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Genetics of Reproductive Development in Forage Legumes

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ABSTRACT

Temperate forage legumes include members of the *Trifolium*, *Medicago* and *Lotus* genera. Aspects of reproductive development such as flowering time and seed yield are important breeding objectives for commercial seed producers. The two most advanced model species for legume genetics and genomics *Medicago truncatula* (barrel medic), and *Lotus japonicus* are close relatives of cultivated species, and provide important sources of information for breeding improvement through translational genetics and genomics. Genetic control of floral initiation is poorly understood in forage species, but genes regulating flowering time and floral development have recently been identified in a number of model species, providing the basis for functional evaluation in forages. Some of these genes appear to be genuinely orthologous, exerting effects on the predicted processes when functionally tested in heterologous systems. Reproductive development genes are hence likely to be conserved between legumes and other higher plant species. Genetic variation for reproductive developmental traits has been assessed in *Medicago*, *Lotus* and *Trifolium* species, and molecular genetic marker-based linkage groups have been used to identify quantitative trait loci. The information presented in this review suggests that comparative genomics between forage and model legumes is now sufficiently developed to allow prediction of the candidate genes contributing to variation for important agronomic traits.

Keywords: flowering, genetics, inflorescence, *Medicago,* meristem, *Trifolium*

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INTRODUCTION

Forage legume species

Forage species provide herbage for grazing, hay and silage production servicing livestock production industries in both tropical and temperate regions of the world. The grazing industries are responsible for generation of dairy, red meat and fibre (wool and leather) products. The most important forage taxa are grasses (belonging to the Poaceae family) and legumes (belonging to the Fabaceae family). Individual species are either cultivated separately or in combination as mixed swards. Co-cultivation permits exploitation of the complementary properties of grass and legume species, such as differential digestibility, protein content (especially of rumen bypass proteins), water soluble carbohydrate content and most significantly, the capacity of legume species to reduce the requirement for exogenous fertilisation due to biological nitrogen fixation.

The major forage legumes of temperate pasture agriculture include white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.), subterranean clover (*T. subterraneum* L.), bird's foot trefoil (*Lotus corniculatus* L.) and lucerne/alfalfa (*Medicago sativa* L.), while minor species such as sainfoin (*Onobrychis viciifolia*), Caucasian clover (*T. ambiguum*), greater lotus (*L. uliginosus*) and serradellas (*Ornithopus* spp.) continue to be evaluated and selected for niche agronomic use. Tropical forage legumes include round-leafed cassia (*Chamaecrista rotundifolia*), siratro (*Macroptilium atropurpuerum*) and members of the genera *Stylosanthes*, *Centrosema* and *Desmodium*. In addition, the two legume species which have been adopted as international models for genetic and genomic studies are closely related to the major temperate forage species: *Medicago truncatula* Gaertn. and *Lotus japonicus* L. In addition to use in a variety of molecular and genetic studies, *M. truncatula* (barrel medic) is also a minor forage legume crop in its own right. *M. truncatula* is adapted to the warm temperate conditions of hot dry summers and mild moist winters of the Mediterranean region (Lesins and Lesins 1979), and has been integrated into rotations with cereal crops in Mediterranean-type climatic zones of mainland Australia. In this

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system, *M. truncatula* self-regenerates from persistent seed after the cropping phase (Puckridge and French 1983).

Taxonomy and genetics of temperate forage legumes

The clover species are members of the *Trifolium* genus of the Trifolieae tribe of the cool-season Galegoid clade in the Papilinoideae sub-family of the legume family Fabaceae (Doyle and Luckow 2003). The most closely related genus is *Melilotus* (sweet clovers) and the genus *Medicago*, including alfalfa and other medic species, is also part of the Trifolieae. As a consequence, *M. truncatula* shares a very recent common ancestor with alfalfa and a relatively recent ancestor with the clovers and sweet clovers. Translational genomics based on whole genome sequencing of *M. truncatula* (Young *et al*. 2005; Zhu *et al*. 2005) is therefore anticipated to be highly efficient for members of the Trifolieae. Members of the *Lotus* genus, including *L. japonicus*, are also Galegoid legumes, but are placed in a separate tribe, the Loteae.

White clover is an allotetraploid species with a fundamental chromosome number of 8 (2n = 4x = 32). The evolutionary origin of white clover has been a matter of dispute over many years, although two diploid species, *T. occidentale* D.E. Coombe and *T. nigrescens* Viv., and another allotetraploid species, *T. uniflorum* L., were previously considered to be potential progenitors (Chen and Gibson 1970; Chen and Gibson 1971; Badr *et al*. 2002). More recent molecular phylogenetics studies based on chloroplast DNA and nuclear ribosomal DNA variation have implicated *T. occidentale* and the diploid taxon *T. pallescens* Schreber as the paternal and maternal progenitors, respectively (Ellison *et al*. 2006). White clover is a perennial obligate outbreeding species, with a gametophytic self-incompatibility (SI) system controlled by a series of alleles at a single locus (*S*) (Attwood 1940, 1941, 1942b). Rare instances of self-compatibility have been reported (Attwood 1942a; Yamada *et al*. 1989), presumably due to the presence of self-fertile (S_f) alleles at the SI locus.

Red clover and subterranean clover are diploid species with chromosome constitutions 2n = 2x = 14 and 2n = 2x = 16. Red clover is a biennial obligate outbreeder, while subterranean clover is an annual inbreeder. Alfalfa is a perennial autotetraploid species (2n = 4x = 32) with a partial self-incompatibility system (Barnes *et al*. 1972) while *M. truncatula* and other annual medics are diploid species (2n = 2x = 16). Bird's foot trefoil, like alfalfa, is an outbreeding autotetraploid species (2n = 4x = 24), while *L. japonicus* is an inbreeding diploid (2n = 2x = 12).

Agronomic significance of key traits

The most important reproductive development trait for forage legumes is seed production. Although forage species are predominantly bred for production and quality, the ability to produce economically viable amounts of seed is crucial for the commercial success of varieties. The implementation of seed production technologies has steadily improved yields, but the performance of different species remains highly variable. As an example, during the decade beginning in 1990 in New Zealand (the production zone for over half of the global annual output of certified white clover seed) yields ranged from 100 to 1000 kg/ha, with an average of 300 kg/ha (Mather *et al*. 1996).

For white clover, the number of mature inflorescences is the major contributor to seed yield (SY) (Jahufer and Gawler 2000), and substantial genotypic variation for this and other yield-associated reproductive traits has been observed at the single genotype level (Cain *et al*. 1995) and also between different cultivars (Connolly 1990; Williams *et al*. 1998). Yield per inflorescence is an additional contributory component, and is a product of variation in floret number per inflorescence, floret fertility, and seed size and density (Barrett *et al*. 2005). Profuse flowering and seed production are in general inversely correlated with plant persistence (Gibson 1957; Piano and Annicchiarico 1995). Inflorescences are produced at some, but not all, of the nodes of developing stolons, and the balance between reproductive and vegetative nodes is important for plant persistence. Stolon density, as a measure of such persistence, is negatively associated with SY (Annicchiarico *et al*. 1999), so a strong incentive exists for the independent selection of reproductive performance traits without otherwise impairment of agronomic performance. For example, selection for the strength of the flower stalk (peduncle) significantly improves both inflorescence survival and SY (Marshall 1995).

In red clover, improvements of forage yield and quality have produced cultivars with generally unsatisfactory SY, leading to high production costs and limited commercial success (Taylor and Quesenberry 1996). Improved management practices are capable of limited improvement of SY and complex contributions of various sub-traits were observed (Oliva *et al*. 1994; Montardo *et al*. 2003). As for white clover, inflorescence number was a major determinant of SY, and negative correlations were observed with other agronomic traits such as forage yield and persistence (Steiner *et al*. 1997). These effects were particularly apparent for Mattenklee cultivars developed from locally adapted Swiss ecotypes (Deneufbourg 2004; Herrmann *et al*. 2005).

SY in alfalfa is generally regarded as being of secondary importance compared to forage quality, and is characterised by variable yield, often associated with poor quality (Bolanos-Aguilar *et al*. 2001; Iannucci *et al*. 2002). Environmental conditions such as irrigation in combination with mowing regimes during early growth or early in reproductive development contribute to high SY (Iannucci *et al*. 2002). SY was also shown to be highly correlated with above-ground biomass at harvest, as low yields are generally observed during the year of sowing (Bolanos-Aguilar *et al*. 2002). Significant cultivar x environment effects are often observed, indicating important genotypic effects. Genetic variation for the number of inflorescences, seed number per plant, seed number per inflorescence and seed weight per inflorescence is highly correlated with SY per plant (Bolanos-Aguilar *et al*. 2000; Sengul 2006), and seed weight per inflorescence has been recommended as a selection criterion in breeding practice.

For *L. corniculatus*, most of the available data relates to environmental effects on SY. Unlike other forage legumes, little or no supplemental irrigation is required to achieve maximal SY (Garcia-Diaz and Steiner 1999), even in the humid temperate maritime conditions characteristic of seed producing areas of the Willamette Valley of western Oregon, USA (Garcia-Diaz and Steiner 1999).

MOLECULAR GENETICS OF FORAGE LEGUME REPRODUCTIVE DEVELOPMENT

The identification of genes and proteins that are essential for reproductive development in legumes has been greatly facilitated by identification of similar genes in other plants, primarily *Arabidopsis thaliana*. A comprehensive treatment of the molecular genetics of reproductive development is beyond the scope of this article, as the topic has been extensively reviewed in recent years (Komeda 2004; Putterill *et al*. 2004; Gendall and Simpson 2006). Models derived from arabidopsis have stimulated gene discovery in legume species such as *M. truncatula*, *L. japonicus* and pea (*Pisum sativum* L.). Although the development, morphology and reproductive strategies of legumes differ from those of a cruciferous weed such as arabidopsis, many of the fundamental process appear to be well conserved (**Table 1; Fig. 1**). In particular, recent work has described conservation of aspects of the photoperiod sensing pathway.

Conservation has been revealed by careful analysis of mutant phenotypes, or close examination of gene expression profiles. Several genes have been identified in the photoperiod pathway which promotes flowering in response to

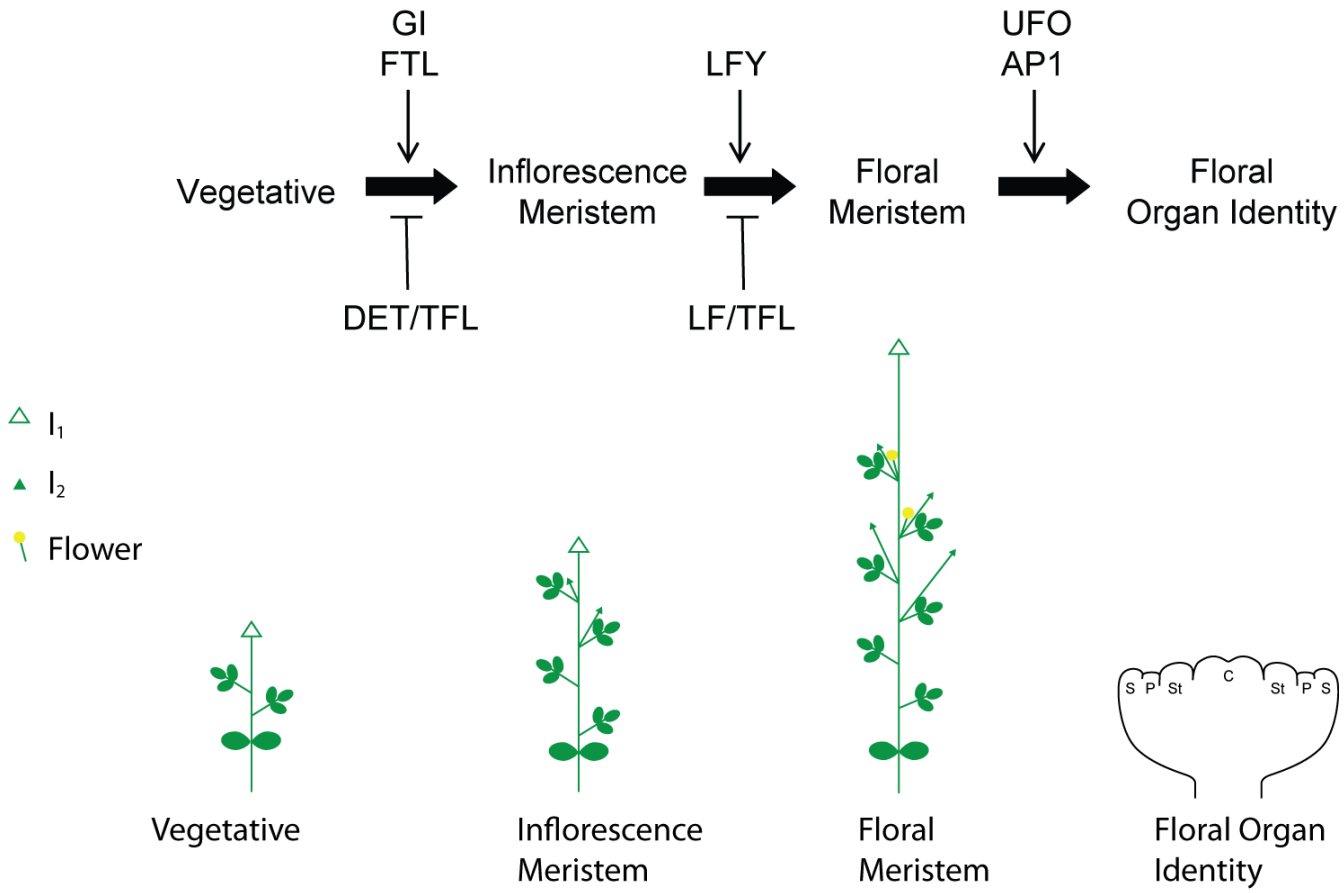

Fig. 1 Model for the action of flowering time regulators in model legumes. The model indicates positive action (promotion) by lines ending in arrows, and inhibition by perpendicular bars. In pea, DET and LF have distinct functions which may be served by a single gene product in other species. I_1 – Primary inflorescence, I_2 – secondary inflorescence.

Table 1 Flowering time gene orthologs and homologs in model legume species.

Pathway	Function	*L. japonicus*	*M. truncatula*	*P. sativum*	*A. thaliana*	References
Photoperiod	Unknown, nuclear localised	*LjGI*[1]	*MtGI*[2]	*LATE1*	*GI*	Paltiel *et al*. 2006; Hecht *et al*. 2007
	Mobile floral inducer (exact function unknown)		*MtFTLa/b/c*[3]	*FTL*[2]	*FT*	Hecht *et al*. 2007
Floral Repressor	Exact function unknown	*LjCEN1*	*MtTFL*[1]	*DET* and *LF*	*TFL*	Foucher *et al*. 2003
Floral Integrators	Transcription factor	*LjLFY/ LjPFM*	*MtLFY*[1]	*Uni*	*LFY*	Hofer *et al*. 1997; Dong *et al*. 2005
	MADS transcription factor	*LjAP1a*[2] *LjAP1b*[2]	*MtPIM*	*PIM/PEAM*	*AP1*	Taylor *et al*. 2002; Dong *et al*. 2005; Benlloch *et al*. 2006
Floral Organ Identity	F-box – protein interaction/degradation	*LjUFO/Pfo*	*MtUFO*[1]	*Stp*	*UFO*	Taylor *et al*. 2001; Zhang *et al*. 2003
	MADS transcription factor		*MtAPETALA*		Unclear (*AP3* or *PI*?)	Penmetsa and Cook 2000

[1] No genetic (mutant) or other functional data – Genome and/or EST sequence data only (Hecht *et al*. 2005).
[2] RT-PCR or *in situ* hybridization data only.
[3] Expression data suggest these are likely to be functional (R. Laurie and R. Macknight – pers. comm.).
Most of the relationships are clearly orthologous as defined by mutant phenotypes. Other relationships are not fully resolved and are based on *in situ* hybridization data, expressed sequence tags (ESTs), or genomic sequences only. Many other flowering time gene homologs have been identified, particularly in the genome sequence of *M. truncatula* (Hecht *et al*. 2005).

light:dark cycles. Two recent papers have described the analysis of legume orthologues of the arabidopsis *GIGANTEA* (*GI*) gene. The exact function of *GI* is unclear, as it encodes a protein with no currently-characterised functional domains, but *GI* function is crucial for the normal regulation of the circadian clock (Fowler *et al*. 1999; Huq *et al*. 2000). Mutations in the pea *LATE BLOOMER 1* (*LATE1*) gene give rise to late-flowering photoperiod-insensitive plants (Hecht *et al*. 2007). The expression of the *M. truncatula GI* (*MtGI*) gene has also been investigated (Paltiel *et al*. 2006). In pea and *M. truncatula*, the expression profile is similar to that observed in arabidopsis, as both *LATE1* and *MtGI* are regulated by circadian rhythms (Paltiel *et al*. 2006; Hecht *et al*. 2007). In addition, the expression of two downstream genes, *CONSTANS-LIKE a* (*COLa*) and *FLOWERING LOCUS T-LIKE* (*FTL*), is affected in the *late1* mutants, in a manner similar to that observed in *gi* mutants of arabidopsis (Hecht *et al*. 2007). Grafting analysis with *late1* mutants indicates that the *LATE1* protein controls the production of a mobile floral stimulus, perhaps a FTL protein (Corbesier *et al*. 2007; Hecht *et al*. 2007). Expression of some of the *M. truncatula FTL* genes also appears to be correlated with flowering time (R. Laurie and R. Macknight, pers. comm.), suggesting that these genes may regulate the transition to reproductive development in legumes.

The function of several *CENTRORADIALIS/TERMINAL FLOWER*-like (*CEN/TFL*) genes also appears to be conserved between dicotyledenous plant species. In Arabidopsis, *TFL* maintains the indeterminate state of the shoot apical meristem, regulates the transition from vegetative to

reproductive development, and is expressed at high levels in inflorescence meristems (Bradley *et al*. 1997). Three *TFL*-like genes have been characterised in detail from *L. japonicus* and pea (Foucher *et al*. 2003; Guo *et al*. 2006). The *L. japonicus* gene *CENTRORADIALIS1* (*LjCEN1*) is expressed in specific sub-domains of inflorescence meristems, suggesting that the *LjCEN1* function may regulate determinacy (Guo *et al*. 2006). Further support for this hypothesis comes from the functional analysis of *LjCEN*. Arabidopsis plants overexpressing *LjCEN* were late flowering, and in one case produced shoots in place of branches, similar to the phenotype of *TFL*-overexpressing plants (Bradley *et al*. 1997; Guo *et al*. 2006). Two *CEN/TFL* homologs have been cloned from pea (Foucher *et al*. 2003). *DETERMINATE* (*DET*) and *LATE FLOWERING* (*LF*) are both important for regulation of shoot apical meristem function in pea, as *lf* mutants have an early flowering phenotype, and *det* mutants produce relatively few axillary flowers and prematurely terminate in a terminal flower (Foucher *et al*. 2003).

The transcription factor *LEAFY* (*LFY*), which promotes flowering in response to a number of different signals (including changes in photoperiod and hormones) and regulates phase transition, appears to be highly conserved in all higher plants (Weigel *et al*. 1992). The *proliferating floral meristem* (*pfm*) mutant of *L. japonicus* has abnormal leaves, and produces sepal-like structures in the lace of flowers of secondary inflorescences (Dong *et al*. 2005). This phenotype is reminiscent of the *unifoliata* (*uni*) mutants of pea (Hofer *et al*. 1997), and analysis of the *LjLFY* gene in *pfm* plants revealed the presence of a premature stop codon (Dong *et al*. 2005). *LjLFY* is expressed in specific domains of the shoot and floral meristems (Dong *et al*. 2005).

A number of genes regulate the normal production of flowers, some having additional roles in the regulation of meristem function and flowering time. One such gene is the arabidopsis MADS-domain transcription factor *APETALA1* (*AP1*), which accelerates flowering and promotes the development of petals (Mandel *et al*. 1992). The *M. truncatula* mutant *proliferating inflorescence meristem* (*pim*) has been attributed to a transposon insertion in the *M. truncatula APETELA1* orthologue (Benlloch *et al*. 2006). The *pim* mutant has flowers with sepals transformed into leaves, and flowers that are transformed into inflorescences (Benlloch *et al*. 2006). Although no *AP1* mutations have been described in *L. japonicus*, two *AP1*-like genes, *AP1a* and *AP1b* have been characterized (Dong *et al*. 2005). These genes are expressed in floral primordia early in flower development and are subsequently restricted to sepals and petals (Dong *et al*. 2005). Mutations in the pea *AP1* orthologue, also called *PIM*, exhibit floral and inflorescence meristem defects (Taylor *et al*. 2002).

In arabidopsis, mutations in the *UNUSUAL FLORAL ORGANS* (*UFO*) gene that encodes a F-box protein involved in protein degradation lead to transformation of petals to sepals and stamens to carpels (Samach *et al*. 1999). The pea mutant *stamina pistilloida* (*stp*) and the *L. japonicus* mutant *proliferating floral organs* (*pfo*) both carry mutations in *UFO* orthologues, and exhibit similar floral organ transformations (Taylor *et al*. 2001; Zhang *et al*. 2003; Dong *et al*. 2005).

In contrast to arabidopsis, significant interest has been shown in long-distance signaling between roots and shoots in legumes. The *klavier* (*klv*) mutant of *L. japonicus* was isolated following a screen for mutants with an increased number of root nodules (Oka-Kira *et al*. 2005). The *klv* mutant is also late flowering, has abnormal pistils and additional flowers (Oka-Kira *et al*. 2005). Although the *KLV* gene has not yet been cloned, its characterization will reveal important aspects of long-distance signaling and the function of meristems at either end of the plant.

TRAIT-DISSECTION ANALYSIS OF FORAGE LEGUME REPRODUCTIVE DEVELOPMENT

Molecular genetic map construction

The development of molecular genetic markers for white clover has been reviewed by Forster *et al*. (2001). A comprehensive set (*c*. 400) of unique white clover genomic DNA-derived simple sequence repeat (TRSSR) markers was developed using enrichment library construction technology (Kölliker *et al*. 2001). Bioinformatic analysis of *c*. 42,000 white clover expressed sequence tags (ESTs) (Sawbridge *et al*. 2003) was used to develop 792 EST-SSR primer pairs (Barrett *et al*. 2004).

Genetic map development in white clover was performed using a combination of TRSSR and amplified fragment length polymorphism (AFLP) markers. The reference mapping population was a F_2 (I.4R x I.5J) family with parental genotypes from fourth and fifth generation inbred lines descended from plants containing the rare self-fertile (S_f) allele. A single F_1 plant was self-pollinated to generate an F_2 population of 150 individuals (Michaelson-Yeates *et al*. 1997). The F_2 (I.4R x I.5J) map contained 135 loci (78 TRSSR and 57 AFLP) on 18 linkage groups (LGs) (two more than the karyotypic number), with a total map length of 825 cM. The extent of map construction was limited by high levels of segregation distortion, affecting 39% of the TRSSR loci, with the majority distorted towards the heterozygous genotypic class (Jones *et al*. 2003). A higher-resolution genetic map largely based on EST-SSR markers was constructed using the F_1 (Sustain 6525-2 x NRS 364-7) mapping family (Barrett *et al*. 2004). A total of 335 EST-SSR and 30 TRSSR primer pairs were polymorphic and permitted assignment of 493 loci to a genetic map containing 16 LGs, with a total map length of 1,144 cM. The EST-SSR markers detected homoeologous locations between the ancestral genomes at high frequency, and provided the basis for standard chromosome nomenclature development.

A comprehensive red clover genetic map was constructed using the F_1 (HR x R130) two-way pseudo-testcross population, initially through the use of 157 cDNA-derived RFLP markers (Isobe *et al*. 2003), and more recently through incorporation of genomic DNA-derived and EST-SSR markers (Kölliker *et al*. 2006) to generate a map containing 1,434 SSR loci across the 7 LGs with a cumulative map length of 869 cM (Sato *et al*. 2005). Macrosynteny was determined between red clover and both *M. truncatula* and *L. japonicus* (Sato *et al*. 2005). A second two-way pseudo-testcross population (F_1[pV x pC]) has been used to construct a map containing 216 AFLP and 42 SSR loci, with a cumulative length of 444 cM (Herrmann *et al*. 2006).

For *M. truncatula*, complementary genetic maps have been developed using two different F_2 populations, A17 x A20 and A17 x DZA315 (Thoquet *et al*. 2002; Choi *et al*. 2004a). The A17 x A20 map was based on 141 sequence based markers, including EST-SSRs, bacterial artificial chromosome (BAC)-end sequences and resistance gene analogues (Choi *et al*. 2004a). The A17 x DZA315 map was generated using 289 markers, including RFLPs, AFLPs, isozymes and gene-based sequences (Thoquet *et al*. 2002). An refined map using a recombinant inbred line (RIL) population of 199 RILs has been derived from the A17 x DZA315 cross, and genotyped with SSR markers (Huguet *et al*. 2004). A large number of additional RILs are currently in development, and will prove extremely informative (M. Delalande and J.-M. Prosperi, pers. comm.).

The *M. truncatula* genetic maps have been subsequently enhanced with functionally-associated genetic markers (Andersen and Lubberstedt 2003; Julier *et al*. 2003) through *in silico* mapping of putative orthologues identified from the *M. truncatula* genome sequence project (Cannon *et al*. 2005; Hecht *et al*. 2005), including many reproductive development genes belonging to the gibberellin-dependent, autonomous, vernalisation-dependent, light-dependent and integration floral induction pathways. Template DNA se-

quences from target genes (including *GAI*, *LD*, *VIP2*, *CRY1*, *CRY2*, *PHY*, *CO*, *GI*, *FT*, *LFY* and *TFL1*) were also used to develop sequence tagged site (STS) markers, with polymorphism revealed by amplicon length or internal restriction site variation (Julier *et al*. 2007).

A *L. japonicus* trait-specific genetic map was constructed using F_8 RILs from a cross between the parental lines GifuB-129 and Miyakojima MG-20 (Gondo *et al.* 2007). Genotypic analysis was performed using 96 SSR markers covering over 90% of the reference genetic map obtained from an F_2 population (Hayashi *et al*. 2001).

QTL analysis

The F_2 (I.4R x I.5J) white clover genetic map has been exploited for quantitative trait locus (QTL) analysis of a number of reproductive morphogenesis and reproductive development traits including flowering date, height of tallest flower, peduncle length and girth, number of flowers, number of florets, number of seeds per flower, fertility score (number of seeds per floret), SY per plant and thousand seed weight (TSW) per plant. Flowering dates were measured at two sites, the Institute of Grassland and Environmental Research (IGER), Aberystwyth, United Kingdom in 1999 and 2001, and at East Craigs (Scotland) in 2001, while other traits were assessed in a single environment (IGER in 1999) (Cogan *et al*. 2006). High trait correlation coefficients were observed for fertility score, seed per flower and SY, but these relationships were not reflected in high correlations with TSW. QTLs for flowering date were detected on LGs 2, 16 and 18 from the IGER 1999 dataset and LG12 from the East Craigs 2001 dataset, with no coincidence between environments. Clusters of coincident QTLs were observed on LGs 2 and 3: for instance, SY and flower number QTLs co-locate in the upper region of LG3. The two SY QTLs were of relatively large effect (accounting for 56 and 61% of the phenotypic variance [V_p], respectively), providing the basis for effective selection for this trait (Cogan *et al*. 2006).

The F_1 (Sustain 6525-2 x NRS 364-7) white clover mapping population was evaluated for SY and the component traits of inflorescence density (ID), yield per inflorescence (YI) and TSW (Barrett *et al*. 2005). Data was obtained from plants grown in the field at Lincoln, New Zealand during three full growing seasons completed in 2002, 2003 and 2004. A total of 11 QTLs were identified on 9 of the 16 LGs. Single SY QTLs were detected for each of the three years, with coincidence between the regions detected from the 2003 and 2004 datasets. SY QTLs accounted for 19.5-23.2% of total V_p. Coincidence was also observed between ID QTLs from 2002 and 2003 on both LGs C2 and E1, and TSW QTLs from 2002 and 2003 on both LGs D2 and G1. Most QTLs for a given trait were observed on only one of a given homoeologous chromosome pair. The largest cluster of QTLs was observed in the lower region of LG D2, including effects for all of the measured traits. The effects of YI and TSW genotype class in this genomic region were inversely correlated, possibly due either to pleiotropy, or repulsion phase linkage. The temporal stability and relatively independent genetic control of traits in this study suggests that substantial improvement through molecular marker-based breeding is achievable.

Eight SY-associated traits were measured in the F_1(pV x pC) red clover population: seed yield per plant (SYP), seed number per plant (SNP), seed yield per head (SYH), seed number per head (SNH), head number per plant (HNP), thousand-seed weight (TSW), number of seeds per 100 florets (PSS) and time of flowering (TOF), expressed as days after first herbage cut (Herrmann *et al*. 2006). Highly significant variation was detected for all eight traits, and heritability values were estimated to range from 0.51 (SYP) to 0.85 (TSW). SYP was shown to be highly significantly correlated with all other traits apart from TOF and TSW. A total of 38 QTLs were detected, with 3-8 QTLs per trait accounting for 33.8%-69.1% of V_p. Between 4 and 9 QTLs were observed on each of the 7 LGs, with clustering of QTLs for highly correlated traits such as SYP, SNP, HNP, SNH, SYH and PSS. Three SYP QTLs were identified on LGs 3, 4 and 6, collectively explaining 33.8% of V_p. Due to the levels of correlation and QTL coincidence, HNP provides a potential indirect measurement for SYP which can be implemented in the field prior to seed maturity.

The *M. truncatula* Jemalong-6 x DZA315.16 cross RIL population was evaluated with variable replication in five environments corresponding to combinations of three locations (Montpellier, Toulouse, Lusignan) and four years (2000-2004) (Julier *et al*. 2007). A core set of 93 RILs was present in each experiment. In addition to multiple aerial morphogenesis traits, flowering date was evaluated in four environments. Significant variation was observed within the experimental population, and flowering date was shown to be negatively correlated with main stem and branch length. Eleven QTLs were detected, with consistent identification of regions on chromosomes 7 and 8 (all environments) and chromosome 1 (2 environments). Up to 59% of V_p was explained by the chromosome 7 QTLs. Coincidence was observed between flowering date QTLs and reproductive development candidate genes: *CRYPTOCHROME*, *PHYTOCHROME A* and *GI* loci were located adjacent to the chromosome 1 QTLs, while *CO* and *FTL* loci coincided with the major chromosome 7 QTLs (Julier *et al*. 2007).

The *L. japonicus* GifuB-129 and Miyakojima MG-20 RIL population was evaluated with replication in the field in Sapporo, Japan, in two successive years (2004 and 2005) (Gondo *et al.* 2007). Reproductive traits included flowering time, flowering degree (FD), seed pod length (POL), seed pod width (POW), seed per pod (SPO) and seed weight (SW). Positive phenotypic correlations were observed between the POL, POW, SPO and SW traits within trials. Heritability values were generally high in 2004, but variable in magnitude between the two years. The FD trait showed a very low correlation coefficient between years. Two flowering time QTLs were located on chromosome 1, accounting for 14.7% and 13.3% of V_p, respectively. A single FD QTL was also detected in a non-coincident location on chromosome 1. Eighteen seed pod trait QTLs were observed on chromosomes 1, 2, 4, 5 and 6. Chromosome 1 contained two independent clusters of POW and SW QTLs and of POL and SPO QTLs, while chromosome 5 contained a cluster of POW and POL QTLs from 2005 and chromosome 6 contained coincident POL and SW QTLs.

Prospects for comparative genetics

To date, trait-dissection studies have been performed for the important forage legume species, white clover and red clover, and the corresponding model species. Comparative genetic analysis permits the alignment of trait-specific genetic maps, identification of putative homologous QTLs (within species) and orthologous QTLs (between species), and evaluation of candidate gene-QTL co-location. The latter activity is highly supported by whole genome sequencing efforts and functional classification of candidate genes in the model species (Young *et al*. 2005; Zhu *et al*. 2005). For Fabaceae species, identification of conserved synteny between the genomes of species such as *M. truncatula*, *L. japonicus*, *Pisum sativum*, chickpea (*Cicer arietinum*), mungbean (*Vigna radiata*) and soybean (*Glycine max*) has been achieved through the use of anchor genetic markers (Choi *et al*. 2004b; Yan *et al*. 2004; Mudge *et al*. 2005). However, intra- or interspecific genetic map alignment in the studies described here is limited due to a dearth of common markers. The two white clover trait-specific maps are non-congruent, although a proportion of loci from both maps have been combined in the genetic maps derived from the F_1 (GA43 x SRVR) population (Zhang *et al*. 2007), and more extensive genotyping of both genomic DNA-derived and EST-derived SSRs is currently being performed for the F_1 ($Haifa_2$ x LCL_2) and F_1 ($S184_6$ x LCL_6) populations (Cogan *et al*. 2007). On the basis of current data, the QTL clusters on F_2

(I.4R x I.5J) LGs 2 and 3 do not correspond to the major regions (LGs C2, D2, E1 and G1) on the F_1 (Sustain 6525-2 x NRS 364-7) parental maps. Similarly, relationships between the white clover and red clover specific maps cannot be inferred due to the disparate nature of the marker sets. Phenotypic analysis of the F_1 (HR x R130) population would permit effective comparison with model species genomes, but has not been performed. However, both empirical (Zhang *et al*. 2007) and computational (George *et al*. 2006) comparative analysis has permitted alignment of the EST-SSR-based white clover genetic map to the *M. truncatula* and *L. japonicus* genomes. A total of 124 SSR-containing ESTs which were assigned to the F_1 (Sustain 6525-2 x NRS 364-7) maps detected putative ortholoci, suggesting a predominant one-to-one relationship between each of the homoeologous groups of white clover and a single *M. truncatula* chromosome (George *et al*. 2006). *M. truncatula* chromosomes *Mt*1, *Mt*5, *Mt*7 and *Mt*8, which contain flowering time QTLs, hence correspond to homoeologous groups E, G, C and B respectively. As F_2 (I.4R x I.5J) LG 12 has been tentatively aligned with group G and hence *Mt*5, orthologous QTLs may be located on these syntenic chromosomes. Flowering time QTLs were also identified on *L. japonicus* chromosome 1 (*Lj*1), which corresponds to segments of both *Mt*3 and *Mt*7. The seed trait QTLs located on *Lj*4, 5 and 6 may also correspond to the equivalent regions identified on F_1 (Sustain 6525-2 x NRS 364-7) LGs D2, E1 and A1, respectively. Although these initial inferences are highly preliminary, more detailed map melding and comparative analysis will permit subsequent refinement.

CONCLUSIONS

The recent advances in the genome sequencing and mutational analysis of model legumes, coupled with the refinement of trait-specific genetic maps in agricultural forage species, provides the basis for rapid identification of agronomically-important genes in these important crops. Enhanced efforts in comparative genomics and genetics, based on the use of common gene-associated polymorphisms, will drive major advances in the understanding of regulation of reproductive development in the legume family. Consequent improvements of traits such as SY will confer substantial benefit to breeders and growers.

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