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Synteny between a major heading-date QTL in perennial ryegrass (*Lolium perenne* L.) and the *Hd3* heading-date locus in rice

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Abstract The genetic control of induction to flowering has been studied extensively in both model and crop species because of its fundamental biological and economic significance. An ultimate aim of many of these studies has been the application of the understanding of control of flowering that can be gained from the study of model species, to the improvement of crop species. The present study identifies a region of genetic synteny between rice and *Lolium perenne*, which contains the *Hd3* heading-date QTL in rice and a major QTL, accounting for up to 70% of the variance associated with heading date in *L. perenne*. The identification of synteny between rice and *L. perenne* in this region demonstrates the direct applicability of the rice genome to the understanding of biological processes in other species. Specifically, this syntenic relationship will greatly facilitate the genetic dissection of aspects of heading-date induction by enabling the magnitude of the genetic component of the heading-date QTL in *L. perenne* to be combined with the sequencing and annotation information from the rice genome.

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Introduction

The control of the transition from vegetative growth to reproductive growth is a fundamental process in plant development with considerable potential for practical application. Consequently, there is a large body of reported research on the molecular biology of flowering in model species and, particularly in grain crops, on the genomic locations of QTLs for heading date and associated characteristics. In crops principally grown for their vegetative organs, such as *Lolium perenne*, other forages and many *Brassicas*, the transition to flowering growth is often associated with a reduction in 'quality' traits in the vegetative parts of the plants. Therefore, it is also important to gain a basic understanding of the flowering processes and to identify QTLs which influence heading date in these species.

The observation that, to a greater or lesser extent, there has been a recognisable conservation of the physical order of orthologous genes between plant species that share common ancestry has had a great influence on our interpretation and application of plant molecular genetics (Tanksley et al. 1992; Teutonico and Osborn 1994; Moore et al. 1995; Van Deynze et al. 1998). This, combined with the recent publications of the complete sequences of the rice and *Arabidopsis* genomes (Arabidopsis Genome Initiative 2000; Goff et al. 2002), has provided a powerful set of tools for undertaking genetic analyses in plant species whose genomes are not as well characterised or as amenable to molecular approaches as model species, but which may have considerable scientific or economic significance. In addition, comparative genetic and physiological analyses of crop species may provide insights into the possible properties of genes and genomic regions of model species.

There have been a number of studies which have identified QTLs for heading date and photoperiod sensitivity in rice, wheat and barley (for example: Laurie et al. 1995; Li et al. 1995; Xiao et al. 1996; Yano et al. 1997, 2000; Lin et al. 1998; Worland et al. 1998; Yamamoto et al. 1998, 2000; Börner et al. 2002a, 2002b). As with most

other traits studied in economically important crops, the ultimate aim is to be able to manipulate the trait and associated characteristics, both through marker-assisted selection and through the identification and cloning of the underlying genes. However, accurately locating QTLs and so, in genomic terms, the search-space for these underlying genes, can be a major problem. Two of the chief reasons for this are (1) the problem of recognising the true genotype/phenotype relationship in the presence of considerable environmental influences, and (2) the possible presence of complex epistatic effects. However, comparisons of chromosomal organisation and QTL location between crop species can indicate areas of genomes which seem to have analogous functions (Teutonico and Osborn 1994; Xiao et al. 1996; Osborn et al. 1997; Kole et al. 2002). Presumably, these conserved regions also indicate the presence of underlying gene(s) of generic importance. Where these 'cross-species' QTLs can be identified, there may be the possibility of cross-referencing between the genetic map of a non-model species and the sequenced genome of a model species (Osborn et al. 1997; Kole et al. 2001). The study presented here identifies a genomic region with a major effect on heading date in the forage crop *L. perenne* and identifies the orthologous region in the fully sequenced rice genome as a first step to identifying the underlying effective gene(s).

Materials and methods

Plant materials

The F_2 *L. perenne* mapping population (180 genotypes) was derived from self-pollinating an F_1 hybrid obtained by crossing individuals from partially inbred lines developed from the two agronomically contrasting cultivars, Perma and Aurora (Turner et al. 2001).

Heading-date evaluation

One hundred and sixty-six F_2 genotypes were grown as spaced plants and induced to flowering under field-conditions at Aberystwyth, UK. Heading date was scored as number of days from 1 April until there was a minimum of three inflorescences emerging from the flag leaf.

Molecular marker analysis

DNA was extracted from the plant material as described by Hayward et al. (1998) and using the QIAGEN DNEasy Plant Mini Kit (QIAGEN, Crawley, UK). The majority of the molecular marker data was collected as described by Armstead et al. (2002). Additional markers were as follow: microsatellites M16-B, M4-136 and M15-185 were described by Kubik et al. (1999); LPSSRH01A07, LPSSRK01A03 and LPSSRK01A11 by Jones et al. (2001); and RYE 5, 6, 12 and 14 and Uni-001 were obtained from Jan de Riek, DvP, Melle, Belgium. STS markers B6101–B6107 were described by Skøt et al. (2002) and OSE, PRO, ADH, OSW and CAT were described by Lallemand et al. (1998). RFLP probe Fp41 was obtained from P. Canter (IGER, Aberystwyth, UK), M65-1 and LtCO were obtained from I. Donnison (IGER, Aberystwyth, UK), INV1:2 and AlkInv were obtained from J. Gallagher (IGER, Aberystwyth, UK) and analysed according to Armstead et al. (2002). Additional AFLP markers were produced as described by Armstead et al. (2002). STS markers LpF1–LpF4 were assayed on 96 individuals from the F_2 population. They were amplified using primers designed on the basis of conserved regions in target sequences from rice, wheat and barley; the rice sequences were either mapped RFLP probes in rice or physically located near mapped RFLP probes in the rice genome (Table 1). Thermal cycling was performed beginning with 1 min at 94°C, followed by 10 cycles of 1 min at 94°C, 1 min at 65°C (with the temperature reduced by 1°C per cycle), 3 min at 72°C, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C and 3 min at 72°C. Amplification products were electrophoresed on agarose gels. LpF2 and LpF3 were scored directly on the basis of segregating size polymorphisms of the amplification products; LpF1 and LpF4 alleles were scored as CAPS markers after appropriate restriction enzyme digestion of the amplification product (LpF1, *Dde1*; LpF4, *Rsa1*). Primers used for the amplification of LpF1 for the CAPS analysis were designed directly on the *L. perenne* sequence.

Table 1 Primer sequences and target sequence origins for markers LpF1—LpF4

Lolium perenne marker	Polymorphism type	Primer sequences 5′–3′	Rice BAC GenBank accession number	Target sequences[a]	Closest rice markers
LpF1	CAPS	ACTATGGGCGTTTATGTGCG[b] GTATCATCTGAACCAAGGCG[b] CGTCCAAGCTTTATGCTCTGG[c] GCTTGGAACTGAGGTGTTCTCAG[c]	AP000399	Rice: GRMG00000006747 Barley: GA BG415252 Wheat: GA BE606973	R1952 G8008[d]
LpF2	SIZE	CTTCACTGAGCATGGTTCTTC CTCCCCAGCTTAGCATTGTCACCCT	AP004806	Rice: GRMG00000073640 Wheat: GA BE446622	R2967[d] R1962
LpF3	SIZE	TCATGTACGCCGACTTCTAC TGAGGTGGATGCCGTCCCAG	AP004844	Rice: GRMG00000071822 Barley: GA AV938402 Wheat: GA BU100288	RM3414 RM5815 Hd3a C764
LpF4	CAPS	GAGCGGTACCGCAGCTGCAACT TTATTAACATCTTGCAGTTGTTGTT	AB026295	Rice: GRMG00000007107 Barley: GA BE193581 Wheat: GA BJ245890	G30 C1032[d] R2634

[a] GRMG-predicted gene sequence obtained from <exref type="URL">http://www.gramene.org</exref>, *GA* GenBank accession number
[b] Primer pair based on rice/barley/wheat homology
[c] Primer pair based on *L. perenne* sequence
[d] Rice marker sequence forms part of rice target sequence

Genetic analysis

The molecular marker data were analysed as an F_2 population using JOINMAP 2.0 (Stam and Van Ooijen 1995) with the Kosambi mapping function. Regions of significant segregation distortion were identified using the χ^2 tests in JOINMAP 2.0. Comparative mapping information for rice chromosome 6 was obtained from Monna et al. (2002), the Gramene database (http://www.gramene.org) for the Cornell 1994 RFLP map and the RGP database (http://rgp.dna.affrc.go.jp/publicdata/geneticmap98/geneticmap98.html) for the high-density RFLP map.

QTL analysis

QTL analysis was carried out using MapQTL version 4.0 (Van Ooijen et al. 2002) using interval and MQM mapping. Co-factors for MQM mapping were identified using the automatic co-factor selection option within MapQTL version 4.0.

Results

Heading date

Heading date in the *L. perenne* F_2 mapping population varied from a minimum of 33 days to a maximum of 73 days (mean = 48.5; n=166), with the distribution skewed towards a lower heading date (Fig. 1). The original parents of the mapping family were not available for heading-date evaluation, but field tests over 3 years of similar inbred materials derived from the two varieties 'Perma' and 'Aurora' gave mean heading dates of 68±8 and 26±14 days-to-heading, respectively. This is consistent with the range of heading dates observed in the mapping family, as illustrated in Fig. 1, and indicates that the observed heading-date response in the mapping family was not atypical.

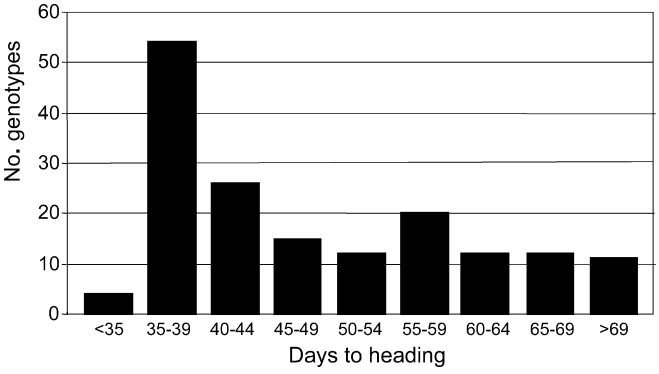

Fig. 1 The distribution of number of days-to-heading in the F_2 *Lolium perenne* family

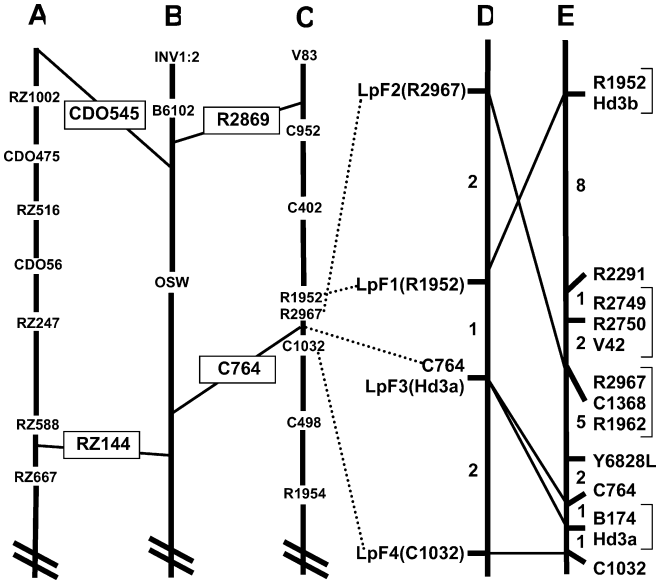

Fig. 2A–E Genetic maps illustrating syntenic region between *L. perenne* L7 and rice chromosome 6. **A** Section of rice chromosome 6 from Cornell RFLP 1994 map. **B** Section of *L. perenne* F_2 L7 map. **C** Section of rice chromosome 6 RGP high density RFLP map. Probe names in *boxes* linking **A/B** or **B/C** indicate probes mapped in both *L. perenne* and rice. **D** Enlarged section of *L. perenne* L7 map, including markers LpF1-LpF4. Distances are given in centiMorgans. Probe name in *brackets* indicates closest mapped marker in rice illustrated in **E**. **E** Section of chromosome 6 rice map taken from Monna et al. (2002) indicating relative positions of heading-date loci *Hd3a* and *b*. *Numbers* indicate the number of plants in which recombination between adjacent markers occurred (see Monna et al. 2002)

Genetic mapping

Genetic mapping of the *L. perenne* F_2 population identified seven linkage groups (L1–L7) and covered a total of 628 cM (Table 2). This map is an extension of that reported earlier by Armstead et al. (2002) and can be considered to give good coverage of the *L. perenne* genome and to be consistent with the International Lolium Genome Initiative (ILGI) reference map (Jones et al. 2002). Significant deviations (P<0.05) from expected segregation ratios for individual markers were identified on all linkage groups, with L5 and L7 being particularly affected (Table 2). These regions of segregation distortion extended for the complete genetic distance of L7 and the majority of the genetic distance of L5.

Comparisons of the relative positions of RFLP probes CDO545, RZ144, R2869 and C764 in *L. perenne* and rice indicated a region of synteny between *L. perenne* L7 and rice chromosome 6, which covered the region of the rice genome containing the *Hd3* locus (Yamamoto et al. 1998) or the *Hd3a* and *b* loci (Monna et al. 2002) (Fig. 2). Mapping of markers LpF1–LpF4 in the region of the RFLP marker C764 indicated a likely high degree of synteny in this genomic region between *L. perenne* and rice (Fig. 2). The marker order for LpF1 and LpF2 was different from that which would be predicted from rice; however, the relatively small number of genotypes assayed does not allow a firm conclusion to be drawn as to the precise marker order over the small recombinational distance being analysed. An STS amplified by primers based on the *Hd3a* rice sequence (GenBank accession number BD169090) was also evaluated; how-

Table 2 Genetic linkage map of *L. perenne* F_2 population. Chromosome designations L1–L7 are those which conform to the *Triticeae* numbering (Jones et al. 2002)

L1		L2		L3		L4		L5		L6		L7	
Marker	Distance (cM)	Marker	Distance (cM)	Marker	Distance (cM)	Marker	Distance (cM)	Marker	Distance (cM)	Marker	Distance (cM)	Marker	Distance (cM)
PGI	0	M4-136	0	C30	0	E42M3311	0***	E39M4909	0***	CDO542	0***	INV1:2	0***
CDO580	13	CDO38	10*	E38M4702	15	PSR580	5	GSY60.2	13***	CDO395	10***	B6102b	6***
E36M5508	36	FpAFPD	24	LPSSRK01A11	17***	PSR115	14	F29-1b	16***	E39M4908	27**	R2869	8***
E36M5503	41	FpAFPH	24	LPSSRK01A03	27	Rz395	31	CDO1380.2	17***	E41M5712	34	CDO545	12***
E38M4709	49	E39M5805	26	CDO328	32*	RYE12	35**	CDO127	17***	RZ87	45	OSW	21*
E41M5709	54	BCD855	28	E42M3306	33	BCD808	38	BCD1087	21***	E42M3305	47	C764	29***
E42M3308	60	R3349	29	E39M4907	35	PSR922	41	RZ206	21***	M65-1	49***	RZ144	35***
BCD1072	61	Fp12.1	30	E41M5704	38	C746	42*	PSR574	24***	B6104	52	E40M5909	36***
M16-B	69	Fp12.2	31*	CDO455	39	CDO122	42	CDO412	31***	CDO718	55	E39M4910	36**
OSE	70	Rz395.1	31	F29-1a	42*	E41M5708	46	E40M5907	40	AlkInv.2/3	56*	E38M4710	37***
B6101	72	CDO365	34	PSR394	42	E41M5702	47	B6103b	44***	AlkInv.1/4	58***	PSR690	42***
BCD738	73*	LPSSRH01A07	35	E36M5502	43	E38M4706	48	R2710	65	C37	60	GSY60.1	43***
E42M3309	75*	C451	36*	E40M5901	46*	CDO795	49	E40M5905	69	CDO686	66*	E37M5601	43***
E38M4707	77*	CDO385.2	36	WG889	47	E38M4705	49	E36M5512	72	RYE14	68	CDO385.1	44***
R2953	78	CDO395.2	36*	BCD828	48	ADH	50	RZ404	78***	RYE6	69	E41M5710	45***
CDO202	85	CDO59	37	GOT/3	53*	RZ537	51			RYE5	73	C390	46***
E36M5511	90	E40M5910	38	C250	54*	E36M5505	52			CDO516	85	RZ952	48***
		E42M3310	40	PRO	57***	E36M5506	52					LtCOa	55***
		E41M5713	41	E42M3301	58	Fp41	53					E39M5802	57**
		C145	43	E42M3302	58	CDO1380.1	55					LtCOb	58*
		B6106b	46	E36M5509	68	R2702B	58					E41M5701	60*
		E38M4704	46	CDO345	71***	CDO938	59					ACP	86**
		E39M4906	48	C949	77	PSR163	63					CAT	102***
		CDO1417	49	E38M4712	79***	E42M3313	66						
		E41M5703	55	E36M5513	82	E42M3303	68						
		E40M5908	62*	UNI-001	99	E39M4905	68						
		M15-185	64			B6105	87						
		E36M5510	67*			CDO20	88						
		PSR540	71*			E39M4904	95						
		E41M5705	79										

Distorted segregation ratios for individual markers: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

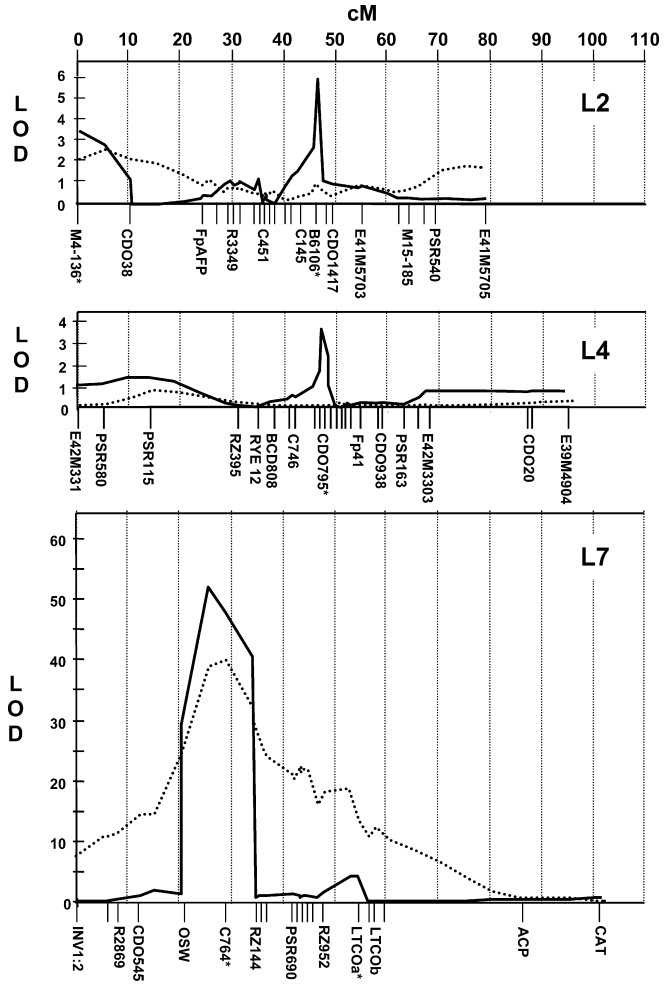

Fig. 3 LOD profiles for the positions of heading-date QTL on *L. perenne* linkage groups L2, L4 and L7, identified using interval mapping (*dotted line*) or MQM mapping (*solid line*). The names of some markers are omitted for clarity (see Table 2 for complete list). * Marker used as a co-factor in MQM mapping

ever, no size or sequence polymorphism in the amplification product was identified and, therefore, the relative map position could not be ascertained.

QTL analysis and genotype/trait association

Figure 3 illustrates the relative position of QTLs with LOD≥3 identified in this *L. perenne* population. Using interval mapping, only a single QTL with an LOD score ≥3 was identified; the peak of this was associated with the C764 region of L7 (maximum: 29 cM, LOD 39, 70% variance explained). However, the magnitude of this QTL, combined with the distorted segregation seen on L7, was such that 75% of the genetic distance of L7 was associated with LOD scores ≥3. For the MQM mapping, the following markers were identified as co-factors: L2, M4-136 and B6106; L3, LPSSRK01A11; L4, CDO795; L6, E39M4908; and L7—C764 and LtCOa. This analysis

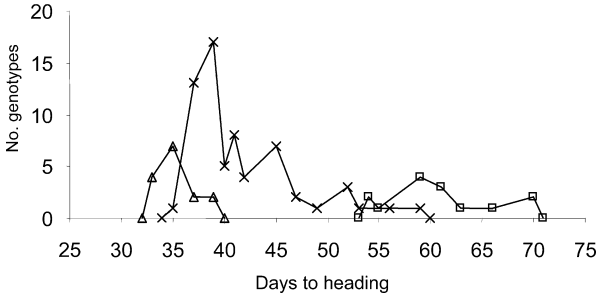

Fig. 4 The relationship between days-to-heading and genotype for marker C764 (L7) in the *L. perenne* F_2 mapping family. △ Homozygous (aa), × heterozygous (ab), ❑ homozygous (bb)

(Fig. 3) improved the resolution of the QTL associated with the C764 region of L7 (maximum: 26 cM, LOD 52, 44% variance explained) and also identified a second QTL on L7 associated with the LtCOa region (maximum: 55 cM, LOD 4, 2% variance explained). Additional QTLs with LOD scores ≥3 were identified on L2 (maximum: 46 cM, LOD 5.9, 5.6% variance explained) and L4 (maximum: 48 cM, LOD 3.5, 1% variance explained).

Figure 4 illustrates the genotype/trait association between C764 (three genotype classes: *aa*, *ab*, and *bb*) on L7 and days-to-heading. The observed segregation ratio for this marker was 0.4:2:1.2 (n=121) which differed significantly (P<0.001) from the expected 1:2:1 and indicated that approximately half as many homozygous early flowering genotypes were recovered as expected. This was consistent with the degree of segregation distortion that was identified for all of the markers on L7, the cause of which is unknown. There was a clear separation of the two homozygous classes in terms of the number of days-to-heading, with the majority of the heterozygotes showing a phenotype skewed towards earlier, rather than later, heading. Overall, this indicated the action of a dominant gene, or closely linked dominant genes, of major effect(s) segregating in the genomic region of C764.

Discussion

A number of aspects of the *Lolium*/*Festuca* complex of plant species make it a useful system for the study of floral transition and heading date: (1) genotypes from this complex exhibit a wide range of phenotypes in terms of the induction to flowering; (2) they represent a natural gene bank of flowering-type variation that can be derived from both geographically and ecologically diverse situations; (3) many of the different species within this group of grasses are inter-fertile, allowing the production and analysis of specific experimental populations (Thomas et al. 1994; Humphreys et al. 1996; Armstead et al. 2001); and (4) the recent publications of anchor-marker RFLP linkage maps for *L. perenne* (Armstead et al. 2002; Jones et al. 2002) allow for the alignments of these genetic maps with the *Triticeae* and rice.

In rice, there are a number of different QTLs which have been associated with heading date and the photoperiodic response which have been located on 8 of the 12 chromosomes (Li et al. 1995; Xiao et al. 1996; Yano et al. 1997, 2000; Lin et al. 1998; Xiong et al. 1999; Ishimaru et al. 2001). Up to six QTLs on different chromosomes have been identified in a single segregating population (Yu et al. 2002). We are unaware of any published study, prior to the present one, designed to locate QTLs for heading date in *L. perenne*, so the complexity of the genetic control of the heading-date response in this species is, as yet, not fully evaluated. However, linkage disequilibrium studies on heading date in *L. perenne* have identified markers which map to the same region of *L. perenne* L7 as the largest QTL in the present population (Leif Skøt, unpublished observation), so it is likely that this region may have wider significance, at least within the *Lolium*/*Festuca* complex.

As illustrated in Figs. 2 and 3, the genomic region associated with the major QTL on L7 shows a high degree of synteny with the *Hd3* region of rice chromosome 6, and it is likely that orthologous genes are involved. However, even though MQM mapping greatly improved the resolution for the likeliest position of a single QTL in this region, the magnitude of the effect and the distorted segregation on L7 means that it is not apparent whether this is just a single QTL, or a number of tightly linked QTLs. A possible candidate for a second effective gene in this region might be the *L. perenne* equivalent of *Hd1*, a CONSTANS-like gene which underlies a major heading-date QTL mapped to rice chromosome 6 (Yamamoto et al. 1998; Yano et al. 2000). Although the precise equivalent genomic region of the *Hd1* locus has not, as yet, been identified in *L. perenne*, general syntenic relationships between rice and *L. perenne* would suggest it might also be located on L7.

The second, though considerably smaller, QTL located on L7 was associated with marker LtCO. Interestingly LtCO, which mapped as duplicate loci *LtCOa* and *b* in the present population, is also a CONSTANS-like, flowering-associated cDNA sequence, which was isolated from *L. temulentum* (Donnison et al. 2002). As with the location of the major QTL on L7, there is also possible equivalence here with rice; the rice QTL associated with *Hd5* maps to rice chromosome 8, and general syntenic relationships between rice and *L. perenne* suggest that the *Hd5* equivalent genomic region in *L. perenne* may be located in this region of L7.

In addition to the QTL on L7 which was associated with the majority of the variance, there were smaller significant QTLs on L2 and L4 (Fig. 3). QTLs for heading date have been identified in *Triticeae* species associated with chromosomes 2, 4 and 7 (Laurie et al. 1995; Lundqvist et al. 1997; Börner et al. 2002a). Also, the C145 region of *Lolium* L2 shows a degree of synteny with the region of rice chromosome 7 associated with the heading-date QTL *Hd4* (Yano et al. 1997), so there may be orthologous genes in these regions as well.

The identification of a highly significant QTL for heading date in *L. perenne* in a region of the genome which shows strongly conserved synteny with rice allows for the possibility of extending the present work towards identifying candidate genes from the sequenced rice genome. Potentially flowering-associated genes from this region have already been identified in rice (the target sequence for marker LpF2 codes for a CONSTANS-like zinc finger protein (Monna et al. 2002) and a BLAST search of the target sequence for LpF4 indicates that it codes for a MADS box-type protein (Jack 2001). *Hd3a* itself—which is physically on the same BAC (GenBank accession number AP004844) as C764 and the LpF3 target sequence—codes for a *FLOWERING-LOCUS-T* (FT) orthologue (Hayama et al. 2003; Yano 2003). If the marked segregation for heading date seen in the present *L. perenne* family can be replicated in a larger family based on selected F_2 genotypes, then it should be possible to fine map this region and so test more accurately genotype/phenotype associations over relatively small recombinational distances. In particular, this might clarify the number of effective genes both in the immediate C764 region [Monna et al. (2002) suggest two loci, *Hd3a* and *Hd3b*] and on the whole of *L. perenne* L7. Additionally, as QTLs for heading date have also been identified on chromosomes 7 of wheat and barley (Laurie et al. 1995; Worland et al. 1998; Börner et al. 2002b) to which *Lolium* is more closely related than it is to rice, cross-comparisons between *L. perenne*, other members of the *Lolium*/*Festuca* complex and the *Triticeae* may help to accurately identify regions of the genome and underlying genes which influence heading date in these important crops.

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