Leaf Trait Coloration in White Clover and Molecular Mapping of the Red Midrib and Leaflet Number Traits

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ABSTRACT

White clover (Trifolium repens L.) is a highly outcrossing heterozygous allotetraploid species, for which classic inheritance studies have been inconclusive. With the aid of molecular markers, it is now possible to study the genes controlling morphological traits. The objectives of this study were to catalog the leaf marks in white clover and map the location of leaf morphological traits based on cosegregation with molecular markers. A mapping population segregating for eight morphological traits consisting of leaf marks and number of leaflets was developed and phenotyped at two different locations during the summer and winter seasons. A confirmation population, derived by selfing one of the mapping population parents, was produced and phenotyped in one location at two different times of the year. Through the use of previously published simple sequence repeat (SSR) marker maps, linkages between the mapped molecular markers and genes for three different morphological traits was identified. The red midrib and red fleck traits were found to be controlled by two closely linked dominant genes on linkage group (LG) B1. The trifoliolate trait is controlled by at least one gene on LG H1. The identification of molecular markers linked to loci affecting leaf morphology traits resolves conflicting hypotheses on the genetics of these complex traits and has potential for molecular breeding improvement of white clover.

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Abbreviations: LG, linkage group; LOD, logarithm of the odds; PCR, polymerase chain reaction; SSR, simple sequence repeat.

HITE CLOVER, *Trifolium repens* L., is an allotetraploid (2n = 4x = 32) legume that is believed to have resulted from the hybridization of *T. occidentale* Coombe (Ellison et al., 2006) and a second, currently unknown *Trifolium* species (Hand et al., 2008). Pollination in this species is controlled by a game-tophytic self-incompatibility system that Atwood (1942) determined was regulated by a single locus with many different alleles, including a rare allele that confers self-compatibility (*Sf*). Due to the tetraploid genome and outcrossing nature of the species, white clover is highly heterozygous.

There are many different leaf marks and other morphological traits found within white clover, many of which have been the subject of genetic studies. For consistency, the original genetic nomenclature has been maintained for each trait, but the notation has been modernized to that of Quesenberry et al. (1991). The lack of leaf mark (Fig. 1a) is recessive to the presence of all leaf marks (Brewbaker, 1955; Carnahan et al., 1955). The most common leaf mark is the multiallelic white V mark (gene symbol *V*; Fig. 1b) on the upper epidermis of each leaflet (Brewbaker, 1955). This trait is highly variable, with a range of marks from a single V mark to a V mark with a yellow tip (*Vby*; Fig. 1c). The marginal

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mark (*Vm*; Fig. 1d) (Lenoble and Papineau, 1970) is rarely seen in naturalized populations.

Other leaf marks found in white clover contain anthocyanin pigments. Some examples are the redspot leaf mark (Vr2; Fig. 1e) (Hovin and Gibson, 1961), red leaflet mark (Vrl; Fig. 1f) (Corkill, 1971), red midrib mark (Rm; Fig. 1g) (Carnahan et al., 1955; Corkill, 1971), red leaf mark (Rl; Fig. 1h) (Carnahan et al., 1955; Corkill, 1971), and red fleck mark (Rf; Fig. 1i) (Carnahan et al., 1955). The identification of these leaf marks is based on somewhat vague written descriptions in the literature and on a black and white photograph published by Corkill (1971). Both the descriptions and the photograph are inadequate to properly identify these marks.

Although it is agreed that all leaf marks described above are dominant traits, there is disagreement regarding the genetic control of these traits. Carnahan et al. (1955) and Brewbaker (1955) concluded that the presence of the various leaf marks is controlled by one of two different genes (V and R) that each contain multiple alleles, such that Rm, Rl, and Rf would all be different alleles of the R locus. In contrast, Corkill (1971) observed low recombination frequencies between the leaf marks within each locus and concluded accordingly that the R and V loci each consist of a series of tightly linked genes. Thus, under the Corkill hypothesis, Rm, Rl, and Rf represent linked (but different) loci, collectively known as the R locus.

Clover leaves differ not only in their leaf marks but also in their number of leaflets. White clover typically has trifoliolate leaves (Fig. 2a), but multifoliolate (greater than three leaflets per leaf) genotypes exist within naturalized populations. The multifoliolate leaves of white clover are traditionally collected as good luck charms, with the four-leaf clover (Fig. 2b) recognized worldwide as a symbol of good fortune. When this trait is combined with a mutant elongated petiolule, the leaf morphology is altered from palmate to pinnate (Fig. 2c). Despite the popularity of the four-leaflet trait in clover, it has not been possible to determine its genetic control. Ford and Claydon (1996) determined that the trait was mostly recessive but were not able to observe any Mendelian segregation in the progeny. Thus, the information available on the genetic control of the multifoliolate trait is limited to that available from other Trifolium species. Knight (1969) studied the multifoliolate trait in crimson clover (T. incarnatum L.) and found that there were two types of multifoliolate leaves: one that was strongly influenced by environmental variation and one that was not. The environmentally conditioned multifoliolate trait inheritance could not be determined, but the non-environmentally controlled multifoliolate trait was found to be a single gene recessive trait (Knight, 1969). In red clover (T. pratense L.), the multifoliolate trait was first studied by Simon (1962), who determined it was conditioned by homozygous recessive alleles at one of two loci.

Also studying red clover, Jaranowski and Broda (1978) determined that the multifoliolate trait was controlled by homozygous recessive alleles at three loci, and Taylor (1982) determined it was a quantitative recessive trait.

White clover genetics are complicated by allotetraploidy, extensive heterozygosity, and a highly outcrossing reproductive system. Therefore, homozygous lines are not available for inheritance studies. Furthermore, many of the morphological traits under study are highly influenced by environment. For example, many of the traits containing anthocyanin are best observed at temperatures below 10°C (Carnahan et al., 1955). As a result, some traits such as the red leaflet (Vrl) trait are not visible in the summer (Davies, 1963). In addition, the multifoliolate trait in white clover was found to be environmentally conditioned in a germplasm source registered by Baltensperger et al. (1991), which supports Knight's (1969) observations in crimson clover. By looking at these traits at the molecular level, the environmental effects on each trait can be separated from the gene itself. As such, mapping morphological traits found in white clover with molecular markers may be a more effective way to determine the inheritance of these traits, many of which have been studied for nearly a century without satisfactory conclusions.

The development of white clover genetic maps based on molecular markers (Barrett et al., 2004; Jones et al., 2003; Zhang et al., 2007) has allowed some important agronomic traits to be mapped (Barrett et al., 2005a, b; Cogan et al., 2006) and macrosynteny between white clover linkage groups (LGs) and chromosomes of the model legume *Medicago truncatula* Gaertn. to be determined (George et al., 2008). In the 2004 white clover simple sequence repeat (SSR) map, the red fleck mark (Rf) was mapped as the R locus onto LG B1 (Barrett et al., 2004). The parents used to create the mapping population in that study were forage genotypes and, as such, had limited morphological diversity for additional leaf marks.

The objectives of the research described here were to inventory the leaf marks expressed in white clover (many of which are shown in Fig. 1) and map the location of genes for leaf morphological traits based on cosegregation with molecular markers from an existing white clover linkage map.

MATERIALS AND METHODS

Plant Materials

Two phenotypically distinct white clover genotypes were used as parents to develop a mapping population (Fig. 3). One parent, GA02-56 (hereafter referred to as GA43 in keeping with its name in the literature), is an agronomic genotype out of the cultivar 'Durana' (Bouton et al., 2005) that was also used as a parent for construction of the genetic map of Zhang et al. (2007). This genotype has trifoliolate green leaves, the intermediate white V mark (Vi), and the red fleck leaf mark (Rf). The second genotype, 05-O-34, contains several traits of ornamental value (Tashiro et al., 2009). This genotype has multifoliolate leaves, the



Fig. 1. Different leaf marks found in white clover. The gene symbols are as originally proposed by the authors that described them, but the notation has been modernized as described by Quesenberry et al. (1991).

a: Palmate trifoliolate leaf with full V, low (VI)



b: Palmate multifoliolate leaf with full V, low (V/) c: Pinnat







Fig. 2. Different leaf morphologies found in white clover. The gene symbols for the leaf marks present on each leaf are indicated in parentheses.



Fig. 3. The white clover genotypes used as parents to create the mapping population. a) Ornamental-type parent 05-O-34; b) Agronomic-type parent GA43.

marginal mark (*Vm*), red leaflet (*Vn*), red midrib (*Rm*), and red fleck (*Rf*) leaf marks. This genotype is also self-compatible (*Sf*). A reciprocal pseudo-testcross mapping population (Grattapaglia and Sederoff, 1994) was made consisting of 178 F_1 's resulting from reciprocal crosses between the two parents. Due to the self-compatibility present in 05–O–34, the F_1 progeny derived from its seed were tested for hybridity by using 40 SSR marker primers. Only those individuals that had markers derived from both parents were used in the mapping population. Individuals that were the result of selfing were incorporated into a confirmation population that was developed by selfing the 05–O–34 parent, resulting in a total of 141 individuals.

Morphological Trait Evaluation

The 178 individuals in the mapping population were grown in 12-cm pots using potting mix made up of equal parts Fafard #3 potting soil (Conrad Fafard, Inc., Agawam, MA), river sand, and farm soil [Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults)] and grown in a University of Georgia greenhouse until the foliage from each individual had filled out the pot. Each individual was scored for the presence and/or absence of each morphological trait in the greenhouse on 22 Aug. 2007 and 29 Mar. 2008 (Table 1). For leaflet number, individuals were scored as either being trifoliolate or multifoliolate. An individual with at least one multifoliolate leaf on the evaluation date was scored as multifoliolate. The 141 individuals that were obtained by selfing the 05-O-34 parent were grown as described above, scored for each trait in the greenhouse on 12 Aug. 2008 and 31 Mar. 2009 (Table 1), and used for confirmation of mapped traits and hypothetical genotypes.

Cuttings of both parents and each individual in the mapping population were obtained and used for replicated field trials. Rooted cuttings of both parents and 140 individuals in which 05-O-34 was the maternal parent were planted at the University of Georgia Plant Sciences Farm (Oconee County, GA) in Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults) soil with a pH of 5.9. Of these 140 individuals, 89 were used for trait mapping. The ramets were planted on 75-cm centers in a randomized complete block design, with four blocks of each genotype, on 6 Dec. 2007. Rooted cuttings of both parents and 89 individuals in which GA43 was the maternal parent were planted at the Plant Sciences Farm on 18 Apr. 2008 with the same experimental design as described above. Morphological data for each individual in each block were scored for each trait on 2 July 2008 (Table 1). Individuals for which 05-O-34 was the maternal parent (89) were also scored on 26 Mar. 2008, and those for which GA43 was the maternal parent (89) were scored on 19 Mar. 2009 (Table 1).

SSR Amplification and Amplicon Detection

DNA was extracted from young leaves of both parents and each genotype in the mapping population using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA). DNA quantification for each sample was performed using a TBS-100 mini-fluorometer (Turner Biosystems, Sunnyvale, CA). After quantification, each sample was diluted to 10 ng μ L⁻¹ and treated with 0.05 U Longlife RNase (G Biosciences, Maryland Heights, MO). From the original 343 primer pairs used by Zhang et al. (2007) to create their linkage map, 96 were selected based on their even distribution

in the different LGs and screened for polymorphism between the two parents of the current mapping population.

A total of 78 primer pairs (81%) were polymorphic, which translates to a marker spacing of around 20 cM, with between 3 and 6 SSR markers per LG. Fluorescently labeled SSR fragments were amplified as described by Zhang et al. (2007) using either 96- or 384-well polymerase chain reaction (PCR) plates, with the exception of the source of the PCR reagents, which were obtained from Promega (Madison, WI). After PCR, plates with different fluorescent tags were pooled together for fragment analysis using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) also as described by Zhang et al. (2007). Simple sequence repeat fragments were visually scored with GeneMapper 3.7 or 4.0 software (Applied Biosystems, Foster City, CA) as dominant markers as described by Zhang et al. (2007). Once the initial LGs were drawn as described below, an additional 48 primer pairs selected from the LGs of interest were screened for polymorphisms between the mapping population parents. From the 48 additional primer pairs evaluated, 41 (85%) of them were polymorphic. Twentyfour of the 41 polymorphic primer pairs were selected based on the number and quality of alleles scored in the parents and used to screen the mapping population as described above. The additional scored alleles were then added to the previously screened marker and phenotypic data to develop LGs with enhanced marker saturation. Markers found to be linked to phenotypic data in the mapping population were screened as described above in the confirmation population.

Linkage Map Development

Linkage maps were developed using the Kosambi mapping function of JoinMap 3.0 (Van Ooijen and Voorrips, 2001) and drawn using MapChart 2.2 (Voorrips, 2002) with each locus coordinate rounded to the nearest whole number. Simple sequence repeat fragments that segregated in a 1:1 ratio in the mapping population were used to create single-parent LGs for each parent as described by Zhang et al. (2007). Consensus maps for LG B1 were developed using bridging loci as described by Barrett et al. (2004). The leaf morphology traits in white clover leaves are either present or absent and therefore were mapped as qualitative traits. All the traits except red fleck leaf mark (Rf) are present only in the ornamental parent and were coded as $np \times nn$ as per software instructions for markers segregating in the first parent. Because the red fleck leaf mark (Rf) is present in both mapping parents and segregating in the mapping population, it was coded as $hk \times hk$ as per software instructions for markers segregating in both parents. In an effort to reduce the effects of genotype × environment interaction on morphological trait expression in white clover, data for the traits were collected and analyzed by the different date and location combinations separately. When mapping the morphological traits, those showing no obvious environmental influence were pooled and the data were mapped as a single dominant trait. The traits showing strong environmental effects were mapped separately based on each individual date (summer and winter) and location (field or greenhouse) combination. After initial linkage map development, the additional marker data were added to the original data and linkage maps were created as described above with a logarithm of the odds (LOD) score ≥ 5.0 .

Table 1. Frequencies of the white clover leaf marks observed within the F_1 mapping population (reciprocal pseudo-testcross between 05-O-34 × GA43) and the S₁ confirmation population (selfing of ornamental parent 05-O-34).

	F₁ mapping population [†]				S_1 confirmation population		
Trait	Summer field	Summer greenhouse	Winter field	Winter greenhouse	Summer greenhouse	Winter greenhouse	
Intermediate white V (Vi)	93	96	89	98	39	41	
Red fleck (Rf)	122	107	113	123	113	116	
Red midrib (<i>Rm</i>)	89	87	81	87	111	110	
Red leaflet (RI)	3	0	40	31	1	43	
Marginal mark (Vm)	81	80	68	80	101	100	
Trifoliolate leaf	44	71	96	101	26 [‡]	55	
Multifoliolate leaf	134	107	62	77	114	85	

[†]Mapping population size consisted of 178 individuals, except during the winter field phenotyping, in which 20 individuals had died in all four blocks, so the population consisted of 158 individuals at this date.

⁺In the confirmation population, leaflet number data failed to be recorded for one individual during each phenotyping date.

In the confirmation population that was developed by selfing of parent 05-O-34, segregating markers were used to create LGs using the F_2 population type in JoinMap 3.0. As in the mapping population, each phenotypic marker was either pooled or mapped individually based on evaluation date and location. Linkage maps were created as described above based on an LOD score ≥ 5.0 .

RESULTS

During phenotypic evaluations of the mapping and confirmation populations it was noted that, while the red fleck (Rf) trait was observed by itself, whenever the red midrib (Rm) trait was present, the red fleck trait was always also present. Since the inheritance of R locus traits in white clover was unclear, the two R locus traits evaluated in this study were tested using both the Carnahan et al. (1955) single-gene hypothesis and the Corkill (1971) linked-gene hypothesis. In both populations, the inheritance data for the two traits controlled by the R locus, red midrib (Rm) and red fleck (Rf), failed to conform to a model in which a single gene controls both phenotypic characters (data not shown). Instead, the two traits were found to be controlled by dominant alleles at two different genes which seem to be simply inherited and tightly linked (r = 0.23%) based on frequency of recombination (Fehr, 1987) for the two genes in the confirmation population (Table 1). Expression of the red midrib (Rm) trait was stable across all environments, with the exception of two genotypes in the mapping population (Table 2). Since this was most likely due to scoring error, the two genotypes were scored as missing data for this trait. Expression of the red fleck (Rf) trait was also relatively stable across most environments, except during the summer greenhouse data collection period, when expression was significantly lower (Table 3). Data for this trait fit a single gene model when $p \ge 0.05$ across all environments, except in the case of summer evaluations of the mapping population in the greenhouse but did not fit a single gene model when $p \ge 0.1$ for half of the environments. Therefore, there is less confidence about a single gene inheritance model for the red fleck (Rf) trait than there is for the red midrib (Rm) trait. Future studies in controlled environments should determine whether the growth temperature is causing the variation in segregation between the crossed population and the selfed population. In an outcrossing species such as white clover, it is common to see skewed segregation patterns (Barrett et al., 2005a). The outcrossing nature of white clover could also explain the fewer-thanexpected recessive genotypes and the increase in heterozygotes observed in the selfed population.

The gene conditioning the red midrib (Rm) trait is linked to markers segregating in the ornamental parent on what corresponds to LG B1 (Fig. 4a) of the map described by Zhang et al. (2007). Phenotypic data for this trait were pooled for mapping due to their high penetrance within the population. Since the red fleck (Rf) trait is segregating in the mapping population but inherited from both the ornamental and forage parents, this trait was mapped by creating a consensus linkage map with markers segregating in both parents (Fig. 4b). The gene conditioning the red fleck (Rf)trait is also linked to markers segregating on LG B1.

The data for both red midrib (Rm) and red fleck (Rf)traits were each originally mapped separately based on date and location. The separate data for each trait mapped to a similar location on the same LG (data not shown). Since the phenotypic data for the red fleck trait mapped so closely on the same LG, the data were pooled and the linkage map recreated. The pooled red fleck (Rf) trait data mapped 1 cM above the red midrib (Rm) trait data on LG B1 of the consensus map (Fig. 4b). The two traits are flanked on either side by molecular markers ats041 and RCS3084. In the confirmation population, both traits also mapped to LG B1, with both traits mapping to the same location on the LG (Fig. 4c). In the confirmation population, the two traits are flanked on each side by molecular markers BG232 and ats099. In both cases, the morphological traits were mapped to the interval flanked by the common markers ats075 and ats099.

The phenotypic data for leaflet number in both populations showed strong environmental influence. Therefore,

Table 2. Segregation of the white clover red midrib (*Rm*; Fig. 1g) trait within the F_1 mapping population (reciprocal pseudo-testcross between 05-O-34 × GA43) and the S_1 confirmation population (selfing of ornamental parent 05-O-34). Chi-squared (χ^2) goodness-of fit test for separate gene hypothesis based on assumed genotypes of the mapping population parents.

Population	Environment	Assumed genotype [†]	Red midrib	Green midrib	Expected ratio	χ^2	${\it p}$ value for χ^2
F ₁	Summer greenhouse	Rmrm x rmrm	87	91	1:1	0.06	0.8065
F ₁	Summer field	Rmrm x rmrm	89	89	1:1	0.00	1.0000
F ₁	Winter greenhouse	Rmrm x rmrm	87	91	1:1	0.06	0.8065
F ₁	Winter field [‡]	Rmrm x rmrm	81	77	1:1	0.06	0.8065
S ₁	Summer greenhouse	Rmrm ⊗	111	30	3:1	0.77	0.3802
S ₁	Winter greenhouse	Rmrm ⊗	110	31	3:1	0.47	0.4930

[†]Assumed genotype based on the separate gene hypothesis of Corkill (1971).

*During the winter field phenotyping, 20 plants had died in all blocks in the field, so the chi-square values were tested against a population of 158 instead of 178.

Table 3. Segregation of the white clover red fleck (<i>Rf</i> ; Fig. 1i) trait within the F ₁ mapping population (reciprocal pseudo-test-
cross between 05-O-34 × GA43) and the S ₁ confirmation population (selfing of ornamental parent 05-O-34). Chi-squared (χ^2)
goodness-of fit test for separate gene hypothesis based on assumed genotypes of the mapping population parents.

Population	Environment	Assumed genotype [†]	Red fleck	No red mark	Expected ratio	χ^2	p value for χ^2
F ₁	Summer greenhouse	Rfrf x Rfrf	107	71	3:1	19.34	< .0001
F ₁	Summer field	Rfrf x Rfrf	122	56	3:1	3.28	0.0701
F ₁	Winter greenhouse	Rfrf x Rfrf	123	55	3:1	2.69	0.1010
F ₁	Winter field [‡]	Rfrf x Rfrf	113	45	3:1	0.68	0.4096
S ₁	Summer greenhouse	$Rfrf \otimes$	113	28	3:1	1.61	0.2045
S ₁	Winter greenhouse	$Rfrf \otimes$	116	25	3:1	3.43	0.0640

[†]Assumed genotype based on separate gene hypothesis of Corkill (1971).

[‡]During the winter field phenotyping, 20 plants had died in all blocks in the field, so the chi-square values were tested against a population of 158 instead of 178.



Fig. 4. Linkage maps indicating the location of the loci conditioning the red midrib and red fleck traits on linkage group (LG) B1 of the map described by Zhang et al. (2007). Linkages are based on segregation in the mapping population (reciprocal pseudo-testcross between $05-O-34 \times GA43$) and confirmation population (selfing of ornamental parent 05-O-34). *Rm* = Red midrib trait; *Rf* = Red fleck trait. a) Single parent linkage map of LG B1 showing the location of the red midrib locus based on segregation in the ornamental parent within the mapping population. b) Consensus map of LG B1 showing the location of the red midrib locus and red fleck locus based on segregation linkage map of LG B1 showing the location of the red midrib locus and red fleck locus based on segregation linkage map of LG B1 showing the location of the mapping population. c) Confirmation linkage map of LG B1 showing the locus based on segregation within the confirmation population.

the data from each collection date and location were mapped separately. While attempting to map the gene(s) controlling multifoliolate expression, which is believed to be a recessive trait, the data did not segregate with any markers from the ornamental parent. In contrast, when the dominant trifoliolate trait data were used to map the gene(s), they segregated with markers inherited from the agronomic parent. The gene(s) responsible for trifoliolate leaves (Fig. 5) is linked to molecular markers segregating in the GA43 parent on what corresponds to LG H1 of the map described by Zhang et al. (2007). Although the gene(s) controlling the trifoliolate trait always maps to the same LG, the data collected in the winter and in the summer map to locations 30 cM apart (Fig. 5). Within the winter phenotypes, the data collected in both the greenhouse and the field map to locations 19 cM apart. Molecular marker RCS2681 maps to the same location as the winter field data. Simple sequence repeat marker TRSSRA02C02 maps 2 cM above the winter greenhouse data. Summer data from the greenhouse maps 28 cM away from data collected in the field.

Although molecular markers distributed throughout all white clover LGs were evaluated, the density of markers in some LGs was too low and therefore insufficient to detect marker-trait linkages for all of the traits segregating in the mapping population. The red leaflet (*Vrl*) trait, which was only visible during the winter evaluations, failed to segregate with any of the molecular markers used in this study. Likewise, the marginal mark (*Vm*) and the intermediate white V mark (*Vi*), although visible during all collection dates, did not segregate with any of the molecular markers evaluated.

DISCUSSION

Before this study, only one white clover morphological trait had been placed on the white clover linkage map, namely the R locus on LG B1 (Barrett et al., 2004). The results from this study made it possible to refine the location reported by Barrett et al. (2004), to map two additional traits, and to conclusively determine the nature of the R locus. The use of molecular markers permitted the development of both single-parent and consensus maps for assignment of these trait-specific genes and to overcome the historic difficulties associated with mapping traits in heterozygous genotypes.

Mapping of separate genes for red fleck (Rf) and red midrib (Rm) in the same population made it possible to clarify the confusion that has been associated with the genetic control of these traits. Barrett et al. (2004) followed the Carnahan et al. (1955) premise that red leaf marks are conditioned by a single gene, named R, with all of the different morphotypes due to different alleles of that gene. The R locus mapped by Barrett et al. (2004) is actually the red fleck (Rf) trait, as determined by their description of this phenotypic trait.

H1-Forage



Fig. 5. Single parent linkage map of linkage group (LG) H1 showing the location of the trifoliolate leaf trait based on segregation in the GA43 parental map from the mapping population (reciprocal pseudo-testcross between 05-O-34 × GA43). Sum = summer; Win = winter, GH = greenhouse; Fld = Field; 3Leaf = trifoliolate leaf trait.

In this study, the two dominant alleles of the genes that control red fleck and red midrib expression, Rf and Rm, are linked in coupling phase in the ornamental parent, which explains the observation that all individuals with red midrib leaf mark also possess the red fleck leaf mark. In the forage parent, the dominant red fleck leaf mark allele (Rf) and the recessive red midrib leaf mark allele (rm) are also in coupling linkage, resulting in individuals with the red fleck trait but without red midrib leaf mark in the mapping population. The differences in linkage between the two traits between the two populations are therefore a result of the difference in the parental genotypes used to develop the two populations. The distinct mapping of both traits, in conjunction with the inheritance data, confirms Corkill's (1971) assertion that the *R* locus actually comprises a series of linked genes.

With the mapping of the trifoliolate leaf trait, the location of at least one gene responsible for leaflet number in the species has been discovered and highlights the complexity of this trait. The popular multifoliolate trait in white clover is controlled by a recessive gene(s), and its expression is also strongly influenced by the environment. As such, the data for the trifoliolate trait were mapped separately. The trifoliolate trait segregated with molecular markers located on LG H1 of the map described by Zhang et al. (2007). The difference in mapping location of this trait between summer and winter evaluation dates is likely due to the environmental effect on multifoliolate expression but may also be an artifact of the population size and the low marker density or it might mean that different loci control leaflet number in the summer and winter.

Because the basal species of Trifolium often have pentafoliolate leaves (Ellison et al., 2006; Zohary and Heller, 1984), it is believed that the genus Trifolium originated from multifoliolate ancestors and that the number of leaflets was reduced during evolutionary time (Eames, 1961; Jaranowski and Broda, 1978; Zohary and Heller, 1984). The presence of a dominant locus that inhibits the expression of multifoliolate leaves, leading to trifoliolate leaves in white clover, supports the premise that leaflet number suppressors in the Fabaceae in general, and Trifolium in particular, resulted in lower leaflet number (Eames, 1961; Zohary and Heller, 1984). There is another trait which sometimes appears in white clover populations in which the petiolule of the middle leaflet is elongated. It was noted in this study that whenever the multifoliolate trait and the elongated petiolule were expressed together, the resulting leaves were frequently pinnately compound (Fig. 2c) rather than palmately compound (Fig. 2b). These multifoliolate pinnate leaves bear an even greater resemblance to the typical leaf morphology of legumes (Eames, 1961).

As more molecular markers become available in white clover and the LGs become more saturated, it should be possible to map the other morphological traits described here and further clarify the inheritance of these traits. During the development of their molecular map, Zhang et al. (2007) showed that SSR markers from related legume species could be successfully utilized as markers in white clover. Accordingly, the complete sequencing of the closely related (George et al., 2008) reference species *Medicago truncatula* Gaertn. genome (www.medicago.org; verified 24 Mar. 2010) will facilitate the development of additional molecular marker resources and comparative mapping efforts that may be useful to identify the gene(s) responsible for the observed variation in many ornamental and agronomic traits within white clover populations.

In summary, the advent of molecular marker-based maps for white clover means that the tools are finally in place to start addressing long-standing questions on clover genetics and evolution. It was possible to identify the location of two new morphological traits utilizing molecular markers from previously published linkage maps in white clover. The successful mapping of the red midrib trait in a population that also contains the previously mapped red fleck trait resolves the conflicting hypotheses of earlier researchers studying R locus inheritance in white clover. The successful mapping of at least one gene responsible for trifoliolate leaflet number in the species highlights its complexity and brings white clover breeders and researchers one step closer to unlocking the genetic mechanisms behind multiple leaflet expression in white clover and fixing this trait for breeding purposes.

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References

- Atwood, S.S. 1942. Oppositional alleles causing cross-incompatibility in *Trifolium repens*. Genetics 27:333–338.
- Baltensperger, D.D., D.S. Wofford, and W.H. Anderson. 1991. Registration of Fl-Ml white clover germplasm. Crop Sci. 31:853.
- Barrett, B., A. Griffiths, M. Schreiber, N. Ellison, C. Mercer, J. Bouton, B. Ong, J. Forster, T. Sawbridge, G. Spangenberg, G. Bryan, and D. Woodfield. 2004. A microsatellite map of white clover. Theor. Appl. Genet. 109:596–608.
- Barrett, B., C. Mercer, and D. Woodfield. 2005a. Genetic mapping of a root-knot nematode resistance locus in *Trifolium*. Euphytica 143:85–92.
- Barrett, B.A., I.J. Baird, and D.R. Woodfield. 2005b. A QTL analysis of white clover seed production. Crop Sci. 45:1844–1850.
- Bouton, J.H., D.R. Woodfield, J.R. Caradus, and D.T. Wood. 2005. Registration of 'Durana' white clover. Crop Sci. 45:797.
- Brewbaker, J.L. 1955. V-leaf markings of white clover. J. Hered. 46:115–123.
- Carnahan, H.L., H.D. Hill, A.A. Hanson, and K.G. Brown. 1955. Inheritance and frequencies of leaf markings in white clover. J. Hered. 46:109–114.
- Cogan, N.O.I., M.T. Abberton, K.F. Smith, G. Kearney, A.H. Marshall, A. Williams, T.P.T. Michaelson-Yeates, C. Bowen, E.S. Jones, A.C. Vecchies, and J.W. Forster. 2006. Individual and multi-environment combined analyses identify QTLs for morphogenetic and reproductive development traits in white clover (*Trifolium repens* L.). Theor. Appl. Genet. 112:1401–1415.
- Corkill, L. 1971. Leaf markings in white clover. J. Hered. 62:307-310.
- Davies, W.E. 1963 Leaf markings in *Trifolium repens*. p. 94–98. *In* C.D. Darlington and A.D. Bradshaw (ed.) Teaching genetics in school and university. Philosophical Library, New York.
- Eames, A.J. 1961 Morphology of the angiosperms. McGraw-Hill Book Company, New York, NY.
- Ellison, N.W., A. Liston, J.J. Steiner, W.M. Williams, and N.L. Taylor. 2006. Molecular phylogenetics of the clover genus (*Trifolium*- Leguminosae). Mol. Phylogenet. Evol. 39:688–705.

Fehr, W.R. 1987 Genetic principles. p. 26–46. *In* W.R. Fehr (ed.) Principles of cultivar development. McGraw-Hill, New York.

Ford, J.L., and R.B. Claydon. 1996. Inheritance of multifoliolate

leaves in white clover. Agron. Soc. New Zealand Spec. Publ. 11:167–170.

- George, J., T.I. Sawbridge, N.O.I. Cogan, A.R. Gendall, K.F. Smith, G.C. Spangenberg, and J.W. Forster. 2008. Comparison of genome structure between white clover and *Medicago truncatula* supports homoeologous group nomenclature based on conserved synteny. Genome 51:905–911.
- Grattapaglia, D., and R. Sederoff. 1994. Genetic-linkage maps of *Eucalyptus-grandis* and *Eucalyptus-urophylla* using a pseudotestcross- mapping strategy and RAPD markers. Genetics 137:1121–1137.
- Hand, M.L., R.C. Ponting, M.C. Drayton, K.A. Lawless, N.O.I. Cogan, E.C. Brummer, T.I. Sawbridge, G.C. Spangenberg, K.F. Smith, and J.W. Forster. 2008. Identification of homologous, homoeologous and paralogous sequence variants in an outbreeding allopolyploid species based on comparison with progenitor taxa. Mol. Genet. Genomics 280:293–304.
- Hovin, A.W., and P.B. Gibson. 1961. A red leaf marking in white clover. J. Hered. 52:295–296.
- Jaranowski, J.K., and Z. Broda. 1978. Leaf mutants in diploid redclover (*Trifolium pratense* L). Theor. Appl. Genet. 53:97–103.
- Jones, E.S., L.J. Hughes, M.C. Drayton, M.T. Abberton, T.P.T. Michaelson-Yeates, C. Bowen, and J.W. Forster. 2003. An SSR and AFLP molecular marker-based genetic map of white clover (*Trifolium repens* L.). Plant Sci. 165:531–539.
- Knight, W.E. 1969. Inheritance of multifoliolate leaves glabrous leaves and petiolulate leaflet attachment in crimson clover

Trifolium incarnatum L. Crop Sci. 9:232-235.

- Lenoble, M., and J. Papineau. 1970. Note sur une nouvelle marque foliaire chez *Trifoliom repens*. (In French.) Ann Amelior Des Plant 20:485–487.
- Quesenberry, K.H., R.R. Smith, N.L. Taylor, D.D. Baltensperger, and W.A. Parrott. 1991. Genetic nomenclature in clovers and special purpose legumes.1. Red and white clover. Crop Sci. 31:861–867.
- Simon, U. 1962. Inheritance of polyphylly in red clover (*Trifolium pratense*, L.). Crop Sci. 2:258.
- Tashiro, R.M., J.H. Bouton, and W.A. Parrott. 2009. 'Frosty Morning,' 'Patchwork Quilt,' 'Irish Mist' and 'Pistachio Ice Cream' ornamental white clover, *Trifolium repens* L. Hort-Science 44:1779–1782.
- Taylor, N.L. 1982. Registration of gene marker germplasm for red-clover. Crop Sci. 22:1269.
- Van Ooijen J.W., R.E. Voorrips. 2001 JoinMap 3.0, software for the calculation of genetic linkage maps. Plant Research Int., Wageningen, the Netherlands.
- Voorrips, R.E. 2002. MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93:77–78.
- Zhang, Y., M.K. Sledge, and J.H. Bouton. 2007. Genome mapping of white clover (*Trifolium repens* L.) and comparative analysis within the Trifolieae using cross-species SSR markers. Theor. Appl. Genet. 114:1367–1378.
- Zohary, M., and D. Heller. 1984 The genus *Trifolium*. Israel Acad. of Sci. and Humanities, Jerusalem, Israel.