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QTL analysis and trait dissection in ryegrasses (*Lolium* spp.)

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Key points

1. Molecular marker-based genetic analysis permits the dissection of complex phenotypes through resolution of the locations of pleiotropic and interacting genetic factors.
2. Several QTLs for agronomically important characters such as flowering time, winter hardiness and forage quality have been identified in perennial ryegrass by molecular marker-based map analysis.
3. Some QTLs were putatively orthologous to those for equivalent traits in cereals.
4. The identification of co-location between QTLs and functionally-associated genetic markers is critical for the future implementation of marker-assisted selection programs.

Keyword: comparative genomics, flowering time, forage quality, QTL analysis, winter hardiness

Introduction

The majority of traits of interest to pasture grass breeders, such as dry matter yield, forage quality and environmental stress tolerance, show continuous phenotypic variation and are controlled by a variable number of quantitative trait loci (QTL). Substantial advances have been made in the genetic improvement of plant populations through artificial selection of quantitative traits. Most of this selection has been on the basis of observable phenotype, without knowledge of the genetic architecture of the selected characteristics. In major crop species, the development of high-resolution genetic maps has made it possible to identify the chromosomal regions, or in some instances, the individual sequence variants that are responsible for trait variation. There have been relatively few reports to date of QTL analysis for agronomic traits in forage grasses, due to the absence of a sufficiently well developed genetic map. An enhanced molecular marker-based genetic linkage map of perennial ryegrass (*Lolium perenne* L.) has recently been constructed through the activities of the International *Lolium* Genome Initiative (ILGI), using the p150/112 one-way pseudo-testcross reference population (Jones et al., 2002a,b). Two genetic mapping populations of perennial ryegrass have been independently developed as successors to the p150/112 population, and have been aligned to the reference map using common markers (Armstead et al., 2002, 2004; Faville et al., 2005). These genetic maps contain functionally-associated molecular marker information through the inclusion of gene-associated cleaved amplified polymorphic sequences (CAPS) markers, and both restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers from expressed sequence tags (ESTs), respectively. In addition, high- density molecular marker-based genetic maps have also recently been constructed for other species of *Lolium* and *Festuca* (Alm et al., 2003; Inoue et al., 2004a; Warnke et al., 2004; Saha et al., 2005). Genetic markers and maps are consequently available for detailed dissection of complex phenotypes to resolve the locations of pleiotropic and interacting genetic factors. In this chapter, recent results from QTL analysis of the p150/112 reference population and another populations of perennial ryegrass are reviewed, and are related to comparative genomics studies with other

Poaceae species.

Flowering time

The timing of flowering during the year is an important adaptive character that impacts on yield and quality in crop and pasture species. Analysis of the molecular basis of several QTLs in *Arabidopsis*, rice, maize and tomato has revealed that in diverse species, orthologous genetic networks can control related complex phenotypes (Paran & Zamir, 2003). For example, QTL cloning studies has revealed that the major flowering-time QTL *ED1* (early day-length insensitive) of the long-day flowering plant *Arabidopsis thaliana* is a novel allele of the blue-light photoreceptor cryptochrome-2 (*CRY2*) (El-Din El-Assal et al., 2001). This allele increased protein stability as a result of a single amino acid substitution that led to early flowering in short days. The genetic control of flowering time control in *Arabidopsis* has been well characterised through the interaction of photoperiod, vernalisation, autonomous and gibberellic acid-dependent pathways (Mouradov et al., 2002; Hayama & Coupland, 2003; Henderson & Dean, 2004; Sung & Amasino, 2004). In rice, a short-day flowering plant, QTL analysis for heading date has been performed using several types of progeny derived from a single cross combination between *Oryza sativa* ssp. *japonica* and *O. sativa* ssp. *indica* cultivars and identified at least 14 QTLs controlling flowering time (Yano et al., 2001). Three heading QTLs (*Hd1, Hd3* and *Hd6*) were mapped at high resolution using near isogenic lines (NILs) and were isolated by a map-based cloning approach (Yano et al., 2000; Kojima et al., 2002; Takahashi et al., 2001). *Hd1* was shown encode a homologue of the *Arabidopsis* photoperiod pathway *CONSTANS (CO)* gene (Yano et al., 2000). *Hd3a* was shown to be related to the *Arabidopsis FLOWERING LOCUS T (FT)* gene, while *Hd6* was shown to encode the $\alpha-$subunit of casein protein kinase 2 ($CK2\alpha$). Gene interactions in the photoperiod induction pathways of *Arabidopsis* and rice were compared (Izawa et al., 2003).

In cereals, genes that regulate the timing of flowering can be divided three categories based on their interaction with environmental signals: vernalisation response genes (*Vrn*) that regulate flowering using low temperature; photoperiod response genes (*Ppd*) that regulate flowering using day length; and ‘earliness’ factors that appear to be largely independent of these cues (Laurie, 1997). Conserved genomic locations for genes involved in processes such as vernalisation and photoperiodic induction have been identified between species by comparative genetic studies (Dubcovsky et al., 1998; Laurie et al., 2004). A detailed physical and genetic map of the $Vrn\text{-}A^{m}1$ (*Vrn1*) region was constructed for the diploid wheat, *Triticum monococcum* L. and found to be colinear with the corresponding region of rice chromosome 3 (Yan et al., 2003). A candidate gene for *Vrn1* was isolated by positional means, and was identified as a relative of the *Arabidopsis apetala-1* gene class (*AP1*). Allelic variation between spring and winter-type growth habit wheats at the *AP1* gene was observed only in the promoter region, suggesting that variation of gene expression was the causal factor for differences between these two varietal groups. A second vernalisation gene, designated $Vrn\text{-}A^{m}2$, was assigned to the distal region of chromosome $5A^{m}L$ within a segment translocated from homoeologous group 4 (Dubcovsky et al., 1998). Yan et al. (2004) reported the positional cloning of the *Vrn2* gene, which encodes a dominant repressor of flowering down-regulated by vernalisation. Loss of function at *Vrn2*, whether by natural point mutation or deletion, resulted in lines with spring-type growth habit, which do not require vernalisation to flower.

A single QTL for heading date was observed on linkage group (LG) 4 in the p115/120 reference family of perennial ryegrass (Yamada et al., 2004). However, a number of QTL positions for heading date have been reported from the analysis of single mapping populations in other Poaceae species (Hayes et al., 1993; Laurie et al., 1995; Bezant et al., 1996; Börner et al., 2002). The number of QTLs and their relative importance may vary according to the origin of the genotypes used to construct mapping families. Studies on geographical populations of *Lolium* species covering the climatic range from the Mediterranean region to northern and central Europe revealed a regular cline in flowering responses to temperature and photoperiod (Cooper, 1960). The heterozygous parent of the p150/112 mapping population was derived from a cross between eastern European (Romanian), southern European (north Italian ecotypes) and northern European ('Melle' or 'S23') genotypes, and might be expected to represent a variety of response genes. In rice, the major heading date QTL *Hd6*, which is associated with inhibition of flowering under long day conditions, is located on chromosome 3 (Yamamoto et al., 2000) and encodes an α-subunit of CK2 (Takahashi et al. 2001). Comparative genetic mapping studies between rice and wheat based on the colinearity of four common RFLP markers (Kato et al., 1999) have revealed that the rice *Hd6* locus region on chromosome 3 is syntenic with the *VrnA1* region on chromosome 5AL. RFLP-based mapping of the wheat CK2α gene (*tck2a*) probe detected a genetic locus closely linked (by 1.1 cM) to *VrnA1* (Kato et al., 2002). Two putative CK2α genes (*Lpck2a-1* and *Lpck2a-2*) genes have been isolated from a cDNA library constructed with mRNA isolated from cold-acclimated crown tissues of *Lolium perenne* using sequence information derived from the *tck2a* gene. The *Lpck2a-1* CAPS marker was assigned to LG 4 of the p115/120 reference family near to the location of the QTL for heading date, while the *Lpck2a-2* CAPS marker was assigned to LG 2 (Shinozuka et al., submitted). The location of the *Lpck2a-1* locus supports the inference of conserved synteny between perennial ryegrass LG 4, the Triticeae homoeologous group 5L chromosomes and the corresponding segment of rice chromosome 3 (Yamada et al., 2004).

A mapping population consisting of 184 F_2 genotypes from a cross between a genotype from the perennial ryegrass synthetic variety 'Veyo' and a genotype from the perennial ryegrass ecotype 'Falster' was measured for vernalisation response as days to heading under artificially controlled condition (Jensen et al., 2005). In total, five QTLs were identified on LGs 2, 4, 6 and 7. A CAPS marker derived from the putative orthologue of the *Triticum monococcum VRN1* gene co-located with a major QTL on LG 4 for vernalisation response (Jensen et al., 2005). This data further confirms the presence of flowering time gene orthologues and corresponding QTLs on LG 4.

Heading date QTLs were also identified in one of the second generation reference populations, derived from self-pollination of an F_1 hybrid obtained by crossing individuals from partially inbred lines developed from the two agronomically contrasting cultivars 'Aurora' and 'Perma' (Turner et al. 2001). Genetic mapping of the F_2 (Aurora x Perma) population identified seven linkage groups with a total map length of 628 cM (Armstead et al., 2004), extending the studies of Armstead et al. (2002) and consistent with the ILGI reference map (Jones et al., 2002). A major QTL accounting for up to 70% of the variance was identified on LG 7, along with additional small QTLs on LGs 2 and 4 (Armstead et al., 2004). The genomic region associated with the major QTL on LG 7 shows a high degree of conserved synteny with the *Hd3* region of rice chromosome 6.

Two annual ryegrass plants from the cultivar 'Floregon' were crossed with two perennial ryegrass plants from the cultivar 'Manhattan'. From the resultant F_1 populations, two random plants were chosen and crossed to develop a pseudo-F_2 mapping family (ψF_2[MFA-4 x MFB-2]). A total of 235 amplified fragment length polymorphism (AFLP) markers, 81 random amplified polymorphic DNA (RAPD) markers, 16 grass comparative anchor probe RFLPs, 106 SSR markers, 2 isoenzyme loci and 2 morphological characteristics, 8-h flowering and seedling root fluorescence were used to construct a male map 537cM in length and a female map 712 cM in length, each with 7 LGs (Warnke et al. 2004). Two major QTLs influencing photoperiodic control of flowering were identified in locations syntenic with flowering control regions of the wheat and barley genomes (Warnke et al., 2003a,b).

Winter hardiness

Winter hardiness is the outcome of a number of interacting factors that may include vernalisation requirement, photoperiod response, low-temperature tolerance and resistance to snow moulds. An understanding of the genetic basis of these component traits permits more efficient selection based on closely linked molecular marker loci. In the Triticeae cereals, QTL analysis has identified a limited number of conserved genome regions as responsible for the winter hardiness character. The most consistently identified region, on homoeologous group 5 chromosomes, contains QTLs for vernalisation response, low temperature tolerance and photoperiod sensitivity (Pan et al., 1994; Cattivelli et al., 2002). These QTL effects have been described as the effects of single loci. Low-temperature tolerance loci on chromosomes 5A, 5B, and 5D of wheat have been given the locus designation *Fr-A1*, *Fr-B1* and *Fr-D1*, respectively (Sutka & Snape, 1989; Snape et al., 1997; Toth et al., 2003). The vernalisation loci have been assigned a *Vrn* prefix, and orthologues of the *T. monococcum Vrn1* locus map to the homoeologous 5 chromosomes (Cattivelli et al., 2002). The *Vrn* gene nomenclature was standardised and map locations were further refined by Dubcovsky et al. (1998). RFLP analysis demonstrated that vernalisation requirement and frost resistance are controlled by two different, but tightly linked loci (*Vrn-A1* and *Fr-A1*) on chromosome 5A of wheat (Galiba et al., 1995; Sutka et al., 1999). Because of the observed large effect on frost resistance, molecular marker-assisted selection for the *Vrn-A1-Fr-A1* chromosomal region has been proposed as a method for improvement of cold hardiness in wheat cultivars (Storlie et al., 1998). Wheat NILs with different vernalisation alleles have been evaluated for cold hardiness in order to assess the viability of this strategy (Koemel et al., 2004). Close genetic linkage between the major genes influencing winter hardiness and genes regulating cold-induced sugar production was also observed in wheat (Galiba et al., 1997). In addition, QTLs controlling traits associated with winter hardiness, such as field survival and crown fructan content, were mapped to the long arm of chromosome 5H in a cross between ‘winter’ x ‘spring’ barley varieties (Hayes et al., 1993; Pan et al., 1994). Recently, comprehensive measurements of low temperature tolerance and vernalisation requirement were used for analysis of a new ‘winter’ x ‘spring’ barley population, and a QTL for accumulation of proteins encoded by COR (COld Regulated) genes on chromosome 5H (*Cor14b, tmc-ap3*) was coincident with a QTL for low temperature tolerance (Francia et al., 2004). C-repeat binding factor (CBF) family genes were also mapped in this region (Francia et al., 2004). In *A. thaliana* the transcription factors encoded by CBF family genes have been shown to be key determinants of low temperature tolerance (Thomashow, 1999; Thomashow et al., 2001).

No significant QTLs for winter survival in the field were identified in the reference map of p150/112 (Yamada et al., 2004). However, a QTL for electrical conductivity corresponding to frost tolerance (Dexter et al., 1930, 1932) was located close to a heading date QTL in a region is likely to show conserved synteny with chromosomal regions associated with both winter hardiness and flowering time variation in cereals, as described above.

The F_2 (Aurora x Perma) genetic map population (RASP) was also used to identify QTLs for traits relating winter hardiness, as well as sugar content. Snow mould-resistant varieties accumulate higher levels of fructan and metabolise them at slower rates compared to susceptible varieties (Yoshida et al., 1998). Many of the snow mould fungi, such as *Typhula* spp., *Microdochium nivale* and *Sclerotinia borealis*, can co-infect on single plants, and their interactions may obscure the respective effects on plant survival (Matsumoto & Araki, 1982; Matsumoto et al., 1982). The use of fungicides with a limited spectrum of activity may clarify these specific effects. *Typhula* snow moulds such as *T. ishikariensis* and *T. incarnata* generally occur in the deep snow environment of the western region of Hokkaido, Japan, including

Sapporo. In this environment, control of *S. borealis* and *M. nivale* infections with the fungicide iminoctadine-triacetate is an effective method for evaluation of resistance to *Typhula* snow moulds (Takai et al., 2004). Scores of winter survival were measured in the F_2 (Aurora x Perma) population using this control regime, and QTLs for this trait were identified on LGs 2, 4, 6 and 7. Fructan content was also measured by high performance liquid chromatography (HPLC) using crown tissues from plants grown outdoor in December. QTLs for content of high molecular fructan with more than eight degrees of polymerization (DP) were observed on LGs 1, 2 and 4. QTLs for winter survival in LGs 2 and 4 are close to coincident with the QTLs for high molecular weight fructan content.

Two major QTLs for freezing tolerance (*Frf*) and 4 QTL for winter survival have been identified in the closely related pasture grass species meadow fescue (*Festuca pratensis* Huds.) (Rognli et al., 2002). Comparative mapping with heterologous wheat anchor probes indicated that *Frf4_1* on LG4 of *F. pratensis* was orthologous to the frost-tolerance loci *Fr1* and *Fr2* in wheat. The QTLs for winter survival, by contrast, were located on LGs 1, 2, 5, and 6 (Rognli et al. 2002).

Fructosyltransferase genes involved in fructan biosynthesis such as 1-SST, 1-FFT and 6G-FFT were isolated from perennial ryegrass and characterized by heterologous expression in the *Pichia pastoris* system (Hisano et al., in preparation). *Lp1-SST* and *Lp1-FFT* mapped to the upper region of LG 7 in the F_2 (Aurora x Perma) genetic map, but failed to show coincidence with any fructan content QTLs. The *Lp1-SST* gene (Chalmers et al., 2003) was also assigned to the equivalent region of LG 7 as a single nucleotide polymorphism (SNP) locus in the F_1 (NA_6 x AU_6) second generation reference family (Faville et al., 2005). However, *Lp6G-FFT* mapped to LG 3 close to a QTL for low-molecular weight fructan content (Hisano et al., in preparation). As previously described, evidence from comparative genome studies suggests that the upper part of LG4 in perennial ryegrass may contain a region of conserved synteny with the long arms of the Triticeae homoeologous group 5 chromosome. It is possible that allelic variation in regulatory genes such as those for the CBF transcription factor family may contribute to the QTLs for winter survival and fructan content observed on LG 4.

Herbage quality

Quality is the most important of all agronomic traits for pastures due to the nutritive requirements of grass-fed livestock. The genetic control of nutritive value parameters in pasture species has been reviewed (e.g. Casler, 2001), and genetic variation for specific traits has been established. Digestibility is generally considered to be the most important temperate grass nutritive value trait for either live-weight gain (Wheeler & Corbett, 1989) or dairy production (Smith et al., 1997). Deliberate attempts to improve dry matter digestibility (DMD) in forage crop species have led to rates of genetic gain in the range of 1 - 4.7 % per annum as a proportion of the initial population means (Casler, 2001). Progress in simultaneous improvement of yield and DMD in forage grasses has, however, been variable (Wilkins & Humphreys, 2003).

Forage quality may be directly evaluated by feeding trials using animals, but this approach is costly, laborious and limited for small quantities of herbage from breeding experiments. Indirect methods of assessment include *in vitro* digestibility with rumen liquor (Menke et al., 1979; Tilly and Terry, 1963), enzymatic digestion (De Boever et al., 1986) and chemical analysis of cellular components (van Soest, 1963). The development of near infra-red reflectance spectroscopy (NIRS) analysis for prediction of forage quality has facilitated rapid and non-destructive evaluation of samples from plant breeding programs. NIRS has been used to develop calibrations to predict a wide range of forage quality traits (Marten et al., 1984; Smith and Flinn, 1991) including crude protein (CP) content, estimated *in vivo* dry matter digestibility (IVVDMD), neutral detergent fibre (NDF) content (Smith & Flinn, 1991) and water-soluble carbohydrate (WSC) content (Smith & Kearney 2000) in perennial ryegrass. NIRS estimates of DMD and related nutritive value traits have been reported in a range of forage systems (e.g. Carpenter & Casler, 1990; Hopkins et al., 1995; Smith et al., 2004).

Lübbestedt et al. (1997, 1998) published the first QTL analysis devoted to forage quality in maize. QTLs for cell-wall digestibility and lignification traits in maize were also investigated in two recombinant inbred lines (RIL) progeny by Méchin et al. (2001). Cardinal et al. (2003) detected 65 QTLs related to fiber and lignin content in maize. The best options for breeding of grasses for improved digestibility was assessed based on a search for genome locations involved in forage quality traits through QTL analysis (Ralph et al., 2004).

Ground herbage samples from genotypes of the p150/112 population were measured for quality traits such as CP, IVVDMD, NDF, estimated metabolisable energy (EstME) and WSC by NIRS analysis (Cogan et al., 2005). A total of 42 QTLs was observed in six different sampling experiments varying by developmental stage (anthesis or vegetative growth), location or year. Coincident QTLs were detected on LGs 3, 5 and 7. The region on LG 3 was associated with variation for all measured traits across various experimental datasets. The region on LG 7 was associated with variation for all traits except CP, and is located in the vicinity of the lignin biosynthesis gene loci *xlpomt1* (caffeic acid-*O*-methyltransferase), *xlpccr1* (cinnamoyl CoA-reductase) and *xlpssrcad2.1* (cinnamyl alcohol dehydrogenase).

WSC provide the most available source of energy for grazing ruminants. In the F_2 (Aurora x Perma) population (RASP), high molecular fructan constituted the major part of the WSC pool was analyzed samples of spring and autumn in tiller bases and leaves with replication of data over years (*ie* collect one replicate each year for several years) (Turner et al., unpublished). Correlation between traits did not always lead to corresponding cluster of QTL and some traits have no reproducible QTL. Tiller base QTL were identified on linkage groups 1 and 5 and leaf QTL on linkage groups 2 and 6, in regions that had previously been identified as important in analyses of single replicates (Humphreys et al, 2003).

Improvements of herbage quality may also be obtained by alteration of the content and ratios of minerals in grasses, to prevent metabolic disorders. Grass tetany (hypomagnesaemia) is caused by low levels of magnesium in the blood of cattle or sheep. Varieties of Italian ryegrass and tall fescue with markedly levels of magnesium have proved to be very effective in maintaining levels of blood magnesium in grazing sheep (Moseley & Baker, 1991) and cattle (Crawford et al., 1998). Milk fever, caused by low blood calcium, produces animal welfare and production problems that could be addressed by reducing potassium content of forage without reducing calcium and magnesium concentrations (Sanchez et al., 1994). Variation in mineral content in grasses may be strongly influenced by genetic factors and is amenable to QTL analysis. Herbage samples of the p150/112 population from four sampling experiment were analyzed for mineral content (aluminum, calcium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, sodium, sulfur and zinc) by inductively-coupled plasma mass spectroscopy (ICP-MS) and a total of 45 QTLs were identified (Cogan et al., in preparation). QTL clusters were observed on LGs 1, 2, 4 and 5. QTLs for the important trait for control of grass tetany, magnesium content were detected on LGs 2 and 5. Field herbage samples from the F_1 (NA_6 x AU_6) population were also analysed for mineral content by ICP-MS. A total of 14 QTLs were identified on the NA_6 map, and 9QTLs were identified on the AU_6 genetic map. A number of clustered QTL locations showed coincidence between the two different populations.

Morphological traits and other agronomical traits

QTLs were detected for morphological traits such as plant height, tiller size, leaf length, leaf width, fresh weight at harvest, plant type, spikelet number per spike and spike length using the p150/112 genetic map (Yamada et al., 2004). A number of traits were significantly correlated, and coincident QTL locations were identified. For example, coincident QTLs for plant height, tiller size and leaf length were identified on LG 3. The rice *SD1* semi-dwarfing gene, that launched the 'green revolution', encodes a gibberellin biosynthetic enzyme (*GA20ox*), and was assigned to the long arm of rice chromosome 1 (Sasaki et al., 2002). A CAPS marker developed for the perennial ryegrass ortholocus of the *GA20ox* gene was mapped to LG 3 close to the plant height QTL, in a region of conserved synteny with rice chromosome 1 (Kobayashi et al., unpublished). This finding provides further evidence for the utility of the candidate gene-based marker approach.

In Italian ryegrass (*Lolium multiflorum* Lam.), a total of 17 QTLs for six traits related to lodging resistance and heading date were detected by single interval mapping (SIM), while 33 independent QTLs from the male and female parents were detected by composite interval mapping (CIM) (Inoue et al. 2004b). QTLs for plant height were located on LG 1, and for heading date on LGs 4 and 7, potentially in conserved regions with those identified in perennial ryegrass.

Conclusions

This review has demonstrated that although the existing QTL information from forage grasses is relatively underdeveloped compared to other major crops, the recent establishment of detailed molecular genetic maps is rapidly stimulating QTL analysis and trait dissection. The current genetic maps are largely populated by anonymous genetic markers, with limited diagnostic value. The next generation of molecular genetic markers for forage grasses will be derived from expressed sequences, with an emphasis on functionally-defined genes associated with biochemical and physiological processes that are likely to be correlated with target phenotypic traits (Forster et al., 2004; Faville et al., 2005). Comparative genomics with other Poaceae species such as rice, wheat, barley and maize will support the development of such high value molecular markers through orthologous QTL detection and co-location of candidate genes. Accuracy in phenotypic assessment will be essential for precise QTL detection, and may in future be amenable to automated high-throughput analysis, as for genotyping.

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