules with a degree of polymerisation (DP) of 3 from other fructan polymers. Short-chain fructan molecules with a DP of 4–6 have been distinguished as shoulders or pronounced tails on the main fructan peak and integrated separately from long-chain polymers. In this communication, DP3, DP4 and DP5–6 have been described as oligofructans and DP>6 as polymeric fructan. Sugars were detected by refractive index (Shodex RI-71 refractive index monitor) and quantified against authentic standards. All

polymeric fructans were quantified against chicory inulin (Sigma). The relative distribution of polymers of different chain lengths was assessed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Aliquots of 25 μ l were injected onto a Carbowax PA100 (Dionex: 4 \times 250 mm) column at 30 °C and eluted with 1.0 ml/min 100 mM sodium hydroxide and a discontinuous linear gradient from 0 to 1.0 M sodium acetate. Sugars were detected by pulsed amperometry using an ED40 electrochemical detector.

DM production of the individually selected C2 plants in pots, over the full growing season, was determined in the glasshouse broadly as described previously [7], but with seven cuts taken between early March and late October 2009. Single copies of the C2^s, C2^{s-} and C2^{s+} plants were grown in 130-cm pots in a different glasshouse and in different layouts both within and between populations compared with the 2007 experiment. They were again randomised within but not between populations to avoid potential confounding effects from mixing smaller, slower growing C2^{s-} plants with larger, faster growing C2^{s+} plants in the context of a glasshouse growth experiment. If the C2^{s-} plants were shaded and growth was artificially reduced early in the growing season, there would be knock-on effects throughout the experiment. This would artificially accentuate any difference between the populations. The presence of a confounding positional effect cannot therefore be excluded, but the populations were arranged in a small part (approximately 12 % growing area) of a large glasshouse to impose minimal variation in temperature and irradiance. Harvests were taken on March 2, April 15, May 20, July 7, August 3, September 14 and October 26. On each date, all top growth above a cutting height of about 4 cm was removed from the plants with hand shears, placed in labelled paper bags and oven-dried to constant mass at 80 °C without any separation of different tissue types.

Statistical Analysis

Statistical analysis of the field sward data was carried out with the standard menu-driven procedures included in GenStat[®] for Windows[®], version 13.2 [11]. Analysis of variance (ANOVA) was carried out with plot values. Both two-way ANOVA with population and year as fixed effects and block as a random effect, and one-way ANOVA of individual years were used for the DM data, WSC yield and CP yield data as appropriate. Outputs presented include predicted means, *F* test probabilities (*P* values) and least significant differences (LSD) at the appropriate level.

Experiments on individual plants (the glasshouse experiments and spaced plant field trial) were carried out on single copies of the individual plants and so no statistical analyses have been performed. Population means are presented to illustrate general trends.

Genetic Analysis

An experimental set of SSRs was identified with the aim of characterising a framework of markers which were co-dominant, reliable, easily scorable in diverse material and located every 20–25 cM on each chromosome. This marker set was mapped on to the ryegrass WSC F2 mapping family linkage map with JoinMap[®], and marker positions are shown in Fig. 1. Markers with the prefix 'rv' are from ViaLactia Biosciences (see Gill et al. [12]) and used under licence. Extraction of DNA was described by Farrar et al. [7]. SSR markers were run on an ABI capillary sequencer as described by Turner et al. [1].

For the estimation of genetic diversity and differentiation parameters, a set of five populations was considered. These were the four 'LTS' plants, the 30 selected 'C0' plants, the 30 selected 'C2^s' plants, the 30 selected 'C2^{s-}' plants and the 30 selected 'C2^{s+}' plants. Allelic richness per population and *F*_{ST} values between pairs of populations were computed using the software package FSTAT 2.9.3.2 [13]. GENEPOP 4.2 [14, 15] was used to estimate observed (*H*_O) and expected (*H*_E) heterozygosities, inbreeding coefficients (*F*_{IS}), and allelic and genotype diversity per locus and per population. The allelic differentiation between pairs of populations per locus and the LD between pairs of loci in each population were also estimated with GENEPOP. Regions of the genome under significant selection pressure were identified using the infinite island model [16] implemented in LOSITAN [17]. Markers that showed *F*_{ST} higher than 95 % of neutral distribution were inferred to be subject to divergent selection, and markers that showed *F*_{ST} lower than 95 % of neutral distribution were inferred to be subject to balancing selection.

Results

Phenotypic Traits

The content of individual soluble sugars in relation to total WSC content is shown in Fig. 2a. The C2^{s-} population had, numerically, the lowest WSC content but a high proportion of WSC as sucrose and fructose. In relative terms, the C2^{s+} population had the lowest amounts of sucrose and fructose, but much higher fructan content. The C2^{s+} population also had the highest proportion of fructan as large polymers of DP>6 (Fig. 2b). The size of the polymers in the fructan pool can be further assessed by qualitative analysis on HPAEC-PAD. Extracts from one C2^{s-} and one C2^{s+} plant were measured (Fig. 3). The chromatography traces were similar up to 13-min retention time (equivalent to a DP of between 6 and 10; unpublished data), although the detector response indicated slightly higher concentrations in the C2^{s+} plant. After this, the

CHROMOSOME

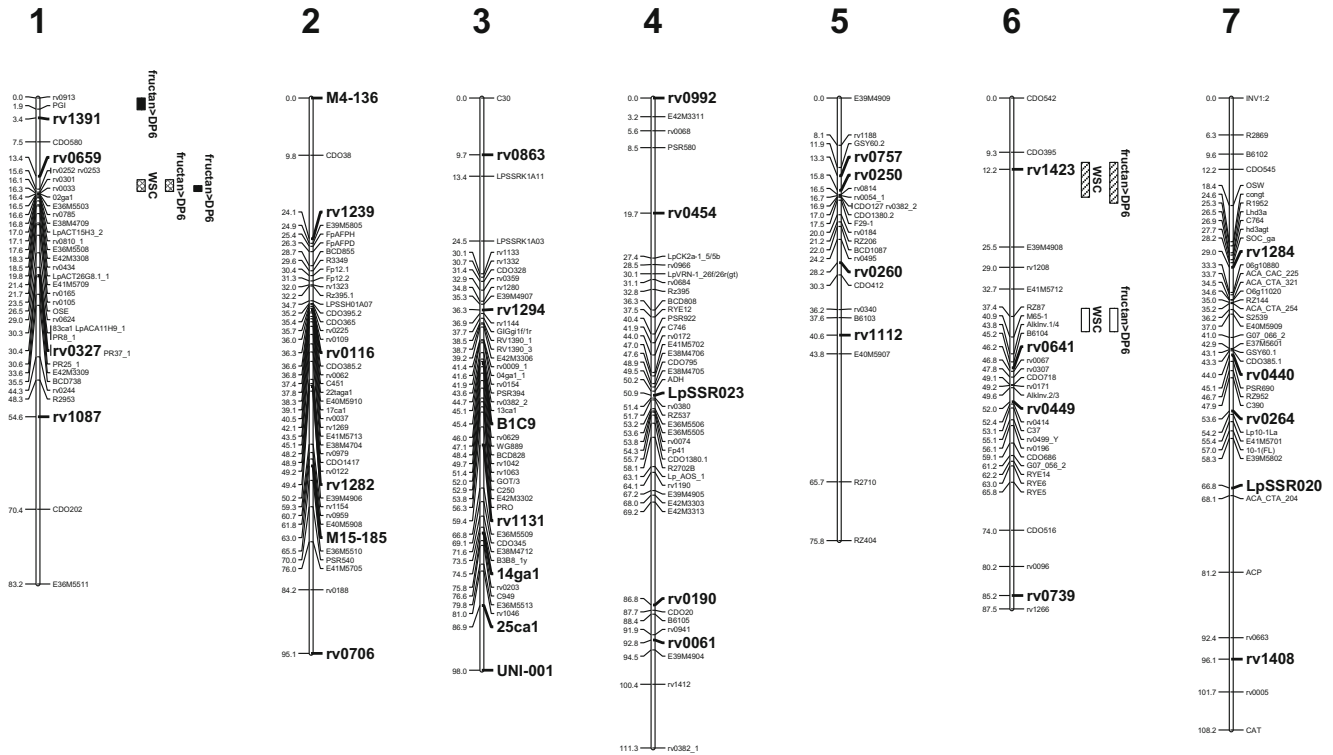


Fig. 1 Linkage map of the WSC F2 mapping family showing the positions of markers in the SSR set used for association studies, and QTL for total WSC and polymeric fructan content. Chromosome designations conform to the Triticeae numbering. Markers included in the analysis are indicated in *larger, bold font*. Fructan and WSC QTL data

are from Turner et al. (2006); *QTL bars* represent the 1 - LOD fall interval from MQM mapping. *Open bars* are leaf QTL in the spring, *hatched bars* are leaf QTL in the autumn, *cross-hatched bars* are tiller base QTL in the spring and *filled bars* are tiller base QTL in the autumn. Total WSC, *WSC*; polymeric fructan, *fructan* > *DP6*

traces were substantially different. The C2^{S-} plant contained virtually no large polymeric fructan whereas the C2^{S+} contained considerable quantities of large polymers which had not completely eluted by the time the column cleanup cycle began after 42 min run time.

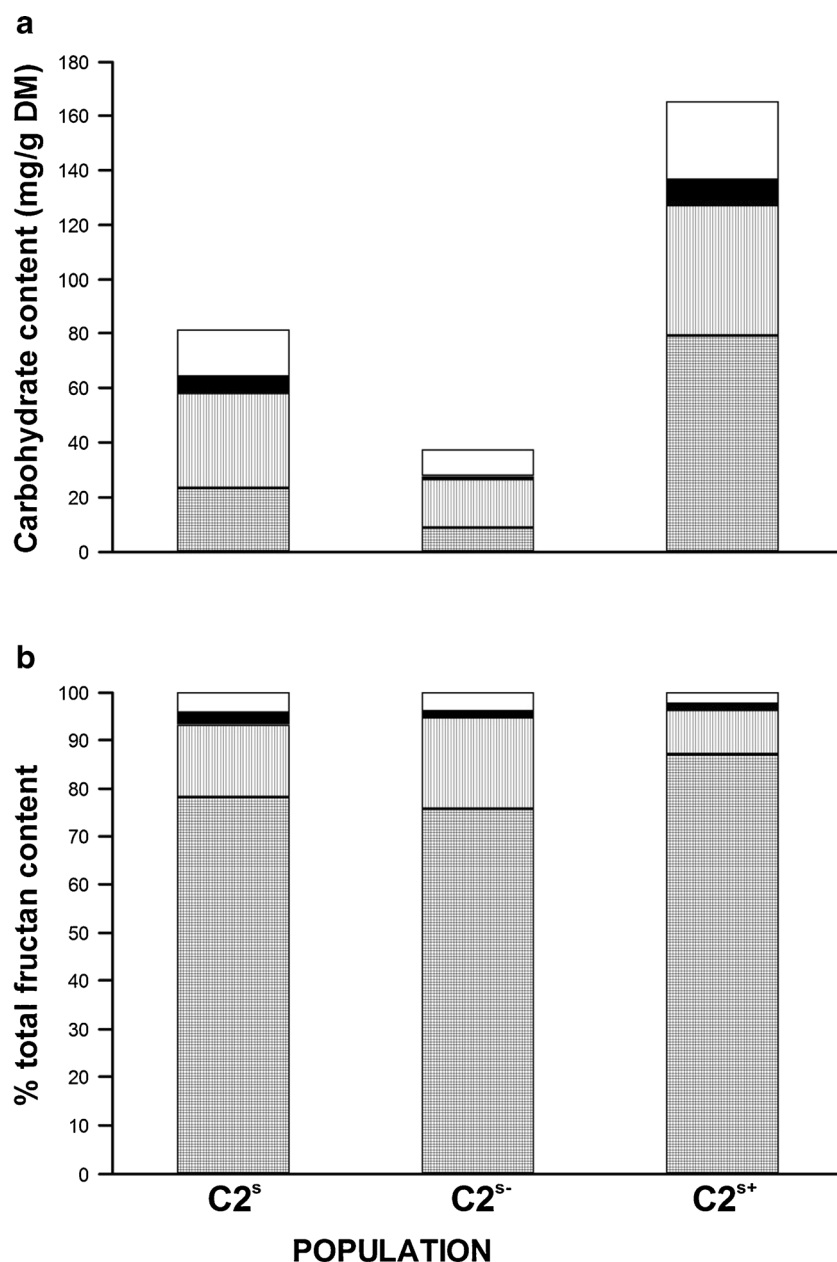
The DM yield of the 90 selected plants from the C2 generation was measured in the glasshouse by seven cuts over the growing season (Fig. 4). In general, the C2^{S+} population had, numerically, higher DM yield than the C2^{S-}, and particularly so for the first three cuts. Overall, this resulted in a trend for higher annual dry matter yield for C2^{S+} (26.6 ± 1.4 g) than for C2^{S-} (22.2 ± 0.8 g). The C2^{S+} population also had higher annual dry matter yield than the C2^{S-} in the field (Table 1). There was a significant (*P* < 0.001) effect of year but no population by year interaction over the course of the field experiment. The C2^{S+} and C2^{S-} populations were significantly (*P* < 0.5) different from each other, although not always from the unselected C2 population, in all 3 years. The overall year means were all significantly (*P* < 0.05) different from each other. The yield advantage of the C2^{S+} population was present throughout more of the year in the field, but particularly early and late in the growing season. However, all the GRASP

populations were lower yielding than commercial reference cultivars.

Nutritional value was only measured (by NIR) in year 1, but significant differences between the C2^{S+} and C2^{S-} populations in WSC content (*P* < 0.05) in the expected direction were observed at all except the first harvest date. WSC content was highest in July in all three GRASP C2 populations. The C2^{S+} population was 18 % higher than the C2^{S-} in May (244.5 mg/g DM WSC and 206.5 mg/g WSC respectively) and 20 % higher in July (286.6 and 239.6 mg/g). Over the course of the growing season, carbohydrate yield was significantly higher (*P* < 0.001) in the C2^{S+} population than in the C2^{S-} population (Table 1). CP content was lower (*P* < 0.05) in the C2^{S+} population on all dates except the fourth harvest in August. It was lowest in May in all three GRASP C2 populations; the C2^{S+} was 15 % lower than the C2^{S-} at this harvest (92.5 mg/g DM CP and 108.1 mg/g CP respectively). However, over the growing season, the higher DM yield of the C2^{S+} was sufficient to result in higher CP yield from this population (Table 1).

Heading dates for the full C2 populations of 300 plants in the field in year 1 were 42.8 days for the C2, with the other

Fig. 2 Soluble sugar content, analysed by HPLC, of all top growth removed by cutting to a 4-cm stubble height for plants of the three C2 selected GRASP populations in pots in the glasshouse in early March. Data are based on single copies of the individual plants and so no statistical analyses have been performed. The population means ($n=30$) are presented to illustrate trends. **a** Sugar content as milligrams/gram DM; fructan (horizontally/vertically cross-hatched blocks), sucrose (vertically-hatched blocks), glucose (solid blocks) and fructose (open blocks). The full height of the blocks represents total WSC. **b** Components of the fructan pool as a percentage of total fructan content (polymeric fructan > DP6, cross-hatched blocks; DP5-6 fructan, hatched blocks; DP4 fructan, solid blocks; and DP3 fructan, open blocks)



two populations slightly later at 43.0 days for both the C2⁻ and the C2⁺. The heading dates for the 30 plants selected from the full populations of 300 plants were 43.6 days for the C2^s, 42.4 days for the C2^{s-} and 38.8 days for the C2^{s+} respectively. This field trial was carried out with single copies of each of the individual members of the populations and so no statistical analyses have been performed.

Genetic Differentiation

The marker set chosen for this study proved reliable and robust. Only two markers (rv0260 on chromosome 5 and rv0706 on chromosome 2) were out of Hardy–Weinberg equilibrium in all populations (data not shown). Observed heterozygosity was

slightly lower than expected heterozygosity in all populations, indicating either the occurrence of null alleles or a slight level of inbreeding (Table 2). Allelic richness decreased in the C2^{s+} population. F_{ST} values demonstrated that clear differentiation of the C2 populations had occurred during the course of the selection experiment. The non-selected population was not different from the C0 population. The positive and negative populations were significantly different from each other, and both were different from the non-selected population and the C0.

Significant P values for linkage disequilibrium were detected between many pairs of marker loci within chromosomes, and also between pairs of marker loci on different chromosomes (Table 3). In the C0 generation, there were more marker

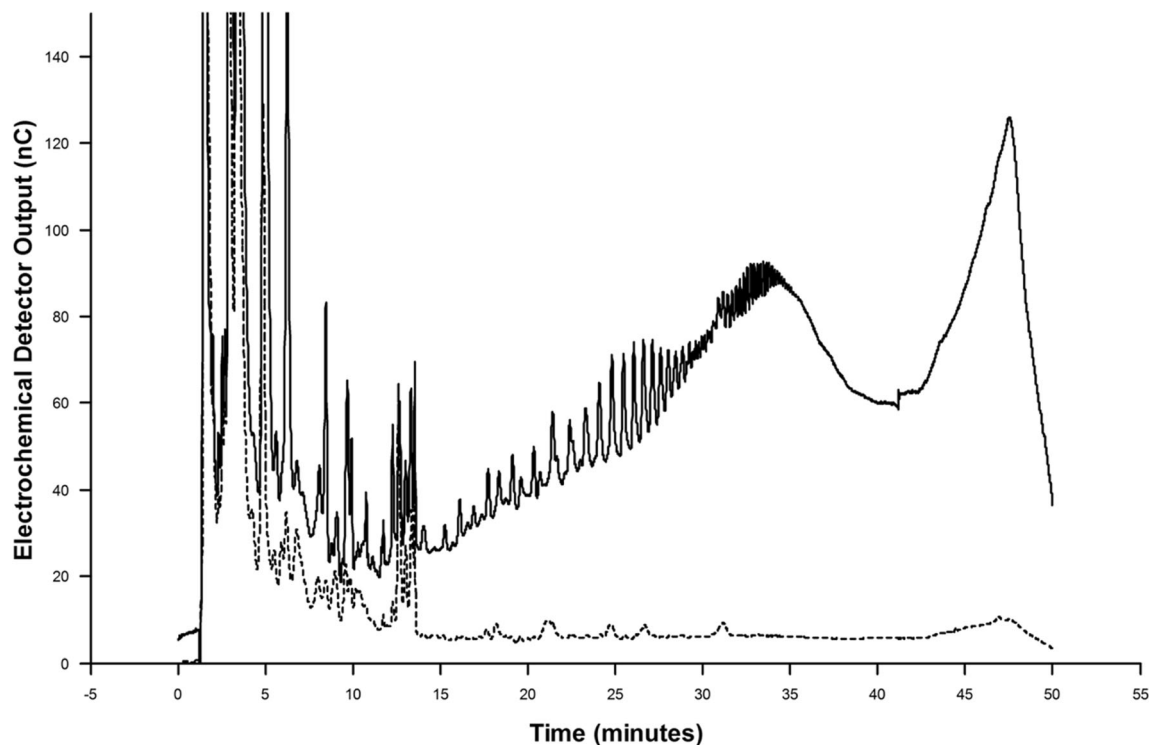


Fig. 3 Chromatograms showing the different length fructan polymers in the WSC pool from two representative plants with contrasting WSC content. C2^{S-} (fine/dashed line) and C2^{S+} (heavy line) analysed at the same sugar concentration, but back calculated to a dry matter basis for presentation

loci pairs in disequilibrium within chromosomes than across different chromosomes. The decay of linkage disequilibrium over generations led to a decrease in the number of marker pairs in disequilibrium by the C2 generation and in particular a 50 % reduction in the number of pairs in disequilibrium within chromosomes. The C2^S and C2^{S-} populations had similar numbers of marker loci pairs in linkage disequilibrium. These were predominantly within chromosomes and adjacent analysed markers or marker pairs with one intervening marker. The more distant pair in the C2^S population was on chromosome 2 (M15-185 and rv0116, 26.69 cM apart). A greater number of marker

loci pairs in linkage disequilibrium were found in the C2^{S+} population, and in contrast, 61 % of these were associations across different chromosomes (Tables 3 and 4). These relationships across chromosomes covered all seven chromosomes, although chromosome 5 was involved in fewest marker pairs (Table 4). Several marker loci, particularly on chromosomes 1, 2, and 6, are located in or close to QTL regions (Table 4).

There was little evidence that any significant genetic drift at the allelic level occurred during the experiment as there were few examples of significant divergence between the C2^S and

Fig. 4 DM yield (g) of plants of the C2 generation maintained in the glasshouse over the growing season. Data are based on single copies of the individual plants. No statistical analyses have been performed and the population means are presented to illustrate trends. Mean DM of the three selected GRASP populations from material removed by cutting each plant to a stubble height of 4 cm: C2^S (triangle), C2^{S-} (square), C2^{S+} (circle). n=30

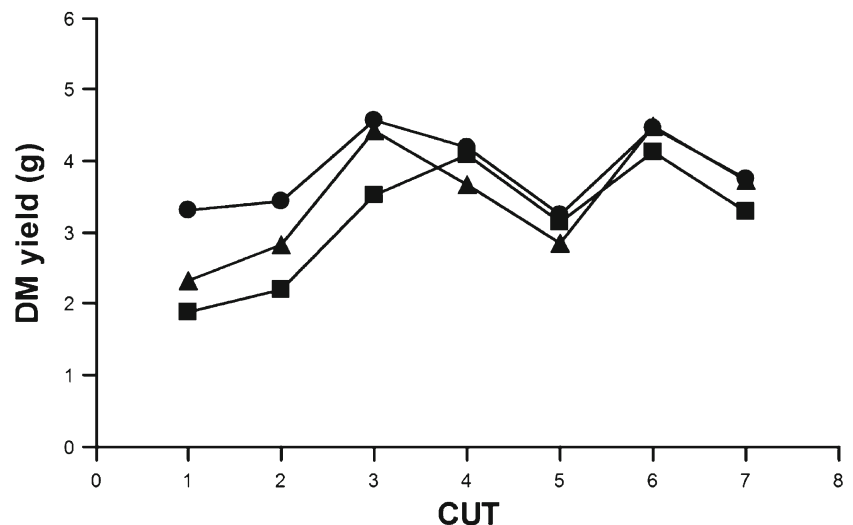


Table 1 Annual dry matter yield and nutritional quality for the C2 GRASP populations in field plots

		GRASP population			<i>P</i>	LSD 5 %	Reference cultivar	
Year		C2	C2 ⁻	C2 ⁺			AberMagic	Premium
Annual dry matter yield (t/ha)								
1	2007	12.24	10.78	12.70	0.011	1.075	18.33	15.79
2	2008	11.38	9.96	12.00	0.010	1.087	15.62	14.10
3	2009	12.32	11.77	13.26	0.009	0.759	16.80	15.34
Mean		11.98	10.84	12.65	<0.001	0.502	16.92	15.08
Annual water-soluble carbohydrate yield (kg/ha)								
1	2007	468.0	375.5	505.5	<0.001	47.0	892.5	641.5
Annual crude protein yield (kg/ha)								
1	2007	271.9	243.8	262.5	0.014	18.4	340.6	293.8

DM yield (t/ha) of forage from small field plots over 3 years. WSC and CP yield for year 1 (kg/ha). Data were summed over six cuts between April and October and analysed as individual years by one-way ANOVA, $n=4$. Significance levels are indicated and LSD is the least significant difference for comparing population means at $P<0.05$. Data for AberMagic and Premium from the same trial are presented as reference cultivars but have been excluded from the statistical analysis

C0 populations (Table 5). However, highly significant allelic differentiation between the positive and negative populations was observed across all seven chromosomes, for 30 out of the 35 SSRs studied. In many, but not all, instances, the divergence between the positive and random selections was more significant than between the negative and random selections. This allelic differentiation led to a similar pattern of genotypic differentiation (data not shown). LOSITAN software was used to identify which loci from this large set of markers with significant allelic differentiation showed significant divergent selection (Table 5). This suggested that markers on chromosomes 2 and 6 were subject to significant selection pressure in

the C2^{s+} population and markers on chromosomes 1, 2 and 6 in the C2^{s-} population. Marker 14ga1 on chromosome 3 also showed a significant selection effect.

Genetic Diversity at Selected Loci

Eleven contrasting loci were selected, from the C2 population allelic differentiation and LOSITAN results in Table 5, to examine allelic richness and homozygosity in the GRASP populations and in two ryegrass cultivars (AberElan and AberMagic) from the Aberystwyth breeding programme. The four markers with low differentiation and no significant selection effect were on chromosomes 3, 4, 5 and 7 (Table 6). The seven markers with the most significant allelic differentiation, negligible genetic drift and (except for rv1391) significant selection effects were located on chromosomes 1, 2, 3 and 6. The most common allele in the initial plant material (the LTS plants) was present in the WSC F2 mapping family for all these eleven marker loci. Within the GRASP selection experiment, in general, the non-selected markers had a lower number of alleles and showed no loss of alleles by the C2 generation and few changes in the frequency of the most common allele. There were only small differences between the three C2^s populations. The level of homozygosity was relatively high but little affected by selection. In contrast, the SSRs showing selection effects had a higher number of alleles, although comparison of allele number with degree of allelic differentiation for the full marker set showed only a low, although significant, correlation ($r=0.386$; $P=0.035$). Within the GRASP selection experiment, the markers showing significant differentiation by the C2 generation accumulated particular alleles (all of WSC F2 mapping family origin and not necessarily the most common allele from the earlier generations) in the C2^{s+} population. This led

Table 2 Population differentiation

	Population			
	C0	C2 ^s	C2 ^{s-}	C2 ^{s+}
Number of individuals	30	30	30	30
Allelic richness	2.956	2.894	2.839	2.434
Expected heterozygosity (H_E)	0.667	0.658	0.641	0.528
Observed heterozygosity (H_O)	0.552	0.498	0.552	0.466
F_{IS}	0.172	0.243	0.138	0.117
F_{ST}	C0	0	0.0082	0.0656**
	C2 ^s		0	0.1073**
	C2 ^{s-}			0
	C2 ^{s+}			

Description of the initial C0 generation and the three selected populations from the C2 generation. Allelic richness (A) from FSTAT, and expected heterozygosity, observed heterozygosity and F_{IS} from GENEPOP. F_{ST} values for population pairs (FSTAT) with significance levels are indicated (** $P<0.01$)

Table 3 Summary of linkage disequilibrium in the C0 and C2 selected populations

	Marker pairs in linkage disequilibrium		Population			
			C0	C2 ^s	C2 ^{s-}	C2 ^{s+}
Numbers of marker pairs in linkage disequilibrium with <i>P</i> values less than 0.05 from GENEPOP output for the C0 population and the three selected C2 populations (C2 ^s , C2 ^{s-} and C2 ^{s+}). <i>n</i> =30	Total marker pairs Within a chromosome		37	25	22	31
	total		28	14	15	12
	with the next analysed marker		16	11	9	9
	with the next but one analysed marker		9	2	4	2
	more distant		3	1	2	1
On different chromosomes		9	11	7	19	

to a higher homozygosity for these loci in the positive selection compared with the negative and random selections. In some cases, this was accompanied by the complete loss of alleles: for example M4-136 alleles 185 and 200 were not found in the C2^{s+} population.

Table 4 Location of marker pairs in linkage disequilibrium across chromosomes for the C2^{s-} and C2^{s+} selected populations

Population	Locus 1		Locus 2	
	Marker 1	Chromosome	Marker 2	Chromosome
C2 ^{s-}	rv1391 ^a	1	SSR020	7
	rv0659 ^a	1	B1C9	3
	rv0659 ^a	1	rv0641 ^a	6
	rv0116 ^a	2	rv1284	7
	M15185	2	rv1284	7
	UNI-001	3	rv0449 ^a	6
	rv0190	4	SSR020	7
C2 ^{s+}	rv0327	1	rv0641 ^a	6
	rv0327	1	rv0449 ^a	6
	rv1087	1	rv1239 ^a	2
	rv1087	1	rv0116 ^a	2
	rv1087	1	rv0706	2
	rv1087	1	rv0863	3
	rv1239 ^a	2	rv0992	4
	rv0116 ^a	2	UNI-001	3
	14ga1	3	rv1284	7
	25ca1	3	rv1408	7
	UNI-001	3	rv0641 ^a	6
	UNI-001	3	rv0449 ^a	6
	UNI-001	3	rv1408	7
	rv0992	4	rv0250 ^a	5
	rv0190	4	rv0250 ^a	5
	rv0190	4	rv1423 ^a	6
rv0190	4	rv0641 ^a	6	
rv0992	4	rv1284	7	
rv0449 ^a	6	rv1408	7	

^a Loci located in or close to QTL regions (from Tumer et al. (2006))

Allele number was lower in AberMagic than in AberElan for 5 of the 11 markers studied (Table 6). Four of these were markers showing selection effects in the GRASP experiment. This was combined with increases in the frequency of homozygous genotypes for the most common allele for these markers. However, in regard to homozygosity, the difference between markers from the SIG and NOT SIG groups from the GRASP experiment was less clear, and homozygosity was higher in AberMagic for most of the markers examined. The most extreme example of allele loss and increased homozygosity in AberMagic was for rv1391 on chromosome 1.

Discussion

Farrar et al. [7] have already described the positive selection of the C2^{s+} population with high sugar content from the low baseline in the C0 generation. The difference between the C2^{s+} and C2^s populations was greater than between the C2^{s-} and C2^s populations, suggesting that the ‘positive’ selection was more intense than the ‘negative’ selection. The data presented here to examine the component sugars of the total WSC pool in these plants are from single copies of the individual plants and for just the last generation at the end of the selection experiment, but comparable results have been observed for the previous C1 generation. Calculation of total WSC content for the three C2^s populations from HPLC data by summing the individual named peaks showed trends that repeat the effects from the previous analyses [7], except for a relative underestimation of the WSC content of the C2^{s+} population. This might have arisen from the accumulation of sugars which were unidentified peaks on the HPLC traces or of sugars that are not detected by a refractive index monitor. Within the identified sugar peaks, the main effect appeared to be an increase in the proportion of WSC present as high molecular weight polymeric fructan in the C2^{s+} selection. Therefore, at a molecular level, the selection may have been achieved through changes in polymeric fructan metabolism. This would minimise the osmotic consequences of storing high concentrations of sugar. This study has suggested that,

Table 5 Marker differentiation between pairs of populations from the C2 generation

Marker	C2 ^{s+} /C2 ^{s-}		C2 ^{s+} /C2 ^s		C2 ^{s-} /C2 ^s		C2 ^s /C0		
	<i>P</i>	F _{ST}	<i>P</i>	F _{ST}	<i>P</i>	F _{ST}	<i>P</i>	F _{ST}	
rv1391	1	0.00000	0.347696	0.00897	0.083971	0.00073	0.115391	0.50268	-0.00654
rv0659	1	0.00000	0.158655	0.23035	-0.00361	0.00261	0.110816	0.66133	-0.01331
rv0327	1	0.00000	0.190718	0.00000	0.087636	0.00000	0.157548 (0.999)	0.61292	-0.00588
rv1087	1	0.00000	0.218757	0.57712	0.005336	0.00000	0.200552 (1.000)	0.04502	0.020236
M4136	2	0.00000	0.312359 (0.977)	0.00000	0.161017	0.00297	0.054642	0.67420	0.001478
rv1239	2	0.00000	0.360489 (0.975)	0.00000	0.257853 (0.994)	0.30653	-0.00067	0.04290	0.037254
rv0116	2	0.00000	0.353839 (0.996)	0.00000	0.184365 (0.983)	0.00250	0.075329	0.02988	0.046916
rv1282	2	0.00000	0.531444 (0.999)	0.00006	0.240937	0.00188	0.144485 (0.985)	0.02647	0.069186
M15185	2	0.00000	0.370913 (0.985)	0.00000	0.28062 (0.994)	0.00121	0.060059	0.01079	0.058434
rv0706	2	0.00000	0.187992	0.00283	0.104483	0.00609	0.061885	0.06863	0.010002
rv0863	3	0.00000	0.256943	0.00000	0.132814	0.00002	0.061133	0.05345	0.015053
rv1294	3	0.00000	0.193139	0.02958	0.049254	0.05210	0.037699	0.35081	0.000585
B1C9	3	0.00020	0.079867	0.00287	0.05082	0.49755	-0.00624	0.34797	0.017258
rv1131	3	0.31981	-0.00267	0.19864	0.004312	0.96775	-0.01601	0.12981	0.027514
14ga1	3	0.00000	0.267126	0.00000	0.231003 (0.998)	0.48220	-0.01242	0.56421	0.004349
25ca1	3	0.00030	0.092065	0.00647	0.058148	0.36553	0.00013	0.44469	-0.00351
UNI-001	3	0.00000	0.118323	0.00006	0.09051	0.03222	0.001246	0.19623	0.010563
rv0992	4	0.00004	0.123302	0.00000	0.097078	0.01252	0.038794	0.32395	0.00829
rv0454	4	0.00017	0.077622	0.00196	0.050224	0.17449	0.016122	0.38211	-0.00339
SSR023	4	0.00000	0.116066	0.00000	0.121297	0.07896	0.006157	0.36884	-0.00534
rv0190	4	0.00000	0.26608	0.00077	0.131379	0.00995	0.021937	0.70876	-0.00709
rv0061	4	0.67280	-0.01308	0.07876	0.041943	0.24718	0.014377	0.84807	-0.01693
rv0757	5	0.00000	0.040856	0.00050	0.042359	0.00029	-0.01641	0.58134	0.003156
rv0250	5	0.01613	0.186876	0.01177	0.055929	1.00000	0.042822	0.53819	-0.00581
rv0260	5	0.04379	0.028602	0.01914	0.036401	0.00029	0.07516	0.40317	-0.00115
rv1112	5	0.00022	0.068498	0.04209	0.079685	0.00000	0.112738	0.07291	0.022513
rv1423	6	0.00000	0.465522 (0.999)	0.00000	0.362913 (0.999)	0.00012	0.056105	0.00585	0.035114
rv0641	6	0.00000	0.233325	0.00696	0.071427	0.00409	0.052481	0.21925	0.003053
rv0449	6	0.00000	0.291457	0.01017	0.039972	0.00000	0.132811 (0.991)	0.72664	-0.01038
rv0739	6	0.00000	0.139397	0.00000	0.227833 (0.985)	0.00027	0.07516	0.66580	-0.00971
rv1284	7	0.00097	0.086796	0.00255	0.051365	0.07950	0.026573	0.23903	0.006912
rv0440	7	0.00273	0.045173	0.46900	-0.00262	0.00891	0.047494	0.04435	0.0321
rv0264	7	0.00000	0.18113	0.00478	0.054113	0.00162	0.072771	0.16568	0.011362
SSR020	7	0.00398	0.083861	0.00139	0.11579	0.05466	0.021833	0.01770	0.041197
rv1408	7	0.06105	0.002364	0.25538	-0.00913	0.80120	<u>-0.0153(0)</u>	0.21895	0.001715

P values are for allelic differentiation between selected populations ($n=30$) from GENEPOP output. F_{ST} values are from LOSITAN. Markers identified as being under divergent selection by LOSITAN are highlighted (bold type) and the *P* value corresponding to *P* (Simul F_{ST}<sample F_{ST}) is given in parentheses. Markers identified as being under balancing selection by LOSITAN are highlighted (italic type and underlined) and the *P* value corresponding to *P* (Simul F_{ST}<sample F_{ST}) is given in parentheses. Markers are arranged in the order they occur on the linkage map

when appropriate variation is present in the source population, phenotypic expression of high WSC may be associated with increased fructan content.

The selection for high WSC was only accompanied by a likely increase in biomass yield during the early part of the growing season, although DM production was numerically higher in the C2^{s+} on all harvest dates. Overall, this early

growth was sufficient to increase annual DM yield, as there did not appear to be any comparable increase in yield of the other populations later in the growing season. The DM data were for single copies of the individual plants in the glass-house for just 1 year, and the possibility of positional effects cannot be completely eliminated. However, the trends in both this DM yield data and the WSC data discussed above are

Table 6 Allelic and genotypic diversity in the GRASP populations and in breeding material

Selected marker Chromosome	rv1131 3	rv0061 4	rv0250 5	rv440 7	rv1391 1	rv0327 1	M4-136 2	rv0116 2	l4gal 3	rv1423 6	rv0759 6
GRASP populations											
Differentiation effect	NOT SIG	NOT SIG	NOT SIG	NOT SIG	SIG	SIG	SIG	SIG	SIG	SIG	SIG
Selection effect	NONE	NONE	NONE	NONE	NONE	SIG	SIG	SIG	SIG	SIG	SIG
Number of alleles											
LTS	4	2	3	4	3	5	5	5	5	5	4
C0	4	2	3	4	3	5	5	5	5	5	4
C2 ^S	4	2	3	4	3	5	5	5	5	5	4
C2 ^{S-}	4	2	3	4	3	4	5	5	4	4	4
C2 ^{S*}	4	2	3	4	3	4	3	5	4	3	3
WSC F2 alleles	186	176	243	129	173	127	166	283	154	137	204
	221	212	250	133	181	137	187	299	172	148	206
Present in LTS	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Most frequent allele(s) (size and frequency (%))											
LTS	221 (38)	176 (63)	243 (63)	129 (25)	173 (38)	137 (33)	187 (50)	283 (50)	154 (25)	137 (38)	206 (38)
				131 (25)	181 (38)				172 (25)		
				162 (25)							
C0	221 (41)	176 (61)	243 (70)	129 (40)	173 (62)	137 (38)	187 (52)	283 (42)	154 (30)	137 (31)	202 (38)
											206 (38)
C2 ^S	186 (48)	176 (59)	243 (62)	131 (41)	173 (67)	137 (38)	187 (38)	287 (36)	164 (34)	137 (38)	202 (43)
C2 ^{S-}	186 (52)	176 (71)	243 (60)	162 (30)	173 (33)	129 (47)	183 (33)	308 (56)	164 (38)	137 (60)	206 (38)
					181 (33)						
C2 ^{S*}	186 (53)	176 (75)	243 (70)	129 (33)	173 (90)	137 (57)	187 (80)	283 (64)	154 (71)	148 (75)	206 (71)
				131 (33)							
% homozygous genotypes for allele high in C2 POS											
C2 ^S	31	38	40	14 (129)	55	17	17	7	3	0	17
				31 (131)							
C2 ^{S-}	32	48	33	12	13	3	7	0	0	0	20
C2 ^{S*}	30	53	40	17	83	32	60	39	48	57	50
Breeding material											
Number of alleles											
AberElan	4	2	4	3	3	4	6	4	6	2	4
AberMAGIC	3	2	4	4	1	4	2	2	2	3	4
Most frequent allele(s) (size and frequency (%))											
AberElan	217 (33)	212 (75)	242 (74)	143 (82)	163 (47)	135 (50)	201 (30)	288 (36)	175 (36)	135 (51)	199 (54)
AberMAGIC	181 (53)	212 (96)	246 (58)	143 (44)	174 (100)	127 (58)	193 (66)	294 (52)	175 (52)	145 (56)	199 (59)
Frequency (%) of most common AberElan allele in AberMAGIC	40	96	41	44	0	28	0	48	52	4	59
% homozygous genotypes for most frequent allele											
AberElan	10	58	55	10	39	30	19	13	7	19	30
AberMAGIC	25	98	40	19	100	34	48	26	25	29	24

Data for GRASP populations ($n=30$) were taken from GENEPOP, genic and genotypic diversity output. Data for breeding material ($n=96$) LTS *Lolium* test set, WSC F2 mapping family, NOT SIG not significant

strongly supported by the data from the mini-swards in the field over 3 years. Early spring growth can be associated with early flowering, but there was no indication of major changes in heading date in the second-generation GRASP populations. The yield differences may not have been dramatic, but they do demonstrate the important point that high WSC accumulation does not necessarily draw carbon supply away from growth processes which would conflict with yield. Overall, this may explain why it has been possible to successfully select for both yield and high sugar during ryegrass breeding programmes at IBERS, Aberystwyth, without introducing any unwanted changes in heading date.

The current SSR studies with this plant material have provided information on the genetic basis of the phenotypic response. Other workers have also demonstrated that SSR markers can be less neutral than might be expected during selection experiments. In wheat, long-term improvement (from 1845 to 2004) of a range of quality and productivity traits resulted in significant allelic changes in nearly half the SSRs tested [18]. In maize, there were changes in the allele frequencies of SSRs during selection; some markers were flanking known QTL but others were spread across the genome [19]. These widespread unexplained effects may be a reflection of the number of unknown, small QTL with cumulative effects that exist for many quantitative traits. In the current work, allelic differentiation within the SSR set was observed on all chromosomes. WSC content is a quantitative trait with expected multi-locus control. Nevertheless, these results could indicate multiple control loci on individual chromosomes or confounding effects due to carry over by linkage during the small number of rounds of recombination during the experiment. However, in general, limited (at best perhaps 1 kb) linkage disequilibrium would be expected in outbreeding species like the forage grasses [20], and linkage disequilibrium decay has been shown to be rapid in other studies with ryegrass [21, 22]. During the current experiment, linkage disequilibrium within a chromosome approximately halved between the C0 and C2 generations, with the decreases larger as the distance between markers increased. At the same time, linkage disequilibrium between markers on different chromosomes increased substantially in the positive selection, reinforcing the suggestion that this population was subject to high selection pressure and that at least some of the shifts detected here were brought about by selecting for high WSC.

Nine of the 35 SSRs used in this study are on chromosome regions that have previously been shown to be linked to WSC or fructan QTL [1]. Five of these nine markers showed a significant LOSITAN selection effect, but so did six markers not previously associated with QTL in our work. QTL markers were no more likely to show strong allelic differentiation across all population comparisons within the C2 generation than non-QTL markers (9.1 % compared with 11.5 %). However, all QTL markers except rv0250 on chromosome 5

showed a strong allelic differentiation in the C2^{S+}/C2^{S-} comparison, although so did 14 out of the 26 other markers. Eighteen of the 26 marker loci pairs in disequilibrium across chromosomes in C2^{S+} and C2^{S-} included one marker in a QTL region, although only one pair involved two markers in QTL regions. Together, these results confirm the importance of the QTL segregating in the IBERS WSC F2 mapping population in regulating WSC content, and also indicate the probable existence of other, as yet unknown, QTL for WSC present in the ryegrass genome. The LOSITAN analysis suggests that some of these QTL may be located on chromosomes 1 and 3. Chromosome 3 would seem to be a likely candidate for a major QTL as WSC QTL have been reported on chromosomes 3 and 7 in perennial ryegrass [23], but the current data do not accurately define the position of this putative QTL. Further work is needed to characterise these new QTL more fully and identify the possibility of underlying candidate genes of fructan metabolism. No association with genes of fructan metabolism has been demonstrated for the WSC/fructan QTL previously reported for perennial ryegrass [1].

Changes in allele frequencies during selection are commonly associated with decreases in allelic richness [18]. Loss of alleles, along with increases in the homozygosity of the remaining alleles, was observed in the GRASP selection experiment for most of the markers showing a significant LOSITAN selection effect. These changes were greatest in the C2^{S+} population, providing further supporting evidence for a stronger positive selection than negative selection. The number of alleles was lower in AberMagic than in AberElan for three of the six 'selection' markers, but only one of these was a QTL marker in the WSC F2 mapping family. Additionally, one of four non-selected markers showed reduced allelic richness. The most extreme example of low allelic richness and high homozygosity in AberMagic was for rv1391. This has been mapped near the isozyme PGI at the top of chromosome 1, which is associated with WSC and fructan QTL [1]. If reduced allelic richness is used as evidence for a region of the genome under selection pressure during cultivar production, then, overall, this suggests that while there may be some common elements to the genetic control of WSC in the plant material in the IBERS breeding programme and the experimental mapping population, there are also considerable differences. These may result from the different backgrounds of the original plant materials.

To the best of our knowledge, no QTL study in perennial ryegrass has succeeded in explaining the major part of phenotypic variation for a quantitative trait, and the percentage variation explained by individual QTL has been low, although the high heritabilities for many of these traits indicate strong genetic control. For plant morphology/productivity traits, the percentage variation explained has only rarely been greater than 20 % [24–27]. The same is true for plant quality traits including WSC content [1, 23]. In conjunction with this,

marker selection has confirmed individual QTL locations but failed to realise substantial gains in WSC content [28]. Test crosses based on marker indices have been only slightly more successful [29, and Turner, unpublished data]. This is all consistent with polygenic control from multiple loci spread across the genome. Future strategies for marker selection of key traits that may be difficult or expensive to assess in forage grasses will therefore need to be based on genome-wide approaches [20].

In conclusion, the strategy followed here of creating an initial population and selecting phenotypically for a given trait during a number of generations has been further confirmed to produce divergence. It has also facilitated the identification of more QTLs for polygenic traits than previously found in single cross mapping populations. Provided that a range of suitable genetic variation is present, the selection intensity is sufficiently strong and the genotyping sufficiently dense; this shows that this approach can complement QTL mapping studies based on bi-parental populations.

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