Plant Biotechnology Journal

Plant Biotechnology Journal (2013) **11**, pp. 572–581 doi: 10.1111/pbi.12045

Genotyping by RAD sequencing enables mapping of fatty acid composition traits in perennial ryegrass (*Lolium perenne* (L.))

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Received 6 August 2012;
revised 11 December 2012;
accepted 12 December 2012.
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Keywords: Fatty acids, lipids, perennial ryegrass (*Lolium perenne*), mapping, quantitative trait loci, restriction-associated DNA.

Summary

Perennial ryegrass (*Lolium perenne* L.) is the most important forage crop in temperate livestock agriculture. Its nutritional quality has significant impact on the quality of meat and milk for human consumption. Evidence suggests that higher energy content in forage can assist in reducing greenhouse gas emissions from ruminants. Increasing the fatty acid content (especially α-linolenic acid, an omega-3 fatty acid) may thus contribute to better forage, but little is known about the genetic basis of variation for this trait. To this end, quantitative trait loci (QTLs) were identified associated with major fatty acid content in perennial ryegrass using a population derived from a cross between the heterozygous and outbreeding high-sugar grass variety AberMagic and an older variety, Aurora. A genetic map with 434 restriction-associated DNA (RAD) and SSR markers was generated. Significant QTLs for the content of palmitic (C16:0) on linkage groups (LGs) 2 and 7; stearic (C18:0) on LGs 3, 4 and 7; linoleic (C18:2n-6) on LGs 2 and 5; and α-linolenic acids (C18:3n-3) on LG 1 were identified. Two candidate genes (a lipase and a beta-ketoacyl CoA synthase), both associated with C16:0, and separately with C18:2n-6 and C18:0 contents, were identified. The physical positions of these genes in rice and their genetic positions in perennial ryegrass were consistent with established syntenic relationships between these two species. Validation of these associations is required, but the utility of RAD markers for rapid generation of genetic maps and QTL analysis has been demonstrated for fatty acid composition in a global forage crop.

Introduction

Perennial ryegrass (*Lolium perenne* L., 2*n* = 2x = 14) is the most important forage crop in temperate agriculture, due to its high yield and quality of forage. While biomass yield and digestibility are continuing targets for genetic improvement, the significant environmental footprint of livestock agriculture has focussed attention on aspects of forage quality as a route to reducing greenhouse gas emissions from ruminants feeding on forage. A high content of water-soluble carbohydrates in ryegrass forage improves the efficiency of meat and milk production in cattle (Lee *et al.*, 2001; Miller *et al.*, 2001) and may help to reduce emissions of nitrogenous oxide gasses. High-sugar grass varieties, which contribute to this improved efficiency, have been bred successfully (Humphreys, 1989). However, livestock agriculture still leaves a significant environmental footprint in terms of methane emissions from ruminants, particularly from dairy cattle. The energy content of the forage is known to affect the efficiency of the digestion in ruminants (Merry *et al.*, 2006). Lipids contain approximately twice as much energy as carbohydrates and protein per unit of weight, and so represent a potential target for genetic improvement. Furthermore, evidence is emerging that supplying free plant oils can have mitigating effects on methane emissions of ruminants (Beauchemin *et al.*, 2007; Martin *et al.*, 2011). More work is needed to validate this conclusion, but a link between a higher energy content of forage and an increased efficiency of meat and milk production has long been recognized (MacRae and Lobley, 1986; Rooke *et al.*, 1987). In addition, an increased supply of omega-3 fatty acids in ruminant diets has resulted in their increased accumulation in animal products, improving quality and nutraceutical value (Scollan *et al.*, 2001). Thus, there is a driver for establishing the genetic basis for lipid and individual fatty acid content in forage crops and so to realize the potential for genetic improvement of this trait. This approach forms part of a broader strategy for reducing livestock-associated greenhouse gas emissions and increasing the unsaturated fatty acid content in ruminant products (meat and milk) for improved human health.

The genetic basis of lipid biosynthesis has been studied in detail in the model species *Arabidopsis thaliana* (L.) (Hobbs *et al.*, 2004), as well as in crops harvested for their seed oil content. These includes flax (linseed oil; *Linum usitatissimum* (L.)) (Cloutier *et al.*, 2011; Fofana *et al.*, 2004; Green, 1986), oilseed rape (*Brassica napus* (L.)) (Barker *et al.*, 2007), soybean (*Glycine max* (L.)) (Lee *et al.*, 2011), sunflower (*Helianthus annus* (L.)) (Mokrani *et al.*, 2002) and oat (*Avena sativa* (L.)) (Kianian *et al.*, 1999). Briefly, fatty acid biosynthesis proceeds via elongation steps in which acetyl-CoA units are added to increase the length of the hydrocarbon chain in 2-carbon units. Elongation steps proceed to palmitic acid (C16:0), and a further condensation step produces stearic acid (C18:0). A number of desaturase enzymes mediate the conversion of C18:0 to oleic (C18:1n-9), linoleic (C18:2n-6) and α-linolenic acids (C18:3n-3; Barker *et al.*, 2007). These are five of the most important fatty acid constituents in lipids of

perennial ryegrass. The beneficial effects of polyunsaturated fatty acids (PUFA) in human diet are well known (Hooper *et al.*, 2006), so a knowledge of the genetic variation which underpins the variation in constituent fatty acids would be valuable. Quantitative trait loci (QTL) mapping of lipid constituents in oil seed crops such as flax (Cloutier *et al.*, 2011; Ecke *et al.*, 1995), rice (*Oryza sativa* (L.)) (Ying *et al.*, 2012), oilseed rape (Ecke *et al.*, 1995) (Barker *et al.*, 2007), soybean (Csanádi *et al.*, 2001; Hyten *et al.*, 2004) and sunflower (Ebrahimi *et al.*, 2008; Haddadi *et al.*, 2010) has established the quantitative nature of this trait. However, no such information is available for perennial ryegrass. Furthermore, forage and oilseed crops differ fundamentally in that the main lipids in the former are present in leaves either as constituents of cell and organelle membranes (in the form of phospholipids and glycolipids), as compared to predominant storage as triacylglycerol (TAG) in oil bodies of the latter.

The Mendelian basis of quantitative traits provides a genetic framework for the dissection of polygenic traits (Mather and Jinks, 1982) and can pave the way for the identification of causal loci controlling the inheritance of complex traits. This approach has been enhanced by the availability of molecular markers that have allowed the creation of genetic maps. Next-generation sequencing (NGS) technology makes it possible to achieve dense SNP marker coverage of genomes without the need for a reference sequence (Davey *et al.*, 2011; Nielsen *et al.*, 2011). An example of this is restriction-associated DNA sequencing (RAD-seq), which was originally developed as a tool for genetic mapping in fish and fungi (Baird *et al.*, 2008), but the use of which has expanded to many other species, including plants (Chutimanitsakun *et al.*, 2011; Elshire *et al.*, 2011; Pfender *et al.*, 2011; Scaglione *et al.*, 2012). RADseq utilizes the power of NGS platforms to generate high coverage of short contigs adjacent to restriction sites, which can then be interrogated for SNPs between genotypes. The advantage of RADseq and related methods compared to whole-genome resequencing is that a relatively large set of markers can be identified and genotyped across multiple individuals using a fraction of the sequencing capacity required to resequence most complex genomes, and without the need for a reference genome (the sequencing coverage of each 'tag' being sufficiently high to permit *de novo* assembly). With the use of methylation-sensitive restriction enzymes, the number of RAD tags can be reduced such that high numbers of individuals can be genotyped within a single NGS library with a high probability that markers will lie in or near genic regions.

The self-incompatible mating system of perennial ryegrass means that mapping populations are most easily generated using a pseudotestcross design (Simone *et al.*, 1997). Quantitative trait mapping of various traits using pseudotestcross populations has been described in perennial ryegrass and other outbreeding forages (Dracatos *et al.*, 2009; Muylle *et al.*, 2005; Schejbel *et al.*, 2007; Studer *et al.*, 2007). Here, we describe the development of a RAD-based genetic map in an F_1 family of perennial ryegrass for QTL analysis of fatty acid content in leaves. This is, to our knowledge, the first analysis and identification of QTL of fatty acids in perennial ryegrass, including the first determination of heritability of this trait in forage grasses. Additionally, as this analysis was based exclusively on field evaluations over a period of 3 years, it represents a realistic platform for future candidate gene identification and genetic dissection of this complex and important trait.

Results

Phenotypic analysis

The means and standard deviations for the measured traits for each of the 3 years is shown in Table 1. C18:2n-6 and C18:3n-3 constituted by far the largest percentage of the total, and their values were highest in year 2, while the three minor constituents tended to increase for every cut. Table 1 also shows that the five constituents measured here made up nearly all the fatty acids present in the samples. A variance component analysis using the REML model across all six cuts demonstrated a significant effect of genotype and of cut, but little evidence of genotype by cut interaction (Table 2). A ratio of 1.645 or higher between a variance component and its standard error indicates a significant effect ($P < 0.05$; Galwey, 2006). For all traits except C18:1n-9, that ratio was well above this threshold (Table 2). The across-cuts heritabilities ranged between 0.25 and 0.82, but only C18:1n-9 was below 0.5. The within-cuts heritabilities were more variable and on the whole lower—specially for the total, and at the later cuts.

The frequency distribution of the overall means for the traits is summarized in Figure 1. For most of the traits, the two parental means were skewed towards one end of the spectrum. This was particularly striking for C18:3n-3 and C16:0 for which both

Table 1 Mean and standard deviation (mg/gDW) of fatty acid components in the perennial ryegrass Aurora × AberMagic population. The values for each year are means of two cuts

Year	Population	*n*	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	CTOT
All	F1	1095	4.26 ± 0.620	0.39 ± 0.078	0.38 ± 0.139	2.73 ± 0.633	15.02 ± 4.015	25.03 ± 5.253
All	Aurora-17	13	3.92 ± 0.693	0.38 ± 0.103	0.46 ± 0.270	2.65 ± 0.846	12.09 ± 3.708	22.95 ± 8.706
All	AberMagic	13	4.01 ± 0.579	0.39 ± 0.073	0.46 ± 0.133	2.94 ± 0.705	11.61 ± 2.889	22.42 ± 6.123
1	F1	340	3.77 ± 0.513	0.36 ± 0.073	0.35 ± 0.116	2.71 ± 0.447	14.66 ± 2.440	23.41 ± 3.141
1	Aurora-17	3	2.89 ± 0.140	0.32 ± 0.036	0.42 ± 0.082	2.52 ± 0.267	9.12 ± 1.305	16.58 ± 1.059
1	AberMagic	3	3.26 ± 0.544	0.34 ± 0.041	0.33 ± 0.044	2.69 ± 0.420	10.17 ± 2.385	18.29 ± 3.141
2	F1	378	4.20 ± 0.389	0.39 ± 0.060	0.37 ± 0.123	3.14 ± 0.372	18.03 ± 3.193	28.96 ± 4.581
2	Aurora-17	4	3.88 ± 0.428	0.42 ± 0.135	0.58 ± 0.501	3.30 ± 1.047	14.53 ± 4.336	29.59 ± 12.764
2	AberMagic	4	3.91 ± 0.220	0.42 ± 0.075	0.57 ± 0.119	3.56 ± 0.558	13.44 ± 2.874	27.10 ± 8.462
3	F1	377	4.75 ± 0.513	0.42 ± 0.088	0.40 ± 0.167	2.35 ± 0.732	12.35 ± 3.861	22.55 ± 5.128
3	Aurora-17	6	4.45 ± 0.247	0.39 ± 0.102	0.40 ± 0.059	2.28 ± 0.722	11.96 ± 3.311	21.71 ± 4.718
3	AberMagic	6	4.44 ± 0.301	0.40 ± 0.079	0.46 ± 0.117	2.64 ± 0.698	11.11 ± 2.929	21.37 ± 3.741

Table 2 Variance components ± standard error and broad-sense heritabilities (H_B^2) of the five fatty acids (C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2 linoleic acid; C18:3, linolenic acid) and the total (TOT), for each of the six cuts and across all cuts. σ_g^2, σ_t^2, $\sigma_{g.t}^2$, σ_r^2, $\sigma_{g.r}^2$ and σ_e^2 represent the genotype, cut, genotype by cut, replicate and genotype by replicate and error variance components, respectively. Negative values were treated as zero

		Trait					
Cut		C16:0	C18:0	C18:1	C18:2	C18:3	TOT
All	σ_g^2	0.0235 ± 0.00602	0.0010 ± 0.00018	0.0009 ± 0.00064	0.0303 ± 0.00790	1.2258 ± 0.24908	1.4414 ± 0.39168
	σ_t^2	0.2381 ± 0.15118	0.0038 ± 0.00239	0.0007 ± 0.00049	0.2288 ± 0.14519	9.3603 ± 5.93766	13.5114 ± 8.58929
	$\sigma_{g.t}^2$	−0.0145 ± 0.00824	0.0001 ± 0.00010	0.0018 ± 0.00074	−0.0236 ± 0.00748	−1.5640 ± 0.32383	−1.8656 ± 0.69248
	σ_r^2	0.0003 ± 0.00087	0.0001 ± 0.00008	0.0004 ± 0.00067	0.0170 ± 0.02475	0.8416 ± 1.20795	1.4583 ± 2.09690
	$\sigma_{g.r}^2$	−0.0010 ± 0.00505	0.0001 ± 0.00006	0.0024 ± 0.00071	0.0136 ± 0.00680	−0.2418 ± 0.19495	−0.5384 ± 0.38208
	σ_e^2	0.1876 ± 0.01249	0.0019 ± 0.00013	0.0135 ± 0.00091	0.1796 ± 0.01194	8.2347 ± 0.54628	16.4033 ± 1.08969
	H_B^2	0.59	0.81	0.25	0.57	0.63	0.50
Cut 1	σ_g^2	0.0116 ± 0.01644	0.0007 ± 0.00030	0.0014 ± 0.00077	0.0345 ± 0.01999	0.0931 ± 0.59262	0.2644 ± 0.99573
	σ_e^2	0.1205 ± 0.02077	0.0017 ± 0.00030	0.0044 ± 0.00080	0.1138 ± 0.02089	4.5905 ± 0.78930	7.7026 ± 1.31846
	H_B^2	0.13	0.40	0.34	0.32	0.03	0.05
Cut 2	σ_g^2	0.0464 ± 0.02253	0.0016 ± 0.00047	0.0046 ± 0.00197	0.0254 ± 0.01501	1.7102 ± 0.59843	2.1014 ± 0.97977
	σ_e^2	0.1660 ± 0.02432	0.0027 ± 0.00040	0.0140 ± 0.00204	0.1175 ± 0.01720	3.8206 ± 0.55925	7.1369 ± 1.04457
	H_B^2	0.36	0.53	0.40	0.30	0.47	0.37
Cut 3	σ_g^2	0.0415 ± 0.01342	0.0014 ± 0.00029	0.0020 ± 0.00084	0.0417 ± 0.01244	2.2053 ± 0.64389	6.6624 ± 3.07479
	σ_e^2	0.0818 ± 0.01193	0.0010 ± 0.00015	0.0059 ± 0.00086	0.0714 ± 0.01042	3.6349 ± 0.53021	22.3948 ± 3.26662
	σ_B^2	0.50	0.74	0.41	0.53	0.55	0.37
Cut 4	σ_g^2	0.0153 ± 0.01308	0.0017 ± 0.00039	0.0012 ± 0.00226	0.0493 ± 0.01752	0.8434 ± 0.68509	0.8015 ± 0.96525
	σ_e^2	0.1095 ± 0.01610	0.0016 ± 0.00023	0.0206 ± 0.00302	0.1108 ± 0.01636	5.6850 ± 0.83595	8.4355 ± 1.24020
	H_B^2	0.22	0.69	0.10	0.47	0.23	0.16
Cut 5	σ_g^2	0.0199 ± 0.03091	0.0009 ± 0.00029	0.0078 ± 0.00433	0.0186 ± 0.03114	−0.1537 ± 0.98023	−0.5121 ± 1.74081
	σ_e^2	0.2765 ± 0.04063	0.0018 ± 0.00027	0.0331 ± 0.00487	0.2810 ± 0.04123	9.5699 ± 1.40594	17.264 ± 2.53346
	H_B^2	0.13	0.49	0.32	0.12	0.00	0.00
Cut 6	σ_g^2	0.0278 ± 0.01652	0.0010 ± 0.00029	0.0015 ± 0.00130	0.0312 ± 0.01793	0.5242 ± 0.61285	−0.7980 ± 0.77985
	σ_e^2	0.1299 ± 0.01890	0.0016 ± 0.00023	0.0110 ± 0.00161	0.1393 ± 0.02039	5.3634 ± 0.78569	8.2960 ± 1.21306
	H_B^2	0.30	0.56	0.21	0.31	0.16	0.00

parental values were at the lower tail-end of the distribution. The parental values for both C18:2n-6 and C18:0 fell more towards the centre of the distribution. A Spearman's rank correlation test between the phenotypic traits showed that C16:0 concentration correlated significantly with all the other fatty acid components, while C18:0 correlated with C18:1n-9 and C18:2n-6, but not C18:3n-3 (Table 3). The high degree of correlation between C18:3n-3 and the total fatty acid content reflects the fact that C18:3n-3 comprised by far the highest proportion of all fatty acids (Table 1).

Linkage map and QTL analysis

A little over 1000 markers [984 RAD markers generated in this work and 56 previously mapped SSR markers (Turner *et al.*, 2006)] were polymorphic in the parents. It was, however, striking to notice that the majority of RAD markers segregated as a one-way pseudotestcross (backcross-type 1 : 1 segregation) with the heterozygous marker present in Aurora. We initially attempted to construct a map based on the two-way pseudotestcross model in JoinMap (CP designation), but that resulted in extended linkage group (LG) sizes, and clustering of the SSR markers. Furthermore, 77 of the 96 markers classified as hk × hk had extreme segregation distortion, so only 19 of those were usable. In order to identify novel alleles associated with fatty acid content, we used a one-way pseudotestcross segregation analysis to capture the variation in the Aurora parent. This gave rise to a map consisting of seven LGs comprising 434 markers and spanning a total of 505 cM (Figure 2, Table S1). This was aligned with a previously published *L. perenne* genetic map using the common SSR markers (Turner *et al.*, 2006).

Of the 434 markers shown on the map in Figure 2, 197 nonredundant markers were retained in the QTL analysis (see Experimental procedures). Table 4 and Figure 3 summarize the results of the QTL analysis. The QTLs shown in Figure 3 represent data for each year, and only those QTLs that were present in both cuts of any given year are included. Additionally, significant QTLs for the overall data are shown. Table 4 lists the main features of these QTLs, including how much of the phenotypic variance they explain, and the markers closest to the maximum LOD score from the multiple QTL mapping function (MQM) analysis. Notably, QTLs were identified for C16:0 in year 2-derived data on LGs 2 and 7, C18:0 in years 2- and 3-derived data on LGs 3, 4 and 7, C18:2n-6 in years 1- and 2-derived data on LGs 2 and 5, and C18:3n-3 in years 1- and 2-derived data on LG 1, and on LG 7 in year 2-derived data. The QTLs for the total content of fatty acids overlap the C18:3n-3 QTLs and are largely explained by the fact that C18:3n-3 constitutes by far the largest proportion of all the five measured fatty acids (Tables 1 and 3). C18:1n-9 is the only fatty acid for which no significant QTL was detected. Furthermore, overall QTLs for content of C18:3n-3 and the total were identified on LG 1, for C18:0 at LGs 3, 4 and 7 and for C18:2n-6 on LG5.

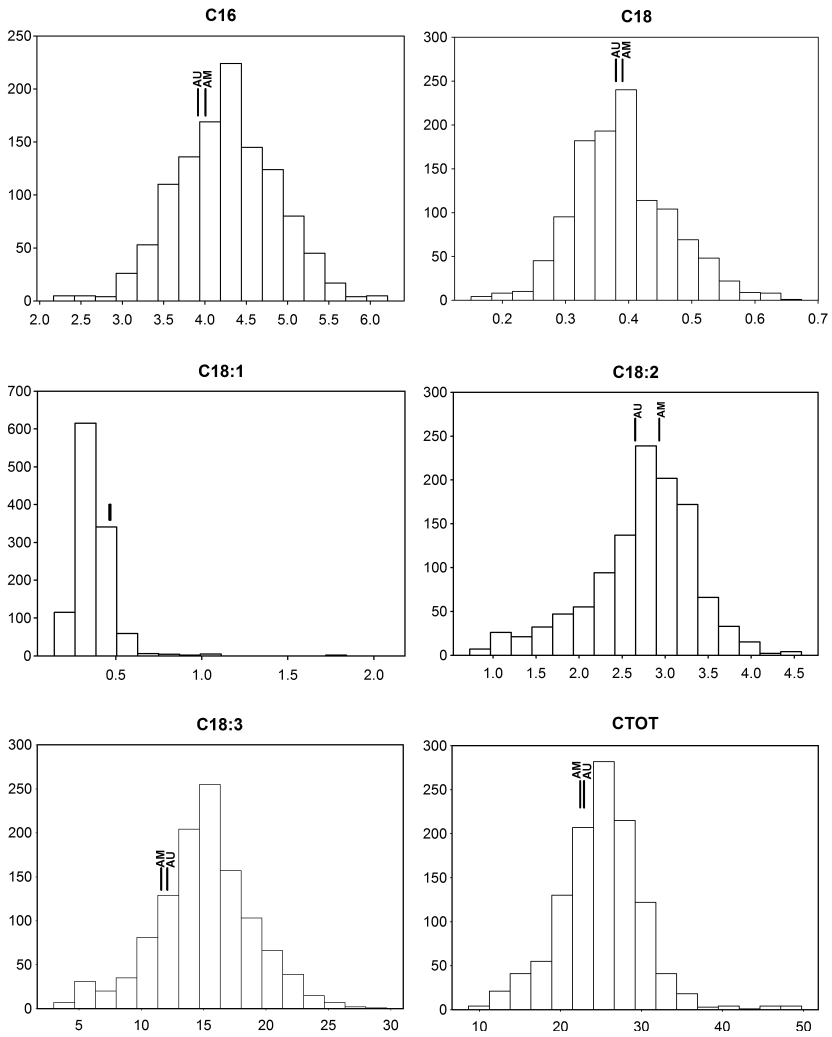

Figure 1 Frequency plots of lipid components in perennial ryegrass. Designations of fatty acids are as follows: (C16 – C16:0; C18 – C18:0; C181 – C18:1n-9; C182 – C18:2n-6; C183 – C18:3n-3; TOT, total fatty acid). All values of fatty acid content are in mg/gDW. The values for Aurora (AU) and AberMagic (AM) are indicated by vertical lines.

Table 3 Spearman's rank correlation coefficients for fatty acid constituent traits in the F1 mapping population based on means across all six cuts

	C16:0	C18:0	C18:1	C18:2	C18:3	CTOT
C16:0		0.44***	0.26*	0.32**	0.56***	0.56***
C18:0			0.24*	0.32**	0.16	0.13
C18:1				0.68***	0.07	0.14
C18:2					0.17	0.28**
C18:3						0.86***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Single marker analysis and RAD marker candidate genes

The Kruskal–Wallis test provided a nonparametric analysis of the significance of individual markers to the phenotype. The marker closest to the peak of the QTL identified in the MQM mapping is listed in Table 4. In order to identify specific genetic markers and their association with the fatty acid phenotypes, we also carried out an association analysis involving all the genetic markers with a minor allele frequency above 5%. A simple linear model (analysis of variance) was used, and we corrected for multiple testing using a false discovery rate test as described by Storey (2002). The *q*-value for genome-wide significance was set at 0.05. The results are summarized in Figure S1, which shows a Manhattan plot of the trait phenotype–genotype associations for each fatty acid at the six cuts. For C18:0, there was good agreement between the single marker analysis and the position of the QTLs on LGs 3, 4 and 7 in years 2 and 3. Similarly, significant C18:2n-6 associations were found on LGs 2 and 5, which is also where the QTLs were located. C18:3n-3 associations and QTLs were identified on LG 1 in both years 1 and 2. The main discrepancies related to single marker associations identified on LG 4 with C16:0 in year 3, cut 2, and on LGs 4 and 5 with C18:2n-6, but for which no significant QTLs were identified.

BLAST searches of RAD markers against rice coding sequences were used to generate comparative alignments between ryegrass and rice. These generated 319 reliable alignments, the majority of which were consistent with previous studies (Devos, 2005; Jones *et al.*, 2002; Yang *et al.*, 2009; unpublished observations), and could therefore be used to delineate regions of the rice genome for candidate gene identification. A full list of markers syntenic to rice, along with their associated trait(s) and alignment positions, is provided in Table S2. While the short length of the RAD sequences (84 bases) reduces the likelihood of imputing function based on similarity to other sequences in the databases, a few putative gene-based sequences were identified among the RAD

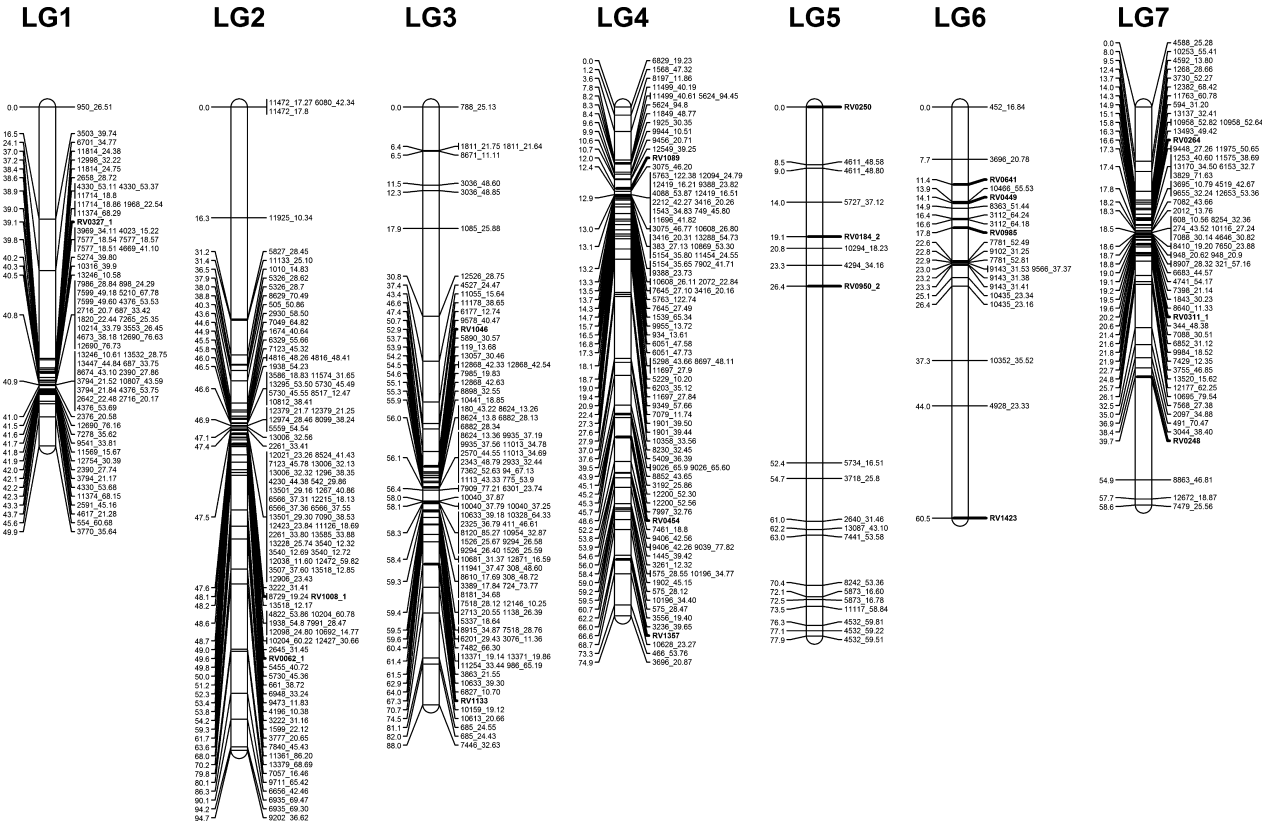

Figure 2 Linkage map of Aurora × AberMagic population using restriction-associated DNA (RAD) and SSR markers. Numbers on the left of the linkage groups are cM. SSR markers are highlighted in boldface. The full list of markers and their positions can be seen in Table S1.

tags. Two RAD tags were of particular interest: the first aligned with a GDSL-like lipase/acylhydrolase from rice chromosome 4 (Os04 g47390). Moreover, this RAD marker (12472_59.82) was located on ryegrass LG 2 (47.5 cM), which is known to be syntenic to rice chromosome 4 (Devos, 2005; Jones *et al.*, 2002; Yang *et al.*, 2009), and it was significantly associated with the variation in C18:2n-6 in the second cut of year 1 with genome-wide significance (q-value = 0.011). In the nonparametric Kruskal–Wallis analysis, the marker was also significantly associated with C18:2n-6 in year 1 ($P < 0.01$) and year 2 ($P < 0.005$). Furthermore, the marker also associated with C16:0 in year 2 ($P < 0.001$) in the Kruskal–Wallis test, although it did not have genome-wide significance with this trait ($q = 0.054$ and 0.143 in year 2, cuts 1 and 2, respectively). A translation of the sequence based on its alignment to the rice gene (Os04 g47390) suggests that the nonsynonymous C/T polymorphism would derive a serine/phenylalanine substitution (Figure 4). The polymorphism was associated with 14% of the phenotypic variance of C18:2n-6 (year 1, cut 2). The second marker (10695_79.54) aligns to a 3-beta-ketoacyl CoA synthase 6 (KCS-6), a very-long-chain fatty acid–condensing enzyme that catalyses the first rate-limiting step in very-long-chain fatty acid biosynthesis (Bannenberg *et al.*, 2009). This marker was also associated with C16:0, but at the qC16_Y2b QTL identified on LG 7. The genome-wide significance of the single marker association was less marked ($q < 0.1$) for this trait. A significant association ($q < 0.05$) was observed with C18:0, and the marker lies within the qC18_Y2c QTL identified for these traits on LG 7 (Figure 3). However, in this case, the G/C polymorphism is not predicted to cause any change in amino acid sequence, suggesting this is not the causative mutation. The marker lies within 6 cM of the closest significant marker for the qC18_Y2c and qC18_Y3b QTL, but is a considerable distance from the closest marker associated with C16:0. The qC16_Y2b QTL is very broad, however, while the qC18_Y2c and qC18:2_Y2b QTLs are clearly defined on LG 7 (Figure 3). It is possible therefore, given the relatively small size of our mapping population, that there is insufficient power to resolve the C16:0 QTL into smaller subcomponents.

Discussion

Restriction-associated DNA sequencing identified a total of 707 unique contigs that enabled the creation of a genetic map based on 434 RAD markers which, in combination with SSR markers, could be successfully assigned to one of the seven ryegrass LGs. The position of the SSR markers that were mapped was in complete agreement with previous work (Turner *et al.*, 2006).

Some of the clustering of the map (Figure 2) may be attributed to sequence variation at the *Sbf*I sites used in RAD library generation (Pfender *et al.*, 2011). Missing values also contribute to this effect. But the reason for the difference in frequency of identification of polymorphic markers within the two parental genotypes is not clear. One explanation could be that AberMagic is a relatively recently developed variety derived from the 11th generation of recurrent selection in the IBERS breeding population. This may have led to fixation of more alleles than would seem likely to have occurred in Aurora. This appears to be supported to some degree by the fact that twice as many SSR markers were polymorphic in the Aurora parent as compared to the AberMagic parent. While any remaining QTL segregation in

Table 4 Identification of quantitative trait loci (QTL) for lipid constituents including LOD score (the significance threshold as determined by permutation test is in brackets), and significance level for the Kruskal–Wallis test and percentage variation explained by the QTL. The names of the QTLs are composed of the fatty acid, the year (Y), and where appropriate a letter distinguishing between QTLs for the same fatty acid in 1 year. QTLs without year indicator are the overall

Trait	LG	Pos. IM (cM)	Closest marker	Single marker association (K–W *P*-value)	Max LOD score significance threshold in brackets	Percentage variation explained by QTL
qC16_Y2a	2	35–60	661_38_72	0.001	2.79(2.4)	12.7
qC16_Y2b	7	0–35	3730_52_27	0.0001	4.14(2.4)	18.2
qC18_Y2a	3	50–77	10040_37_87	0.0005	3.19(2.5)	14.3
qC18_Y2b	4	10–20	934_13_61	0.0001	3.25(2.5)	14.6
qC18_Y2c	7	23–35	7568_27_38	0.0001	3.35(2.5)	14.2
qC18_Y3a	4	10–20	934_13_61	0.005	2.70(2.4)	10.7
qC18_Y3b	7	26–34	7568_27_38	0.001	2.29(2.4)	10.0
qC18a	3	48–66	10040_37_87	0.0005	3.29(2.4)	14.7
qC18b	4	10–20	934_13_61	0.005	2.50(2.4)	10.1
qC18c	7	23–35	7568_27_38	0.0001	3.77(2.4)	18.6
qC18:2_Y1a	2	50–58	4196_10_38	0.0005	2.66(2.4)	12.2
qC18:2_Y1b	5	0–50	4294_34_16	0.0005	4.24(2.7)	19.0
qC18:2_Y2a	2	42–60	9473_11_83	0.005	2.93(2.4)	13.4
qC18:2_Y2b	7	20–32	13520_15_62	0.005	2.51(2.5)	11.6
qC18:2	5	2–40	4294_34_16	0.0005	4.58(2.9)	20.2
qC18:3_Y1	1	15–50	12690_76_16	0.0005	3.98(2.7)	17.6
qC18:3_Y2	1	15–40	6701_34_77	0.01	2.14(2.5)	8.5
qC18:3	1	15–50	12690_76_16	0.0001	4.03(2.6)	17.7
qCTOT_Y1	1	15–50	12690_76_16	0.0005	3.76(2.3)	16.6
qCTOT_Y2	1	37–45	12998_32_22	NS	2.32(2.5)	9.2
qCTOT	1	15–48	12690_76_16	0.0005	3.28(2.7)	14.7

LG, linkage groups.

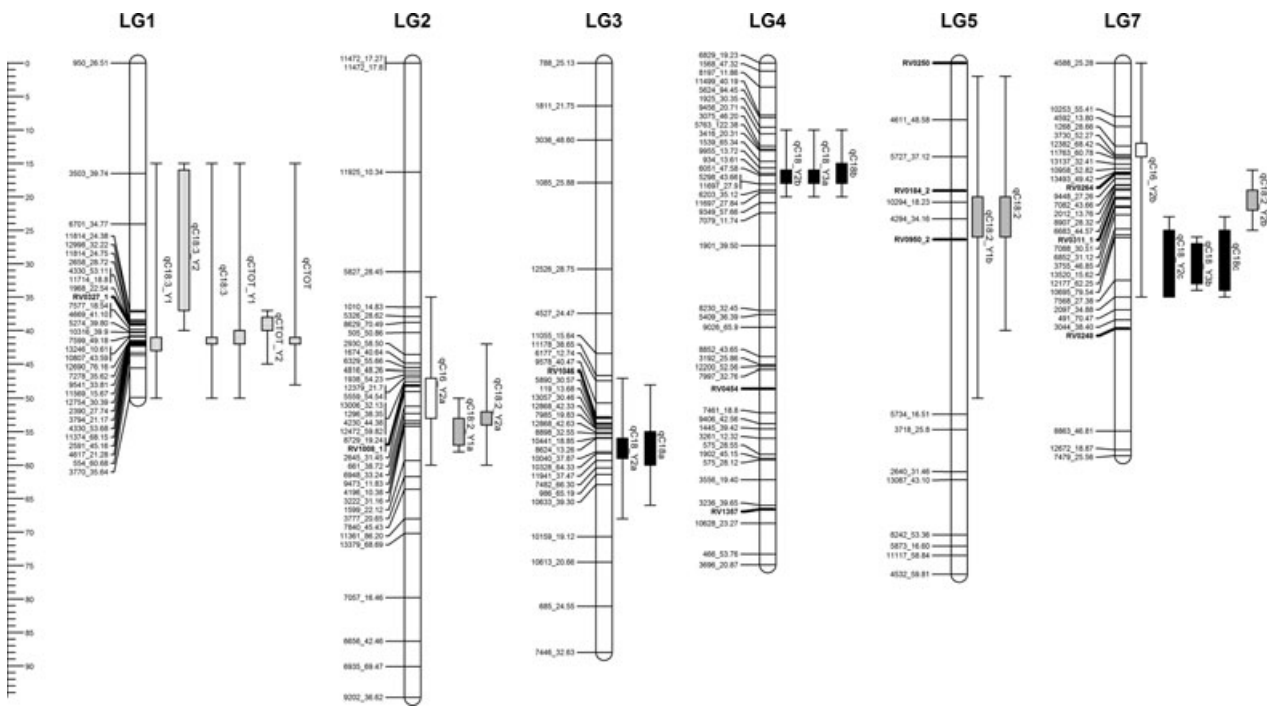

Figure 3 Quantitative trait loci (QTL) of fatty acid constituents at different years. The bars indicate the extent of the QTL by multiple QTL mapping function (MQM) mapping, and the line extensions indicate the IM QTL with approximately 1 LOD score either side of the significance threshold. SSR markers are highlighted in boldface.


```
Lp        LYTAGARKFSIVSPSLVGCCPSQRXAG
          ||.||||||:||||||||||||| ..
Os KGHLKDLYGAGARKFSVVSPSLVGCCPSQRAVAHDTNDLDFHGCSRAAN
```

Figure 4 Alignment of translated sequence of perennial ryegrass restriction-associated DNA (RAD) marker 12472_59.82 with partial translated sequence of the rice GDSL-like acyl-hydrolase/lipase gene (Os04 g47390). The X in the ryegrass sequence indicates the segregating serine/phenylalanine polymorphism.

AberMagic is part of the residual variance, the one-way pseudo-testcross model used here identifies novel alleles not present in the breeding population.

The small difference in phenotypic values of the two parents relative to the distribution in the mapping family for most of the traits (Figure 1) may suggest a degree of transgressive segregation. However, this is more often associated with hybrids between inbred lines. While our results indicate that AberMagic is less heterozygous than Aurora, none of the parents can be described as inbred lines. There is genetic variation within any synthetic population, and it is possible that the specific genotype used here from the AberMagic variety is atypical of the population. Given the relative paucity of segregating markers derived from AberMagic, most of such transgressive segregation would logically be caused by dominance and epistatic effects of heterozygous loci in novel combinations. The continuous distributions seen in Figure 1 also illustrate the environmental contribution to the variation. While there was no statistically significant G × E interaction (Table 2), the effect of cuts on all the fatty acid constituents was highly significant. The variance component analysis bears this out (Table 2). It showed that cuts contributed between 75% and 91% to the explained variance, genotype between 9% and 19%, genotype × cut a maximum of 3%. The exception was C18:1n-9 where cut only contributed 11%, while genotype, genotype × cut and genotype × replication contributed 14%, 29% and 38%, respectively. Environmentally induced effects such as variation in irradiance are likely to contribute to changes in chloroplast membrane structure (Prioul *et al.*, 1980) and thus indirectly affect the content of fatty acids, which are the main building blocks of these structures.

The trait heritabilities across cuts indicate that a sizeable proportion of the variation is heritable with the exception of C18:1n-9 content (Table 2). It was also the only trait for which there was a significant genotype by cut and genotype by replication variance component. It is interesting to note that this was also the only trait for which we found no significant QTLs (Table 4, Figure 3). Lower heritabilities within individual cuts can be explained by the smaller number of replicates (see Experimental procedures), and by large block effects in some cases, particularly in year 3 (Table 2). While the mean values of the total concentration of fatty acids varied between 16 and 30 mg/gDW, the range between the two most extreme genotypes was 10 and 50 mg/gDW (Table 1; Figure 1). The high energy content of lipids compared to carbohydrates and protein emphasizes the scope for enhancing the content of lipids in herbage through genetic improvement. The chloroplasts of plant leaves, including ryegrass, contain a large amount of their lipids in the thylakoid membranes [400 g lipid/kg DM; (Roughan and Batt, 1969)], so an indirect way of increasing lipid content would possibly be to increase chloroplast content in leaves (Powell *et al.*, 2012).

The QTL for C18:2n-6 on LG 2 co-incided with a significant RAD marker (RAD_12472_59.82) representing a nonsynonymous polymorphism in a putative GDSL-like acid hydrolase/lipase gene (Figure 4). It could be associated with the high proportions of C16:0 and C18:2n-6 found in TAG and phospholipids, because lipases play a prominent role in TAG catabolism during germination or in membrane turnover (Buccioni *et al.*, 2012). However, different lipases hydrolyse different fatty acyl chains from lipid moieties, so it is impossible to predict exactly which role this lipase plays in lipid metabolism. It would be useful to obtain the other homozygous genotype in order to establish the degree of additivity and dominance of the alleles. It is interesting to note that numerous GDSL lipases/esterases were also identified within candidate regions of the rice genome delineated by syntenic RAD markers (Table S2), for several of the fatty acids studied. GDSL esterases and lipases are hydrolytic enzymes with multifunctional properties such as broad substrate specificity and regiospecificity. They are responsible for the recycling of membrane lipids, but also the breakdown of diacylglycerol (DAG) and TAG to release long-chain PUFA, which may then be assembled into membrane lipids. As a large multigene family, these lipases may underlie numerous QTL of small effect, explaining the high number of significant marker/trait associations identified here. Similarly, the KCS-6 gene identified within the QTLs for C16:0 and C18:0 lies within 200 kbp of two KCS-5 genes on rice chromosome 8 and is also within 5 Mbp of a known rice candidate gene for C18:0 (C16:0 acyl-ACP-thioesterase) identified by Ying *et al.* (2012). This emphasizes the positive role that genomic selection, that is, the use of all marker effects simultaneously to predict phenotype from genotype (Meuwissen *et al.*, 2001), may play in the future plant breeding of ryegrasses in 'capturing' multiple small QTL in segregating marker space.

Some of the syntenic regions correlate with locations of known QTLs for lipid content in rice. For example, a rice QTL for C18:0, ste8-2 (Ying *et al.*, 2012), maps to positions between 20 and 25 Mbp of rice chromosome 8. Both the MQM mapping and the single marker analysis identified significant QTLs (qC18_Y2c and qC18_Y3b) for this lipid constituent on LG 7 (Figure 3, Figure S1). Ryegrass LG 7 is partly syntenic to rice chromosome 8. The significant RAD markers on ryegrass LG 7 returned BLAST alignments to a region 21–28 Mbp on rice chromosome 8. Ying *et al.* (2012) did not discuss any candidate genes for their QTL, but analysis of model transcripts within the rice annotation reveals genes encoding omega-6 fatty acid desaturase, TAG lipase 2, two putative palmitoyltransferases and a chloroplastic acyl carrier protein. These enzymes tend to be involved in the catabolism of lipids rather than biosynthesis, which is consistent with the observation of Ying *et al.* (2012) that this QTL showed a negative effect. On ryegrass LG 4, we identified a block of significant RAD markers associated with C18:0. This is syntenic to a region of rice chromosome 3 spanning the 24–34 Mbp region and consistent with the positions of the two SSR markers used by Ying *et al.* (2012) to detect a QTL for C18:0. Within this region lies a gene encoding trigalactosyl-diacylglycerol 3 (TGD3), a key enzyme in the accumulation of thylakoid glycolipids. Ryegrass LG 2 has known synteny with rice chromosomes 4 and 7 (Jones *et al.*, 2002; Yang *et al.*, 2009). RAD markers on ryegrass LG 2 associated with qC18:2_Y1a and qC18:2_Y2a span the region

between 19 and 34 Mbp on rice chromosome 4. Ying *et al.* (2012) previously identified a QTL mapped between 21 and 25 Mbp containing the candidate gene beta-ketoacyl-ACP synthase I (KASI), a key enzyme in the elongation of C18:0 to very-long-chain fatty acids (C20 +). The ryegrass QTL region contains a number of other candidate genes, including another copy of GDSL esterase/lipase, stearoyl-ACP-desaturase and acyl-CoA thioesterase, suggesting that this relatively broad QTL region may actually represent several small QTL beyond the power of this analysis to resolve further.

The availability of detailed information about synteny to a reference genome such as rice can be used to facilitate the search for candidate genes underlying QTL and single marker associations. However, some caution is warranted, as the analysis described here pertains to fatty acids in the herbage, and not seed oil storage. The fatty acids we have measured will thus chiefly represent those from the thylakoid membranes in chloroplasts as well as those from other organelles and the plasmalemma. However, the correlation of known QTLs for lipid in a syntenic grass genome with those detected here is encouraging. To our knowledge, this work represents the first identification of QTL and single marker associations with lipid content in leaf material of perennial ryegrass and other similar forage crops, and we have been able to identify significant QTLs for C18:0, C18:2n-6 and C18:3n-3 in 2 of 3 years, and for the overall. The RAD markers used in the genetic map construction enabled us to identify a candidate gene underlying one of the QTLs for C16:0 and/or C18:2n-6. Seasonal and environmental effects play a substantial role, but the significant contribution of the genetic component to the phenotypic variation in fatty acid content is encouraging for the prospects of genetic improvement of qualitative and quantitative aspects of fatty acid content in forage grasses for ruminant nutrition.

Experimental procedures

Plant material

Progeny from a cross between genotypes of an old variety Aurora and a recently developed high-sugar grass variety AberMagic was used in this study. RAD marker and fatty acid analysis was performed on the two parents and 95 progeny. The plants were grown in the field near IBERS as spaced plants, with management cuts every 6 weeks and annual application of 250 kg N/ha. Two replicate clones of each genotype were planted in a randomized design in two blocks. Leaf material was harvested for fatty acid analysis in June and September of 2008, 2009 and 2010.

Analysis of fatty acids

Fatty acid content was determined from 1 g of freeze-dried material using heneicosanoic acid methyl ester (C21:0) as an internal standard (Sigma-Aldrich Co, St Louis, MO) and a one-step extraction-transesterification procedure (Sukhija and Palmquist, 1988). Fatty acid methyl esters (FAME) were separated and quantified using a gas chromatograph (CP-3800; Varian Inc., Walnut Creek, CA) equipped with a flame ionization detector, automatic injector, split injection port and a 100-m fused silica capillary column (i.d., 0.25 mm) coated with 0.2-μm film of cyanopropyl polysiloxane (CP-Sil 88; Varian Inc) using hydrogen as the fuel and helium as the carrier gas. The total FAME profile in a 1-μL sample at a split ratio of 1 : 30 was determined using a temperature gradient programme described by Lee *et al.* (2005). Peaks were identified by comparison of retention times with authentic FAME standards (ME61; Larodan fine chemicals, Malmo, Sweden; S37; Supelco, Poole, Dorset, UK).

RAD marker identification

DNA was extracted from leaf tissue using the DNeasy 96 Plant Kit (QIAGEN, Crawley, West Sussex, UK) and quantified via pico-green detection analysis. RAD library preparation (using the rare cutter *Sbf*I for restriction digest) and sequencing was carried out by Floragenex (Eugene, OR) using an Illumina HiSeq2000 NGS platform (Baird *et al.*, 2008). RAD tags that displayed polymorphism between the parents were screened in the progeny to confirm segregation, and markers with more than 10% missing data in the progeny were excluded.

Genetic map and QTL detection

The genetic map was constructed using the JoinMap4 program (Van Ooijen, 2006). The expected segregation ratios of 1 : 1 for a one-way pseudotestcross or 1 : 2 : 1 for an intercross, depending upon the type of polymorphism, were tested for all the markers by a chi-square test. Most of the markers segregated in a 1 : 1 ratio with the polymorphism present in the Aurora parent. We therefore generated the map as a one-way pseudotestcross rather than a two-way pseudotestcross (Simone *et al.*, 1997). A breakdown of markers used in the mapping process is given in Table 5. A LOD score between 3 and 9 was used to separate markers into LGs. The Kosambi mapping function was used to convert recombination frequency to genetic distance. A goodness-of-fit jump threshold of 5 was used for retaining markers except for LG 7, for which 6 was used in order to retain the marker 10695_79.54. We used the MapQTL5 program for the QTL analysis (Van Ooijen, 2004). A reduced version of the map developed as described above was used. Markers closer than approximately 1 cM were eliminated in chromosomal regions with a high density of markers. The phenotypic data for the five fatty acid constituents (C16:0, C18:0, C18:1n-9, C18:2n-6 and C18:3n-3) and total fatty acids (CTOT) for each of the six cuts, the year means and the overall mean were used as phenotype data. A Kruskal–Wallis analysis of single marker associations was performed, followed by interval mapping (IM) to identify QTLs. The LOD thresholds for genome-wide significance were determined by permutation tests (1000). A QTL was declared if the LOD score was higher than the threshold level ($P < 0.05$; Churchill and Doerge, 1994). The MQM was then implemented following selection of a cofactor at the QTL with the highest LOD

Table 5 Restriction-associated DNA and SSR markers used in the genetic map generation and their segregation classification.

Marker segregation type (JoinMap notation)	Markers heterozygous in parent	Markers used in backcross mapping	Markers used in QTL analysis
lm (Aurora) × ll (AberMagic)	789	434	197
nn (Aurora) × np (AberMagic)	131	NA	NA
hk × hk	96	NA	NA
ef × eg	10	NA	NA
ab × cd	14	NA	NA
Total	1040	434	197

QTL, quantitative trait loci.

score for a given trait, to improve its definition. For traits where more than one QTL was identified, a cofactor was added at the QTL with the second highest LOD score. Estimates of the percentages of phenotypic variance explained by the QTL were obtained from the MQM analysis.

Statistical analyses

The experiment was treated as a split-plot design in time with cuts as subplots. A linear mixed model was used to estimate variance components. The analysis was carried out using REML (restricted maximum-likelihood) analysis in Genstat (14th edition; VSN International Ltd, Hemel Hempstead, UK). For a split-plot design as this one, broad-sense heritability estimates were calculated according to the method of Schwartz *et al.* (2009). For each cut, the heritability was determined according to

$$H_B^2 = \sigma_g^2/(\sigma_g^2 + (\sigma_e^2/r)),$$

where σ_g^2 is the genotypic variance, σ_e^2 is error variance, and r is the number of replicates. Missing values in cut 1 (40) were taken into account by using 1.58 (150/190)*2 as the replication factor, and for the across cuts, a replication factor of 1.92 (1094/1140)*2 was used to account for 46 missing values (Galwey, 2006). The across-cuts heritability was calculated as

$$H_B^2 = \sigma_g^2/(\sigma_g^2 + (\sigma_{g.r}^2/r) + (\sigma_{g.t}^2/t) + (\sigma_e^2/t.r)),$$

where t denotes 'treatments', that is, cuts, $\sigma_{g.t}^2$ is the genotype by cuts interaction variance component, and $\sigma_{g.r}^2$ is genotype by rep interaction. Marker effects were estimated from the variance components obtained from the mixed model analysis of the trait against the marker in question, as a percentage of the total phenotypic variance of the trait.

The association analysis on single markers was carried out using a linear model (analysis of variance) for each marker with every trait. This analysis was performed in R (http://www.r-project.org/), and the q-value was used (Storey, 2002) with a genome-wide significance threshold of 0.05. Correlation coefficients were calculated based on overall means using the nonparametric Spearman's rank correlation test also implemented in R.

Acknowledgements

This work was funded partly by BBSRC institute strategic programme grant BB/J004405/1 and partly by DEFRA (Department for the Environment, Food and Rural Affairs) project LS3649 and through the Sustainable Livestock Production (SLP) LINK programme. The sampling and GC FAME analysis of the grasses was carried out within the ProBeef project funded by DEFRA, EBLEX (English Beef and Lamb Executive), QMS (Quality Meat Scotland) and HCC (Hybu Cig Cymru). We wish to thank John Davies and Phil Evans for sample collections and extraction and John Tweed for GC analysis. RAD marker development was funded by the Institute Directorate via the BBSRC and by the Welsh Government.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Manhattan plots of associations between RAD markers and phenotypic values for each year and cut within year.

Table S1 List of 434 RAD and SSR markers and their position in centiMorgan on the seven linkage groups (chrom.).

Table S2 List of RAD markers (RAD tag) associated with a given trait, their syntenic relationship to homologous genes on the rice chromosomes and their position (Mb) within the rice chromosome.

