End of Project Report 5194

Exploration of flowering control in *Lolium perenne* L.

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PROJECT OUTPUT TO DATE

A1	Stephen Byrne, Emma Guiney, Susanne Barth, Iain Donnison, Luis A. J. Mur and Dan Milbourne (2009). Identification of coincident QTL for days to heading, spike length and spikelets per spike in Lolium perenne L. Euphytica. 166: 61 – 70.
A1	U. C. M. Anhalt, P. (J. S.) Heslop-Harrison, S. Byrne, A. Guillard and S. Barth (2008). Segregation distortion in *Lolium*: evidence for genetic effects. Theoretical and Applied Genetics. 117: 297-306.
B2	Byrne, S., Donnison, I., Mur, L., Guiney, E. Exploration of flowering control in late flowering *Lolium perenne* using gene expression and quantitative genetic approaches. XXVI EUCARPIA Fodder Crops and Amenity Grasses. Perugia, Italy, 3-7 September 2006.
B2	S. Byrne, I. Donnison, Luis Mur, E. Guiney. The investigation of flowering time of late/rare flowering *Lolium perenne*. Agricultural Research Forum, Tullamore, Ireland, March, 2005, page 19.

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Summary

Flowering or heading in *Lolium perenne* (perennial ryegrass) is induced by a period of vernalization, followed by long days at higher temperatures. When heading occurs there is a reduction in the feed quality of the forage and therefore extending the period of vegetative growth or eliminating heading during the growing season will improve the potential of perennial ryegrass in agriculture. Conversely, a better control of flowering time and increased heading will lead to higher seed yield for commercial producers. The aim of this project was to investigate the underlying genetic control of flowering time in perennial ryegrass. An F_1 population was created by cross-pollinating two lines with different heading dates and a genetic linkage map was constructed using Simple Sequence Repeat (SSR) markers. The population and genetic linkage map was then used to identify Quantitative Trait Loci (QTL) associated with heading date, spike length and spikelets per spike. A number of QTL were identified for all traits, some of which had not previously been identified in perennial ryegrass. A Suppression Subtractive Hybridization (SSH) study was also employed to identify genes differentially expressed between an extremely late flowering line and earlier flowering sibling line. Expression analysis of a number of identified genes through floral induction was performed using real time RT-PCR. This revealed a number of transcripts with expression profiles indicative of a role to play in floral induction.

1.0 Introduction

Lolium perenne (perennial ryegrass) is a member of the *Poaceae* family of plants which contains approximately 10,000 species. It has great economic importance owing to its suitability as a forage grass. It has high digestibility and palatability making it ideal for dairy and sheep forage systems and it is particularly suitable for animals with high nutrient requirements (Hannaway *et al*., 1999). The point when flowering structures emerge from the leaf sheath is widely referred to as heading. When heading occurs in *Lolium perenne* there is a marked decrease in the digestibility of the forage. Current perennial ryegrass varieties are classified into three maturity classes according to their heading date. They are regarded as early, intermediate or late and each class has favourable characteristics.

Plants respond to various environmental cues to ensure that reproduction takes place under favourable conditions. In the plant kingdom there is great diversity in how plants respond to these external signals and it is greatly influenced by a plants geographic origin. The majority of temperate grasses are classed as having a dual induction requirement to flower and *Lolium perenne* is among them. The primary induction requirement is a combination of vernalization and short days. Secondary induction is the response to long days and in *L. perenne* increasing day length triggers flowering in plants that have been exposed to primary induction.

The vast majority of our knowledge on the molecular events that take place during flowering comes from studies using the model plant *Arabidopsis thaliana*. In *Arabidopsis* a number of key floral inductive pathways have been identified; the vernalisation, photoperiod and light quality, autonomous and the gibberellin pathways (reviewed in Simpson and Dean, 2002). These pathways converge on what are known as the floral pathway integrators, of which three have been identified: *FT*, *AGL20/SOC1* and *LFY* (Simpson and Dean, 2002). The pathway integrators in turn regulate the meristem identity genes that initiate floral organ development.

The genetic control of flowering time has been well studied in the model dicot Arabidopsis thaliana. However, it is clear from studies in the cereals that not all the mechanisms for control are conserved between species and it has been suggested that the vernalization requirement has evolved independently in temperate cereals (Gendall and Simpson, 2006). It is therefore necessary to study the underlying mechanisms of control in non model species of economic value.

The study described here was initiated after the identification of a number of genotypes with an extremely late flowering phenotype under field conditions. A number of approaches were taken to elucidate the underlying genetic mechanisms of the late flowering phenotype of one of these genotypes. The first approach was to identify genes differentially expressed between the late flowering line and a more normal flowering line with the same genetic background. This was achieved using the differential gene expression technique Suppression Subtractive Hybridisation (SSH). Subtracted libraries were generated after the primary and during the secondary induction requirement to elucidate genes under differential expression between the two lines at the level of the shoot apical meristem. A second objective was to select transcripts under differential expression and monitor their expression profiles through floral induction. This was achieved by performing quantitative RT-PCR of individual

transcripts in tissue from non-induced plants, plants after primary induction and from plants during secondary induction.

A second objective was to identify the genetic basis of the phenotypic difference in flowering time using a quantitative genetics approach. Firstly, this involved developing a mapping population segregating for flowering time and create a genetic linkage map. This was achieved by cross-pollination of the same two lines utilised in the differential expression analysis and genotyping the resulting F_1 progeny with SSR molecular markers. Secondly, the F_1 population was characterised for flowering time and Quantitative Trait Loci (QTL) associated with the trait were identified. An additional objective was to identify QTL for traits putatively associated with seed yield. *Lolium perenne* in general is regarded as having a poor seed yield and increasing the yield is an important target for commercial producers. The traits investigated were the length of the flowering head (Spike) and the number of spikelets on each spike.

The final objective was developing marker systems for a number of genes identified in the differential expression study. These were subsequently mapped to determine if any transcripts co-located with identified QTL. It was envisaged that these approaches would provide an insight into the genetic control of flowering time and provide a basis for future research of flowering control in this important forage species.

2.0 Methods

2.1 Establishment of F_1 population

The genotypes J43 (earlier flowering) and J51 (very late flowering) were cross pollinated to generate an F_1 population segregating for heading date. Seed was harvested from the cross and planted in $36cm^2$ pots. This population was established under glass house conditions with an average temperature of 18^0C.

2.2 Phenotyping the F_1 population

The F1 population from the J43 X J51 cross was transferred outdoors in October 2004 and maintained in $36cm^2$ pots under outdoor conditions up to and including collection of phenotypic data. Plants were monitored on a daily basis from April 1st 2005 for head emergence. The day when three or more heads had emerged from an individual plant was recorded. This initial experiment was not replicated.
The F1 plants were propagated at the end of September 2005 to give three replicates of the FI population. Individual tillers were placed in $36cm^2$ pots and grown up under glass house conditions with an average temperature of 18^0C until they had become established. Potted plants were then transferred outdoors at the end of October and allowed fulfil their primary induction requirement over the winter period. Plants were transferred into a bay of a climate controlled glasshouse in April 2006. The three F1 populations were arranged in a completely randomised design within the bay, which was maintained at a temperature of 18^0C. Plants were given a 20 hour day length, supplied by over head fluorescence lamps from 7am to 7pm and by overhead 60 watt incandescent light bulbs from 7pm to 3am. Plants were monitored daily for head emergence, which was recorded when three or more heads had emerged from an individual plant. The first three heads to emerge were tagged and these heads were

used for the collection of additional phenotypic data once heading in the F1 population was complete. Spike length was recorded by measuring the distance between the bottom of the lowest spikelet to the top of the highest spikelet. Three spikes were measured on each individual plant giving a total of 9 measurements per genotype. Number of individual spikelets per spike was also counted on three spikes per plant giving a total of 9 measurements per genotype.

2.3 Testing of SSR molecular markers

A licence was purchased by Teagasc for the use of a large set of SSR markers developed by the Institute of Grasslands and Environmental Research (Aberystwyth, UK) and ViaLactia Biosciences (Aukland, New Zealand) (Table 2.0). Information on the location and linkage group for a subset of these markers was available and this was used to aid selection of appropriate SSR's for use in our study. In addition, SSR markers from the literature that were publicly available and had been mapped to their respective linkage groups (see Jensen *et al*., 2005 for marker information) were also utilised. In total, 150 SSR markers were identified which evenly covered the seven linkage groups of *Lolium perenne*. Primers pairs for each SSR were synthesised and fluorescently labelled by Applied Biosytems (California,USA). The forward primer of each SSR was labelled with one of four fluorescent dyes, Fam, Vic, Ned and Pet to allow multiplexing of PCR products on the ABI 3100 Genetic Analyzer.

Table 2.0: Identifiers for private SSR markers tested in this study

SSR Identifier	Number Tested	Source
RV**** and 13Ca1	45	ViaLactia Biosciences (Aukland, New Zealand)
LpACA***** or LpACT***** or LpHCA*****	55	IGER (Aberystwyth, UK)

As a first step, each marker was tested using the J43 and J51 parental DNA in order to determine amplification ability and detect polymorphism between the parents. Each PCR reaction was set up in 10µl's as follows; 1µl template DNA, 1µl of 2µM dNTP mix, 0.25µl of 10nM forward primer, 0.25µl of 10nM reverse primer, 1µl of 10X PCR Buffer, 0.08µl of Taq Polymerase (5U/µl) and 6.42µl of sterile ddH_2O. PCR products were then incubated at 65^0C for 30 minutes to avoid Poly A peaks. Products from four SSR reactions using different flourescently labelled primers were then pooled as follows; Vic labelled - 0.5µl, Fam labelled - 1µl, Pet labelled - 2µl, Ned labelled 2µl. Pooling of different amounts depending on the fluorescent dye was carried out to normalise for differences in dye strength. A formamide and sizing standard mix was prepared by adding 0.25µl of Liz-250 sizing standard (Applied Biosystems, USA) to 9.25µl of Formamide (Applied Biosystems, USA) and 0.5µl of the pooled PCR product was then added to this mixture. Samples were denatured at 95^0C for 5 minutes before running on the ABI Genetic Analyzer 3100.

2.4 Application of SSR's to the F1 population and linkage map creation

DNA was isolated from all 219 plants from the J51 and J43 cross along with both parents using a CTAB extraction protocol. Only markers showing good amplification

and polymorphism between the parents were selected for application to the entire mapping population. Markers were analysed on the entire population using the amplification conditions and recipes described above.
Initially markers were scored dominantly as an allele either being absent or present with the aid of GeneMapper 3.7 (Applied Biosystems, USA). Individual parental genetic linkage maps were created using the Kosambi mapping function in JoinMap 3.0 (Van Oijen and Voorrips, 2001). Locus genotype frequencies were calculated in JoinMap for the detection of markers differing significantly from Mendelian segregation ratios. In a second approach markers were scored co-dominantly using codes compatible with JoinMap 3.0 (Table 4.5). Linkage mapping was then repeated using the Kosambi mapping function of JoinMap 3.0 to develop a *Lolium perenne* consensus map.

2.5 QTL Analysis

QTL analysis was performed using MapQTL 4.0 (Van Ooijen *et al*., 2002). The first round of analysis was performed using the individual parental linkage maps. A non parametric Kruskal and Wallis test was performed using non transformed data sets. This test performs analysis on each molecular marker independently. Two criteria were necessary for the detection of a QTL; 1) a marker had to be significant at $P<0.01$ and 2) there had to be an increase in significance towards the marker with greatest significance. The parametric test Simple Interval Mapping (SIM) was then performed with transformed data where necessary. A threshold of LOD ≥ 3 was taken as a cut off for the presence of a QTL. In the second round of analysis simple interval and MQM mapping were performed using the consensus map. A permutation test of 1000 permutations was performed in MapQTL 4.0 for each trait to determine a genome wide significane level of $P<0.05$, above which a QTL was deemed significant. Significant QTL were identified in simple interval mapping and markers closest to the LOD peak for each QTL were selected as co-factors. MQM mapping was then performed and results investigated for shifts in QTL position and appearance of new QTL. In the case of either of these scenarios the co-factors were modified and MQM mapping was repeated. This was continued until no position shifts or new QTL were identified.

2.6 Mapping candidate genes

A number of EST derived markers were also developed to map putative flowering time controls genes identified in the study described in section 2.7. The marker CK2B3 was mapped using a fluorescent labelled primer, which detected size polymorphism within the mapping population. Additional EST derived markers were mapped by identifying SNPs within restriction enzyme sites (Table 2.2).

Table 2.2: Primer sequences and restriction enzyme used to map EST derived CAPS markers.

EST derived CAPs Markers	Forward and Reverse Primer Sequence	Restriction Enzyme
AGO4	F: GGAGCGTTGGAATTTCAATA R: GACACATCCATTCCCAGGAT	RsaI
BHLH	F: GTACCACGTGGGCCTATGA	BanII

	R: GTGTTCGCTGTCACCTCTCA	
Cullin	F: CGTCTGATGTGGCAAAATTC	BstNI
	R: CATATCCGCTGGCTTAATCG	
EARF	F:ACAAGATGAGGCCAGAGAGG	Hinf1
	R:GACCATGGGTTTTCCTACCA	
MYO	F: TCAAAGAGGATGGCATTGCT	HPyCH4IV
	R: TGTTGGGCTTAATGACACGA	

2.7 Suppression Subtractive Hybridization (SSH)

2.7.1 Harvesting Material

The genotypes J51 and J43 were tillered out and grown up in 0.8L pots under glasshouse conditions for six weeks. A total of 58 plants from each genotype were then transferred into controlled growth cabinets (Conviron). The cabinets were set to the following conditions: 8 h day length at 7^0C provided by a mix of fluorescent and incandescent light followed by 16 h of darkness at 6^0C. Plants were subjected to these conditions for 12 weeks, after which the following conditions were imposed: 20 h day length followed by 4 h darkness, all at 18^0C. Day length was provided by 12 h of fluorescent and incandescent light and 8 h of incandescent light alone. Shoot apical meristems were harvested from plants after 12 weeks of primary induction (PI) and on days 3, 4, 5, and 6 of secondary induction (SI). Meristems were harvested under a stereo microscope (Zeiss) in a laminar air flow unit using home-made tungsten dissection needles. Once meristems were removed from the plant they were placed in a Lysing Matrix D tube (Q-Biogen) and immediately stored in liquid nitrogen. Only five meristems were taken from any one plant and they were harvested between 10am and 1pm on each harvest day. Meristems were harvested from lines being compared in alternation; five meristems from each line at a time. A total of 30 meristems were isolated from each line on each of the harvest days.

2.7.2 RNA isolation and cDNA synthesis

Total RNA was isolated using Tri reagent (Sigma-Aldrich), and glycogen (Sigma-Aldrich) was added as a nucleic acid carrier to aid precipitation. Any contaminating DNA was removed by performing a DNase digestion using the DNA-*free* kit (Ambion) according to the manufacturer's protocol. cDNA was synthesised from samples after twelve weeks primary induction and from samples during secondary induction. In the case of secondary induction equal amounts of total RNA was pooled from days 3-6 for each line. Double stranded cDNA was synthesised using the Super Smart cDNA synthesis Kit (Clontech) according to the manufacturer's instructions.

2.7.3 Generation of Subtracted libraries

Suppression Subtractive Hybridisation (SSH) was performed according to the protocol described by Desai *et al*. (2000). cDNA was digested with *RsaI* and tester cDNA was divided into two aliquots and ligated with different adaptors. Both primary and secondary hybridisations were carried out in the presence of excess driver cDNA. Primary and secondary PCR were carried out with the following cycling conditions: 94 ^{o}C for 30 s, 66 ^{o}C for 30 s and 72 ^{o}C for 90 s and the optimum number of cycles was determined empirically. In total four SSH libraries were generated; forward and reverse libraries after twelve weeks primary induction and from pooled samples during days 3-6 of secondary induction. Reverse subtracted libraries were generated by switching the cDNA sample being used as tester.

2.7.4 Library Cloning and Differential Screening

The four subtracted libraries were cloned into pCR 2.1 vector using a TA cloning system (Invitrogen) and transformed into *Escherichia coli* TOP 10 chemically competent cells (Invitrogen). A total of 384 colonies were randomly selected from each library and their inserts were amplified and fixed as dot blots onto nylon membranes. Four replicates were made and each replicate was hybridised with one of four [α-^{32}P]-dCTP radioactively labelled probes. Probe labelling was performed using the Rediprime II labelling kit (Amersham Biosciences). The probes used were the forward and reverse subtracted libraries and the two unsubtracted tester controls generated during the SSH protocol. A total of 480 clones from the four libraries displaying a differential expression pattern were sequenced (AGOWA, Germany). The sequences were trimmed to remove vector contamination and assigned putative function by carrying out BLASTN analysis against the rice genome (The Institute of Genomic Resources; Release 4).

2.8 Expression profiling of candidate genes

cDNA was synthesised as described above using the total RNA. In addition meristem specific RNA was isolated from J43 and J51 glasshouse grown plants which did not receive a floral induction treatment. Primers were designed using Primer3 software to amplify 150-200 bp fragments for 20 transcripts selected from the SSH libraries as potential flowering time candidate genes (Table 3.1). Optimal annealing temperatures were determined experimentally. Real time expression assays were performed with SYBR Green dye (Sigma-Aldrich) on the ABI Prism 7000 Sequence Detection System. Data were normalized to four housekeeping genes using QBase relative quantification software (Hellemans *et al.*, 2007). The house keeping genes used were LpGAPDH (Petersen *et al*., 2004), elongation factor 1-α. (designed in a conserved region of a multiple alignment of the following sequences; *Arabidopsis* (NM_100667.2), maize (AY109326.1), barley (Z50789.1) and rice (AF030517.1)), LpActin (AY014278) and LpTubulin (AY742902). The efficiency of PCR reactions was calculated for each primer set by carrying out serial dilutions of a cDNA template and plotting CT values against the log of the template concentrations. The slope of the line was subsequently used to calculate the amplification efficiency (E) according to; $E=10^{(-1/slope)}$, which was used in the calculation of relative normalized expression in QBase (Hellemans *et al.*, 2007).

3.0 Results

3.1 Linkage Map

The SSR markers were assigned to seven linkage groups (Figure 3.0). The consensus map consisted of 54 SSRs and 6 EST derived markers and had a total map length of 226 cM. A total of 31% of markers on the consensus map showed a degree of segregation distortion with clusters on LG1 and LG7. In addition to the consensus map, individual parental maps were also constructed (data not shown) and there was very good preservation of marker order between all maps.

3.2 QTL identification

QTL analysis was carried out with both SIM and MQM mapping (Table 3.0). Positions of QTL derived from MQM mapping were determined for all traits analysed in the *'J43'* X *'J51'* F_1 late flowering population (Figure 3.0). For days to heading, using the replicated 2006 glasshouse data (DTH2006), five QTL were detected by MQM mapping on LGs 2, 3, 4, 5 and 7 explaining between 6.6 and 17.2% of the variance. Using the 2005 outdoor data (DTH2005), three QTL were detected with MQM mapping on LGs 3, 4 and 5 explaining between 5.6 and 31.0% of the variance.
Three QTL for spikelets per spike were detected on LG2, LG3 and LG4 explaining a total of 33.1% of the variance. Only one QTL was detected for spike length, on the top arm of LG4. As might be expected, given the apparent correlations and probable physiological link between the traits, all of the SPS and SL QTL either mapped very near to or coincided with QTL for DTH. On the top arm of LG2, a QTL for SPS mapped near to a DTH2006 QTL. Similarly, on the top of LG3, a QTL for SPS coincided with a DTH2005 QTL. The most significant cluster occurred on the top of LG4, where overlapping QTL for all three traits measured were found. This cluster included the large effect SL QTL (var. exp. 31.7%, LOD 13.36), in addition to DTH2006 and SPS QTL which explained 6.9% (LOD 5.31) and 10.3% (LOD 4.9) of the phenotypic variance respectively.

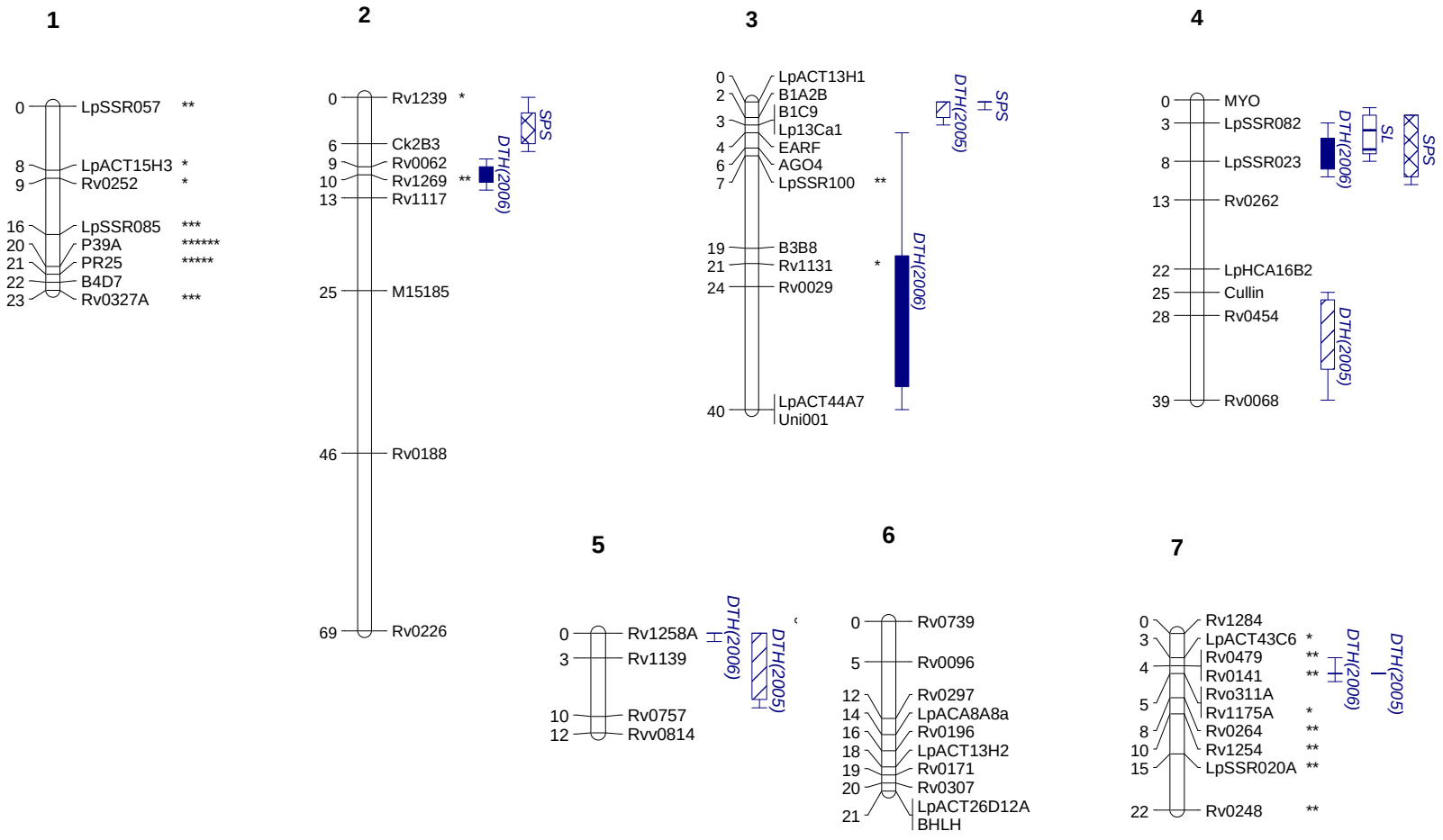


Figure 3.0: Genetic linkage map of *Lolium perenne* showing QTL for the traits, spike length (SL), spikelets per spike (SPS), days to heading in 2005 (DTH2005) and days to heading in glasshouse experiment (DTH2006) on five out of seven linkage groups using MQM mapping on the parental consensus linkage map. Asterisks indicate segregation distortion * P<0.05, ** P<0.01, *** P<0.001. QTL are shown with 1 and 2-LOD support interval. (Taken from Byrne *et al*., 2009).

Table 3.0: Quantitative trait loci detected using simple interval mapping and MQM mapping using a LOD threshold at a genome wide significance level of 0.05 determined by 1000 permutations. Spike length (SL), spikelets per spike (SPS), days to heading in 2005 (DTH2005) and days to heading in glasshouse experiment (DTH2006) *Represents values from a single replication. (Taken from Byrne *et al*., 2009).

Trait	Linkage Group	Simple interval mapping			MQM mapping		
		Position (cM ± 2 LOD)	LOD	Variation explained (%)	Position (cM ± 2 LOD)	LOD	Variation explained (%)
DTH(2006)	2	12.9 (0 – 37.7)	3.77	9.4	10.1 (8 – 12)	4.6	6.6
	3	-	-	-	23.8 (4.0 – 39)	3.61	6.4
	4	8.5 (0 – 23)	4.31	11.2	8.5 (2.5 – 10.2)	5.31	6.9
	5	0 (0 – 9.7)	7.06	22.6	0 (0 – 0.8)	9.12	17.2
	7	-	-	-	5.3 (3.5– 6)	3.71	4.7
DTH(2005)*	3	-	-	-	2.1 (0 – 2.5)	4.81	8.3
	4	-	-	-	28.3 (24.2 – 38.3)	4.77	7.7
	5	-	-	-	2.5 (0-9)	3.56	5.6
	7	5.4(5 – 9.5)	11.92	28.7	5.3 (4.8 – 5.2)	16.5	31
SL	4	2.6 (1.36 – 11)	13.36	31.7	2.6 (0 – 7.9)	13.36	31.7
SPS	2	5.0 (0 – 21.5)	3.86	10.3	5.8 (0 – 7)	5.45	10.9
	3	0 (0 – 19.5)	3.88	9.5	0 (0 – 1)	5.9	11.9
	4	-	-	-	7.6 (1.9 – 10.5)	4.9	10.3

3.3 Suppression Subtractive Hybridisation

A total of 480 inserts were sequenced from the four libraries which resulted in the identification of 144 non redundant sequences with significant homology to rice which have been deposited in GenBank (these can be found with accession numbers *FE905359* - *FE905358* and *FG356121* - *FG356083*).

3.4 Expression profiling of selected candidate genes

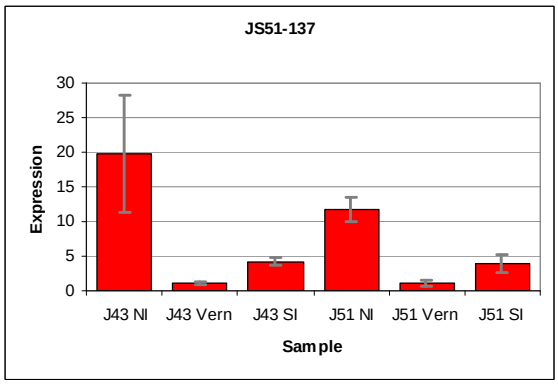
The expression profiles of 20 candidate genes selected from the SSH study (Table 3.1) was profiled through floral induction (Figure 3.1) using real time RT-PCR. The expression levels for both the earlier (J43) and later (J51) flowering lines were characterised in non-induced, primary induced and secondary induced meristems. This allowed the identification of novel expression profiles through floral induction for a number of transcripts.

3.5 Mapping of candidate genes

Six candidate genes from Table 3.1 were mapped. The mapping of these is described in section 2.6. No clear association with any of the heading date QTL was identified. However, these EST based markers will provide useful anchors to the rice genome for future investigations.

Table 3.1: Putative functions associated with 20 transcripts identified from three SSH libraries that were selected for further differential screening using real time RT-PCR. E-values and results of a BLAST search against the rice genome on TIGR.

Transcript ID	Putative Function	E-Value	Accession Number
	Genes with higher expression in line J51 after 12 weeks PI		
JV51-35	Argonaute-like protein	3.20E-32	FE905359
JV51-46	Elongation factor 2, putative, expressed	1.60E-48	FE905360
JV51-55	Drooping leaf protein, putative, expressed	5.00E-65	FE905361
JV51-56	Auxin response factor 3, putative, expressed	3.00E-64	FE905362
JV51-325	expressed protein (bHLH transcriptor like)	2.80E-45	FE905363
JV51-353	Casein kinase II beta-4 subunit, putative, expressed	6.60E-29	FE905364
JV51-370	Histone H3	2.60E-47	FE905365
	Genes with higher expression in line J43 during days 3-6 of SI		
JS43-92	OsMKK1 - putative MAPKK based on amino acid sequence homology, expressed	5.20E-85	FE905346
JS43-148	F-box family protein, putative, expressed	1.30E-94	FE905347
JS43-214	Cullin-4B, putative, expressed	9.80E-51	FE905348
JS43-241	zinc finger family protein, putative, expressed	5.10E-19	FE905349
JS43-254	Aspartic proteinase oryzasin-1 precursor, putative, expressed	1.30E-91	FE905350
JS43-300	Protein kinase domain containing protein, expressed	4.90E-50	FE905351
	Genes with higher expression in line J51 during days 3-6 of SI		
JS51-132	YDG/SRA domain containing protein, expressed	2.30E-29	FE905352
JS51-137	Inositol-3-phosphate synthase, putative, expressed	1.80E-48	FE905353
JS51-276	speckle-type POZ protein, putative, expressed	1.20E-24	FE905354
JS51-282	Methyl-binding domain protein MBD111, putative, expressed	2.50E-29	FE905355
JS51-321	DnaJ domain containing protein, expressed	7.20E-62	FE905356
JS51-344	Phosphoribosylanthranilate transferase, putative, expressed	1.30E-50	FE905357
JS51-352	Mitogen-activated protein kinase kinase kinase 2, putative, expressed	2.60E-30	FE905358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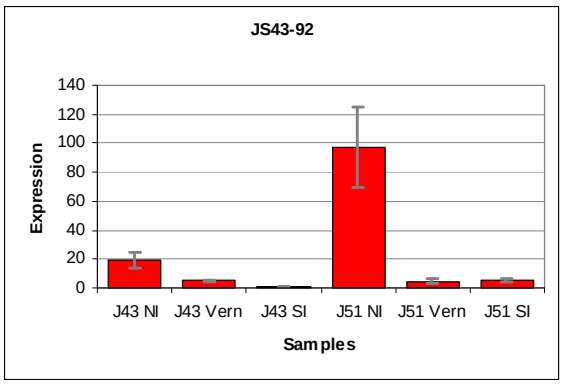
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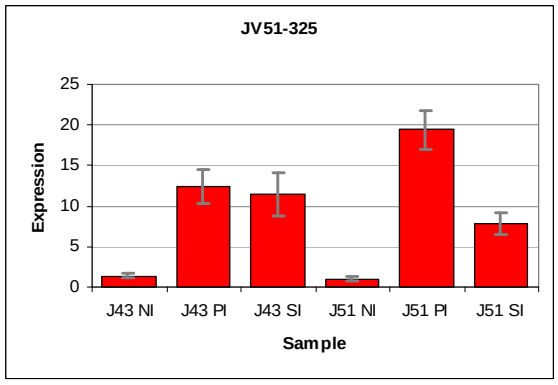
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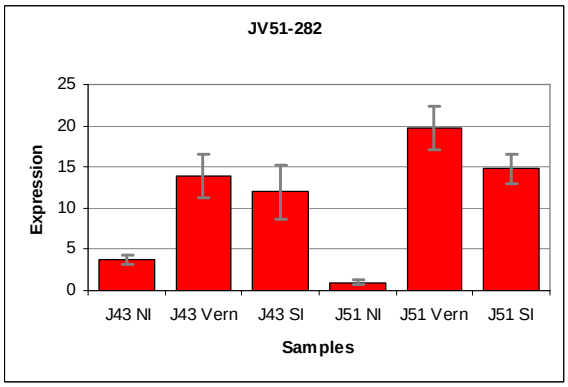
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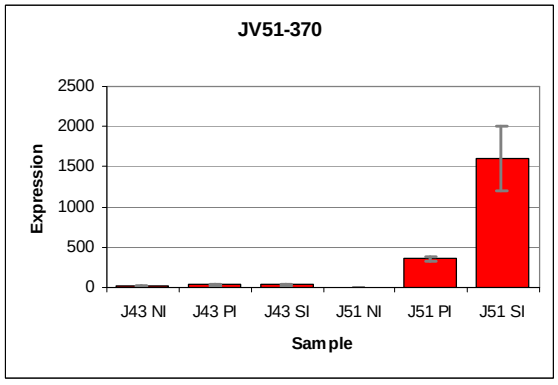
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J51 SI
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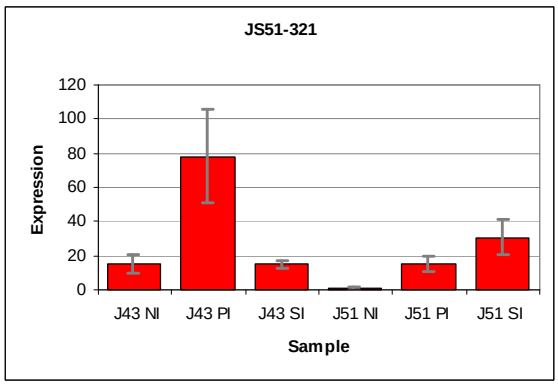
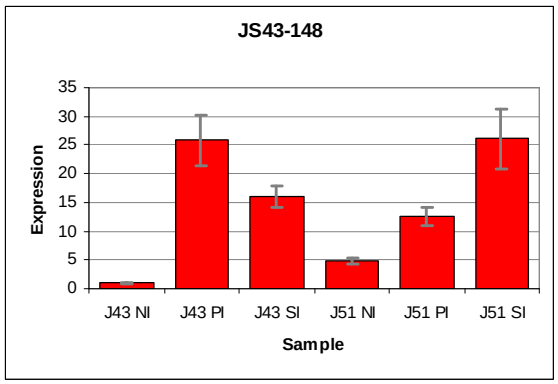
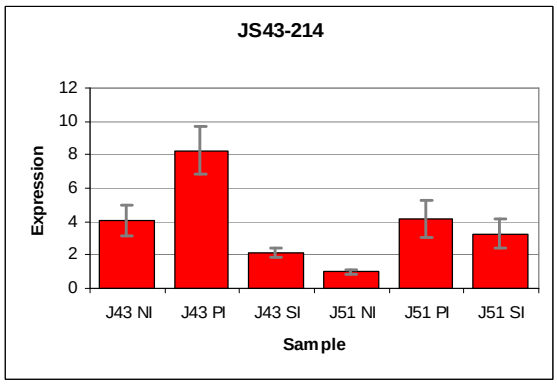
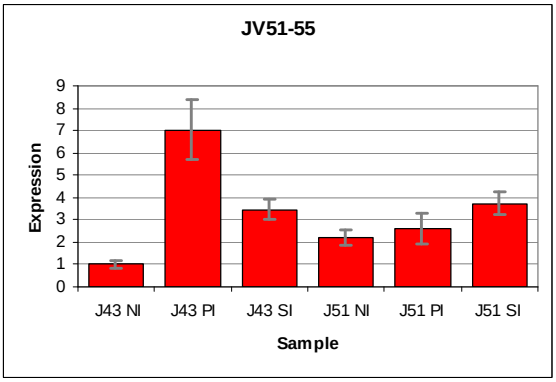
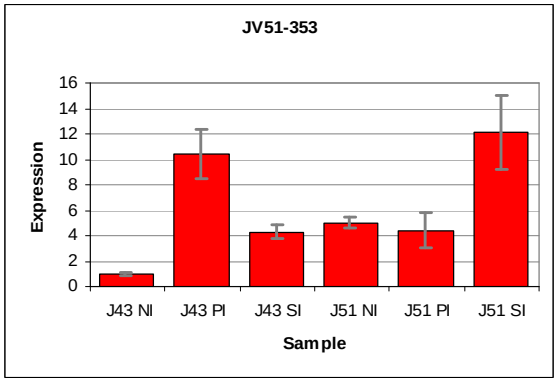
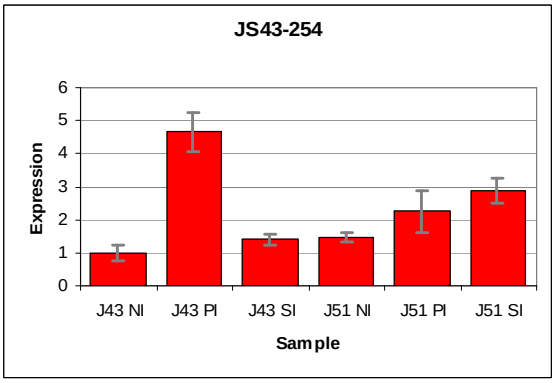

JS51-321
Expression
120
100
80
60
40
20
0
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

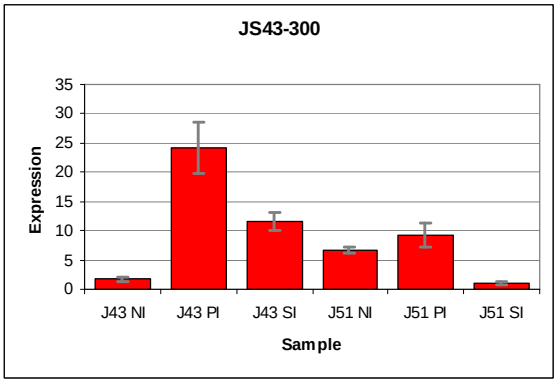

JS43-148
Expression
35
30
25
20
15
10
5
0
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

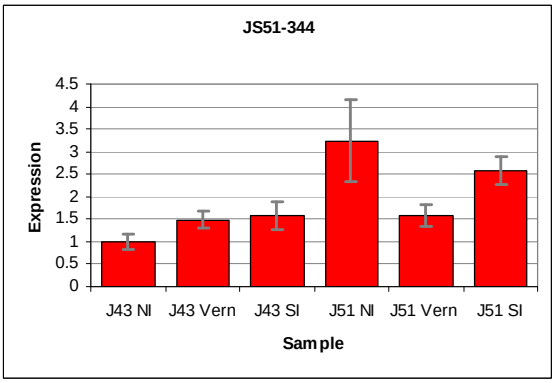

JS43-214
Expression
12
10
8
6
4
2
0
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

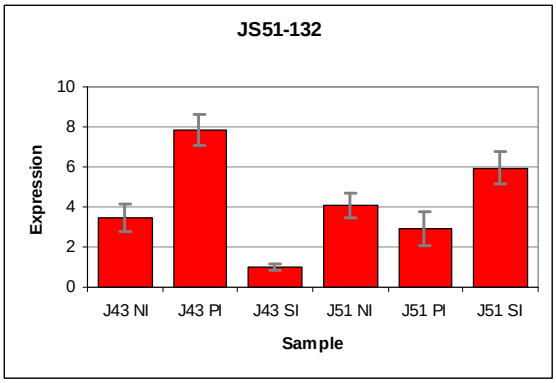

JV51-55
Expression
0
1
2
3
4
5
6
7
8
9
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

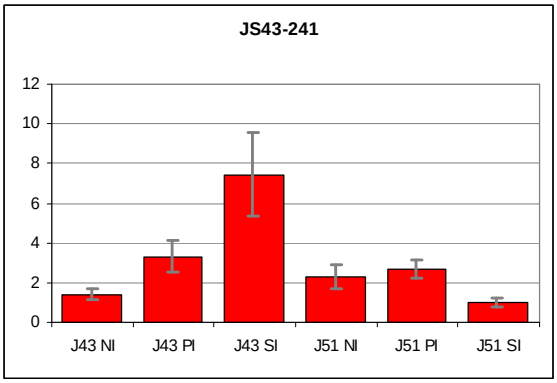

JV51-353
Expression
0
2
4
6
8
10
12
14
16
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

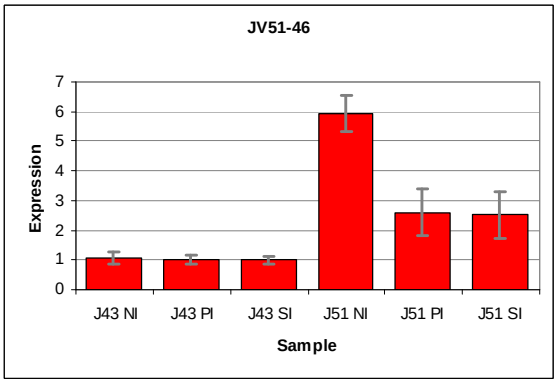

JS43-254
Expression
0
1
2
3
4
5
6
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample


JS43-300
Expression
0
5
10
15
20
25
30
35
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample


JS51-344
Expression
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
J43 NI
J43 Vern
J43 SI
J51 NI
J51 Vern
J51 SI
Sample


JS51-132
Expression
0
2
4
6
8
10
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample


JS43-241
0
2
4
6
8
10
12
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI


JV51-46
Expression
0
1
2
3
4
5
6
7
J43 NI
J43 PI
J43 SI
J51 NI
J51 PI
J51 SI
Sample

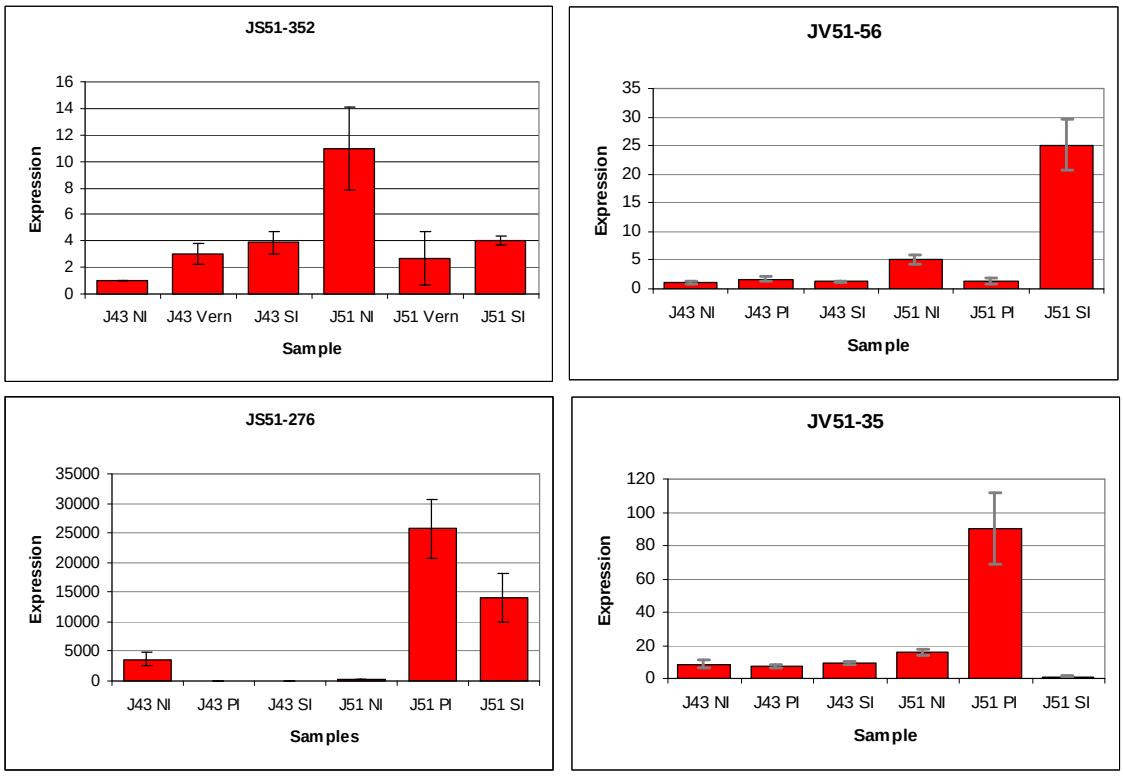


Fig 3.1: Relative quantification of genes describe in Table 3.1, determined by quantitative RT-PCR utilising SYBR green I dye. Relative expression is normalised to the four housekeeping genes as described in section 2.8. NI = Non Induced, PI = Primary Induction and SI = Secondary Induction.

5.0 Discussion

The aim of this project was to investigate the underlying genetic mechanisms involved in the transition from vegetative to reproductive growth in *Lolium perrene*. This transition is of economic importance in terms of both forage quality and seed production. Non flowering material utilized in agriculture would provide high quality feed for ruminants throughout the growing season. Conversely, a better coordination of flowering time and increased flowering propensity may lead to increased seed yield for commercial seed producers. The control of flowering time at the molecular level has been well studied in the model plant *Arabidopsis thaliana*, which has lead to the discovery of a number of well characterized floral inductive pathways. However, studies of the vernalization requirement in the temperate cereals have uncovered key differences in the underlying mechanisms (Gendall and Simpson, 2006). There may also be variance in the control mechanisms between *Arabidopsis* and *Lolium perenne* and it is important that molecular studies be carried out in this significant forage.

Two approaches were undertaken in the study presented here. The first was a gene expression approach utilizing lines with contrasting flowering times. The differential expression technique SSH was employed to identify genes showing differential expression between the two lines in the shoot apical meristem. The decision to study the shoot apical meristem was taken because it is the site of transition from vegetative to reproductive growth. Ideally differential expression is carried out between a mutant and a wild type plant. This increases the chance of identifying the genes responsible for the trait that has been altered in the mutant. However, mutant lines with altered flowering phenotypes were not available in perennial ryegrass. Therefore, the approach followed was to use full sibling lines that displayed no obvious phenotypic difference apart from flowering time. This approach enabled the identification of candidate genes for a role in the primary and secondary induction requirements of perennial ryegrass. Furthermore, the use of an extremely late flowering line facilitated the identification of genes putatively responsible for a delayed flowering phenotype. After differential screening and subsequent sequencing, 155 unique sequences were identified from the four subtracted libraries. These were clustered into functional categories using gene ontology annotations. Of interest were genes associated with the biological process of ‘response to stimulus’ and in particular those associated with a response to environmental cues. This information together with literature searches allowed the compilation of a list of genes putatively involved in the floral transition. The list of 20 genes was further analyzed using real time RT-PCR to confirm differential expression and further characterize their expression throughout floral induction. A number of these genes showed interesting expression profiles, indicative of a role to play in floral induction. However, further investigation and ultimately cloning and functional analysis would be essential to illuminate their specific role, if any, in the floral transition.

The second approach taken in this project was a quantitative genetics approach. A cross was performed between the same two full sibling lines used in the differential expression approach. Primarily, the purpose was to identify QTL associated with heading date and secondly identify QTL associated with characteristics of the flowering head. Additionally the linkage map provided a means of mapping transcripts identified in the differential expression analysis. When the project was conceived very little QTL mapping studies for heading date had been performed in

Lolium perenne. Since then a number of studies have emerged (Armstead *et al*., 2004; Jensen *et al*., 2005; Yamada *et al*., 2004). However, a drawback to QTL mapping is that it only accounts for the variation within the specific mapping population. This justifies the need for performing QTL analysis in multiple mapping populations to identify many possible factors controlling a particular trait. This is evidenced by the identification of QTL for Days to Heading (DTH) that are unique to this study. In addition to scoring for DTH, data was collected on morphological traits of the flower head. The two traits measured were Spike Length (SL) and Spikelets Per Spike (SPS), both of which putatively represent means of improving seed yield. There was a negative correlation between these traits and DTH so that later heading plants had reduced SL and lower SPS. This would have implications for breeding of varieties with extremely late flowering phenotypes if SL and SPS are good indicators of seed yield. One of the most interesting findings in this study was the overlapping QTL for DTH, SL and SPS on LG4. This is the first report of QTL for SL and SPS on LG4 and the similar positions of the QTL may indicate common underlying gene(s) affecting all three traits. The study of Jensen *et al*., (2005) found a major effect QTL on LG4 associated with the vernalization requirement in *Lolium perenne*. This QTL was associated with a marker designed to map an orthologue of the wheat *VRN1* gene. Areas of synteny have been identified between perennial ryegrass LG4 and wheat chromosome 5, containing *VRN1* (Jensen *et al*., 2005; Alm *et al*., 2003). Interestingly, the region containing VRN1 in wheat also has QTL for traits associated with grain yield including spikelets per spike (Kato *et al*., 2000). The QTL for SL on LG4 in our study was a large effect QTL, accounting for 31.7% of the phenotypic variance. This region on LG4 represents an interesting target for further research to identify the underlying genes responsible for these QTL.

This project has resulted in the establishment of a population segregating for heading date and the construction of a genetic linkage. This allowed the identification of QTL associated with heading date and QTL for traits putatively associated with seed yield. The project also generated subtracted cDNA libraries enriched for shoot apical meristem genes putatively involved in the floral transition. This work will form the basis for future studies into the molecular events controlling the transition from vegetative to reproductive growth.

6.0 References

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