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Inheritance of somatic embryogenesis in red clover (*Trifolium pratense* L.)

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Abstract Red clover genotypes capable of regenerating plantlets in vitro from non-meristematic tissue-derived callus are rare. Selection for genotypes capable of somatic embryogenesis identified a clone comprised of a group of plantlets regenerated from a hypocotyl-derived callus culture on L2-based media and another group of plantlets originating from crown divisions of the epicotyl-derived plant. The callus-derived plants of this clone were highly regenerative when reintroduced to callus culture, but the epicotyl-derived plants produced nonregenerative callus cultures. F_1, F_2 and BC_1 populations were evaluated to determine the mode of inheritance of the regeneration trait. Reciprocal crosses did not differ, indicating a lack of maternal effects. Results were compatible with genetic control of regeneration by two complementary genes. We propose the genotype *Rn1-Rn2-* for regenerative plants. Three petiole segment explants were sufficient to evaluate regenerative ability in seedlings. Regenerative ability was often associated with abnormal leaf morphology in a few to several leaves.

Key words Red clover · *Trifolium pratense* · Somatic embryogenesis · Inheritance

Introduction

Red clover (*Trifolium pratense* L.) is a forage legume widely grown for hay, silage, pasture or as a green manure crop. Although botanically a perennial, red clover rarely persists beyond two or three production years (Taylor and Quesenberry 1996). Biotechnology holds promise for increasing the persistence of red clover but usually requires the regeneration of entire plants from cultured cells or tissues. In vitro regeneration in red clover, as in many other species, is genotype-dependent. Reports to date have shown that the frequency of regenerative genotypes from non-meristematic explants is very low: 1% or less (Phillips and Collins 1979; Broda 1984; Wang and Holl 1988; MacLean and Nowak 1989; Repkova 1989).

Studies on the inheritance of regeneration in red clover have produced contradictory results. Keyes et al. (1980) conducted a quantitative genetic analysis of embryogenic response and found significant additive variance while dominance and reciprocal variances were not significant. Keyes et al. (1980) calculated narrow-sense heritabilities of $h^2 = 0.54$ on one protocol and $h^2 = 0.25$ on another. More recent studies have treated somatic embryogenesis in red clover as a qualitative trait. Broda (1984) concluded that red clover regeneration was the result of three recessive genes. Studies by MacLean and Nowak (1989) and Myers et al. (1989) both suggested that the ability to regenerate from red clover hypocotyl callus was highly heritable and controlled by one or more dominant genes. Quesenberry and Smith (1993) increased regeneration frequency in red clover from 4% to 72% during five cycles of recurrent selection, and the authors similarly stated that regeneration was controlled by relatively few genes.

Inheritance of regeneration in other species has been investigated using both quantitative and qualitative genetics. Most of the quantitative studies have dealt with species in which regeneration was confined to regeneration from callus on immature embryos or meristems, whereas qualitative inheritance studies have usually dealt with embryogenesis from callus on non-meristematic explants. There are suggestions that these are two different traits; with the former being a quantitatively controlled trait and the latter a qualitatively

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controlled trait. Genetic control of regeneration from callus originating from non-meristematic explants involving dominant alleles at each of two gene loci has been reported in many species, including alfalfa (*Medicago sativa* L.) (Reisch and Bingham 1980; Wan et al. 1988; Hernandez-Fernandez and Christie 1989; Kielly and Bowley 1992); tomato (*Lycopersicon esculentum*) (Koornneef et al. 1987); *Cucumis sativus* L. (Nadolska-Orczyk and Malepszy 1989); *Sorghum bicolor* (L.) Moench (Ma et al. 1987) and *Gossypium hirsutum* L. (Gawel and Robacker 1990).

An earlier report (MacLean and Nowak 1989) described selection for red clover genotypes capable of somatic embryogenesis from non-meristematic tissue. Hypocotyl segments were placed on callus media, while epicotyls were placed on hormone-free medium. Regeneration was accomplished from hypocotyl callus of three seedlings, but only one of the three regenerative cultures was reliably capable of recurrent somatic embryogenesis when petiole tissue from regenerated plantlets was subjected to the regeneration protocol. Repeated attempts to regenerate plantlets from petiole tissue from the corresponding epicotyl-derived plantlet failed. Regenerated plantlets were designated "F49R", while the non-regenerative epicotyl-derived plants were designated "F49M". The difference in regenerative ability between F49R and F49 M plants indicated that somaclonal variation might be responsible for granting the observed regenerative ability. Progeny analysis showed that the regeneration trait was heritable (MacLean and Nowak 1989). The objective of the study presented here was to determine the nature of inheritance of in vitro regeneration in the selected red clover genotype F49R.

Materials and methods

Germplasm

Red clover is a diploid, cross-pollinating species which is normally unable to produce seed through selfing due to gametophytic incompatibility (Taylor and Smith 1979). Crosses between genotypes were performed by collecting pollen on a small square of fine sandpaper glued to the end of a flat wooden toothpick, then lightly brushing the sandpaper over the stigma of the recipient female parent. All crosses were performed in reciprocal. Seedheads were collected approximately 6 weeks later. Seeds were extracted by hand from dry seed heads. A summary of the progeny populations which were evaluated is presented in Table 1.

Regeneration

Seeds from each cross were scarified lightly with sandpaper and placed in Omnisette® (Fisher Scientific, Nepean, Ontario, Canada) tissue cassettes. Seeds were surface sterilized in the cassettes by rinsing in 2% Liquinox detergent; this was followed by a 30-min wash in running tap water, a brief rinse in 70% ethanol, a 15-min treatment with 2% sodium hypochlorite and three rinses with autoclaved distilled water. Surface-sterilized seeds were plated on hormone-free, sucrose-free B5 medium (Gamborg and Shyluk 1978) solidified with 7 $g l^{-1}$ agar in sterile disposable 96-cell well plates (Microtest III® tissue culture plate, Becton Dickinson Labware, Lincoln Park, N.J.), with one seed per well and each well containing 0.1 ml medium. Germinated seeds were subcultured to 25 × 150-mm culture tubes containing 10 ml hormone-free L2 medium (Collins and Phillips 1982). After 4 weeks three 8-mm-long petiole segments were explanted from each seedling. In the case of 86 BC_1 [(F49R × NRCs) × F49R] seedlings (NCR = non-regenerative control red clover plants), a second group of three-petiole-segment explants were taken 3 weeks later. The objective of taking explants twice from one group of seedlings was to evaluate whether three explants were adequate to determine regenerative ability. The segments were placed in individual wells on L2 medium in sterile

Table 1 Chi-square values from goodness-of-fit tests of ratios of regenerative to non-regenerative progeny from crosses

Generation	Proposed genotypes of parents	Number observed (R : N)[a]	Expected ratio (R : N)	χ^{2b}
F_1				
F49R[c] × NRC[d]	*AaBb* × *aaBb*	66 : 118	3 : 5	0.38 ns
F_2				
N_1[e] × NF_1	*aaBb* × *aaBb*	2 : 91	0 : n	–
NF_1 × NF_1	*Aabb* × *aaBb*	27 : 81	1 : 3	0.00 ns
NF_1 × RF_1[f]	*aaBb* × *AaBb* or *Aabb* × *AaBb*	77 : 107	3 : 5	1.48 ns
NF_1 × RF_1	*aa–* × *AaBB*	18 : 18	1 : 1	0.00 ns
NF_1 × RF_1	*Aabb* × *AaBB*	16 : 15	3 : 1	9.04**
RF_1 × RF_1	*AaBb* × *AaBb*	19 : 10	9 : 7	1.01 ns
RF_1 × RF_1	*AaBB* × *AaB–*	79 : 33	3 : 1	1.19 ns
BC_1				
NF_1 × NRC	*aa–* × *aa–*	1 : 121	0 : n	–
NF_1 × NRC	*Aabb* × *aabb*	0 : 35	0 : n	–
NF_1 × NRC	*Aabb* × *aaBb*	10 : 68	1 : 3	6.17*
RF_1 × NRC	*AaBb* × *aabb*	13 : 43	1 : 3	0.10 ns
RF_1 × NRC	*AaBb* × *aaBb*	67 : 91	3 : 5	1.62 ns
RF_1 × NRC	*AaBB* × *aa–*	33 : 38	1 : 1	0.35 ns
NF_1 × F49R	*aaBb* × *AaBb* or *Aabb* × *AaBb*	47 : 45	3 : 5	7.25**
NF_1 × F49R	*aaBB* × *AaBb*	28 : 7	1 : 1	12.60**
RF_1 × F49R	*AaBb* × *AaBb*	48 : 32	9 : 7	0.46 ns
RF_1 × F49R	*AaBB* × *AaBb*	44 : 15	3 : 1	0.01 ns
BC_2				
RBC_1[g] × NRC	*AaBb* × *aabb*	24 : 79	1 : 3	0.16 ns
RBC_1 × NRC	*AaBb* × *aaBb*	112 : 156	3 : 5	2.11 ns
RBC_1 × NRC	*AaBB* × *aa–*	92 : 98	1 : 1	0.19 ns
Other				
F49M[h] × NF_1	*aaBb* × *aa–*	0 : 24	0 : n	–
F49M × NF_1	*aaBb* × *Aabb*	4 : 12	1 : 3	0.00 ns
F49M × RF_1	*aaBb* × *AaBb*	1 : 16	3 : 5	7.25**
F49M × RF_1	*aaBb* × *AaBB*	5 : 20	1 : 1	9.00**

[a] R, Regenerative; N, non-regenerative
[b] ns, Not significant; *significant at 5% level; **significant at 1% level
[c] F49R, plants regenerated from F49 hypocotyl callus
[d] NRC, non-regenerative red clover plants
[e] NF_1, non-regenerative F_1 (F49R × NRC) plants
[f] RF_1, regenerative F_1 (F49R × NRC) plants
[g] RBC_1, regenerative BC_1 (F_1 × F49R) plants
[h] F49M, non-regenerative epicotyl-derived plants originating from the same cv 'Florex' seedling which produced a highly regenerative hypocotyl callus culture (F49R)

disposable 24-cell well plates (Corning Glass Works, Corning, NY.) with each well containing 2 ml medium, Four-week-old callus tissue was subcultured to SEL medium in 24-cell well plates. After 4 weeks on SEL, the tissue was subcultured to SPL medium in 24-cell well plates. L2, SEL and SPL media components were as described by Collins and Phillips (1982). Regeneration was evaluated after 4 weeks on SPL medium. Any cultures in which fungal or bacterial contamination was evident or suspected were discarded.

Ratios of regenerative to non-regenerative progeny were tested by Chi-square goodness-of-fit tests (Gomez and Gomez 1984). Proposed genotypes were assigned based on progeny segregation ratios.

Presence of abnormal leaves was recorded in a group of 454 BC_1 [(F49R × NRCs) × NRCs] seedlings at the time of explant excision (approximately 5 weeks old). Leaf morphology was tested for independence from regenerative ability by a Chi-square test of independence (Gomez and Gomez 1984).

Results and discussion

Crosses were made between F49M and F49R plants but no seeds were produced, indicating self-incompatibility. Further evidence that F49M and F49R were indeed derived from the same seedling was indicated by DNA fingerprinting (Nelke et al. 1993). It must also be explained that a concerted effort was made to repeat regeneration from F49M callus. This effort spanned several years and was not confined to the normal regeneration protocol but also included experiments with different combinations of auxin treatments, light/dark conditions and warm/cold temperatures. All efforts to date to repeat regeneration from F49M callus tissue have failed in this laboratory.

F_1 generation

A contingency test for reciprocal crosses between F49R and NRCs showed independence between regenerative ability in the progeny and direction of the cross, i.e. whether F49R was the pollen or ovule parent (data not shown). As a result, cytoplasmic inheritance was ruled out, and data were combined over reciprocals in all crosses.

It was assumed that a gene affecting regenerative ability was altered in culture since F49M tissue (derived from the epicotyl of seedling F49) was non-regenerative while F49R tissue (derived from hypocotyl callus of the same seedling) was highly regenerative. This gene was designated "*A*", with the recessive allele(s) *a* failing to express regeneration and the dominant allele *A* allowing somatic embryogenesis to occur. Dominant gene effects were assumed since progeny from crosses between F49R and NRCs included a large proportion (36%) of regenerative plants. A further assumption was that only one allele controlling regeneration was affected in culture; therefore the genotype assigned to F49R was *Aa*. Non-regenerative plants were assigned the genotype *aa*. A cross between the two genotypes would yield an expected 1 : 1 ratio of regenerative to non-regenerative plants. The observed ratio was almost a perfect fit to the 3 : 5 ratio, which inferred that perhaps two complementary genes were responsible. A single-gene model with consideration of incomplete penetrance or lethality was inadequate to explain the progeny data, especially in the F_2 generation. The progeny results were more compatible with a two-gene model.

Crosses among F49R plants and NRC plants produced F_1 progeny which had a regenerative to non-regenerative ratio of 66 : 118 (Table 1). This observed ratio fit the 3 : 5 ratio which would be expected if F49R plants had the genotype *AaBb* and NRCs had the genotype *aaBb*. It should not be assumed, however, that all NRCs were required to have the same genotype. It was also possible for some NRCs to have genotype *aabb*, which would result in a 1 : 3 ratio, or *aaBB*, which would result in a 1 : 1 ratio of regenerative to non-regenerative progeny. When the data were dissected (data not shown), although the total progeny ratio from all NRCs fits the 3 : 5 ratio, two NRCs produced a better fit to the 1 : 3 ratio and one NRC produced a better fit to the 1 : 1 ratio.

F_2 generation (F_1s × F_1s)

In general, crosses among non-regenerative F_1 (NF_1) plants yielded almost no regenerative progeny except when crosses involved one particular F_1 parent. When this particular NF_1 plant was crossed to other NF_1 plants the ratio of regenerative to non-regenerative progeny was 27 : 81 (25%). Crosses between regenerative F_1s(RF_1s) and NF_1s yielded approximately 50% regenerative progeny while crosses among RF_1s yielded nearly 70% regenerative progeny.

A model of two complementary genes would explain why crosses between one particular NF_1 plant and other NF_1 plants produced a significant proportion of regenerative progeny. If the particular NF_1 plant had genotype *Aabb* and other NF_1 plants had genotype *aaBb*, then the expected ratio of regenerative to non-regenerative progeny would be 1 : 3, as was observed. Likewise, if the theoretical genotypes are used, NF_1s crossed to RF_1s would yield regenerative to non-regenerative expected ratios of 3 : 5 (a*aBb* × *AaBb* or *Aabb* × *AaBb*); 1 : 1 (*aa*– × *AaBB*), and 3 : 1 (*Aabb* × *AaBB*). Observed ratios fit theoretical ratios based on possible genotypes for the phenotypes in all crosses except when the putative *Aabb* NF_1 was crossed to putative *AaBB* RF_1s (Table 1). The observed ratio of 16 : 15 did not fit the expected ratio of 3 : 1. Crosses among RF_1s were expected to produce two different regenerative to non-regenerative ratios: 9 : 7 (*AaBb* × *AaBb*) and 3 : 1 (*AaBB* × *AaB*-). In both cases the observed results fit expected ratios (Table 1).

BC_1 generation (F_1s × NRCs)

F_1 plants were backcrossed to different NRC plants than those which comprised their parents in order to prevent inbreeding depression. Similar to the F_2 results, crosses between NF_1s and NRCs usually failed to produce regenerative progeny except when the 1 particular NF_1 plant was a parent. In such cases, the observed ratio of regenerative to non-regenerative progeny was 10 : 68. The observed ratio differed significantly from the expected ratio of 1 : 3 at the 5% significance level (Table 1). It must be noted that when this particular NF_1 plant was crossed to a particular NRC plant, no regenerative progeny were produced. For this reason, this particular NRC plant was assumed to have genotype *aabb*, and ratios involving this NRC plant were tested separately from crosses involving other NRC parents. Results of crosses between RF_1s and NRCs were consistent with genotypes of *AaBb* and *AaBB* for RF_1s and genotypes of *aaBb* and *aabb* for NRCs.

BC_1 generation (F_1s × F49R)

Regenerative to non-regenerative ratios for progeny from backcrosses of RF_1s to F49R were compatible with proposed genotypes of *AaBb* (resulting in a 9 : 7 ratio) and *AaBB* (resulting in a 3 : 1 ratio) for the RF_1 plants and genotype *AaBb* for F49R. However, results from crosses between NF_1s and F49R were difficult to explain. For three of the NF_1s parents, the observed ratio was not significantly different from 3 : 5 (data not shown) which would be expected for genotype *aaBb*. However, when the NF_1 plant earlier designated as *Aabb* was crossed to F49R, the ratio of regenerative to non-regenerative progeny was expected to be 3 : 5 but was in fact 14 : 8. Crosses between F49R and 2 other NF_1 plants also produced a higher than expected ratio of regenerative to non-regenerative progeny (28 : 7). The lack-of-fit may be attributable to inbreeding effects and may or may not involve linkage of a regeneration gene to an *S* allele.

BC_2 generation [(F_1s × F49R) × NRCs]

Results from the BC_2 generation were consistent with proposed genotypes of *AaBb* and *AaBB* for the regenerative BC_1 (RBC_1) parents and *aabb*, *aaBb* and *aa*-- for the NRC parents (Table 1). None of the RBC_1 parents produced progeny ratios consistent with the genotype *AABB*. Fifteen RBC_1 parents were evaluated in the study. The chance of having any *AABB* genotypes in 15 plants is low.

Other crosses [F49M × (F49R × NRCs)]

Crosses between F49M plants and NF_1 plants produced progeny ratios consistent with proposed genotypes of *aaBb* for F49M and *aa*-- or *Aabb* for NF_1s (Table 1). Results from crosses between F49M and RF_1 plants, however, produced lower than expected frequencies of regenerative progeny. There may have been modifying genes in F49M which prevented expression of embryogenesis. Alternatively, the shortage of regenerative progeny in the F49M × RF_1 crosses may have been due to inbreeding since F49M and F49R were nearly isogenic.

Numbers of explants per seedling

Only three petiole segment explants per seedling were subjected to the regeneration protocol and evaluated for somatic embryogenesis. This number may seem small for phenotyping regenerative ability. The number of explants evaluated per genotype in published reports varies greatly. Regeneration from one of only three petiole explants was considered to be a stringent test of regeneration in *Medicago sativa* spp. *falcata* (Groose and Li 1993). In other reports such as Koornneef et al. (1987) a *Lycopersicon* genotype was classed as regenerative if one in ten calli had a shoot bud. Examination of data from the experiment in which seedlings were evaluated twice (explants taken at 5 weeks and again at 8 weeks), however, did not justify increasing the number of explants. Evaluations were repeated on a total of 86 seedlings, and numbers of regenerating explants were recorded. Of the 86 plantlets 67 showed the same numbers of regenerating explants in both evaluations. Only four of the 86 seedlings (4.7%) were evaluated as regenerative in one evaluation and non-regenerative in the other evaluation. With respect to these four seedlings, in each case the genotypes were classified as non-regenerative in the first evaluation and regenerative in the later evaluation. Thirty-two of the plantlets failed to regenerate from any of the explants in either evaluation, while 29 of the seedlings produce regenerating cultures from all three explants in both evaluations. In other words, the response was all (6/6) or nothing (0/6) for 61 of the 86 seedlings (71%). Intermediate classes were relatively rare. The fact that less than 5% were incorrectly classified as non-regenerative in the first evaluation along with the fact that for the majority of seedlings either none or all of the explants exhibited somatic embryogenesis in both evaluations led to the conclusion that increasing the number of explants was unwarranted. More information could be obtained by evaluating only three explants from a large number of seedlings rather than evaluating more than three explants from fewer seedlings. It must be kept in mind, however, that some regenerative plantlets were misclassified as non-regenerative. The reverse case is

unlikely. Therefore, classes of regenerative progeny may be slightly under-represented.

Abnormal leaf morphology

F49R plants transplanted to soil in the greenhouse or field always exhibited at least a few leaves with abnormal morphology. A few regenerated plants had no normal leaves and were grossly abnormal, while most displayed only a few abnormal leaves at the crown. This abnormal leaf characteristic was not detected in F49M plants. It was tempting to speculate that the presence of abnormal leaves in F49R and absence of abnormal leaves in F49M plants was an epigenetic effect due to exposure to plant growth regulators during the regeneration protocol. The phenotype persisted, however, even after the plants were propagated by cuttings. A similar phenotype in white clover regenerants also persisted following vegetative propagation (Pelletier and Pelletier 1971). Abnormal leaves in $F_1 \times NRC$ progeny were observed in seedlings on hormone-free medium, suggesting that the phenotype was the result of genetic rather than epigenetic changes. Abnormal leaf morphology was also reported among red clover regenerants in two previous studies (Phillips and Collins 1980; Wang and Holl 1988).

Regenerative ability and abnormal leaf morphology were not independent in the $F_1 \times NRC$ population according to a Chi-square test (Table 2). Only 38 of the 106 regenerative $F_1 \times NRC$ progeny had no abnormal leaves as 5-to 6-week-old seedlings. Some of these plants would possibly develop the abnormal leaf trait later. On the other hand, only 4 of the 348 non-regenerative $F_1 \times NRC$ progeny expressed abnormal leaves, but it is possible that these 4 plants were misclassified as non-regenerative. The abnormal leaf phenotype appears to be a good candidate for a marker of regenerative ability in this germplasm.

Inheritance of somatic embryogenesis

This is not the first investigation in which enhanced regeneration was observed from explants of plants previously regenerated from culture. This phenomenon was observed in Brussels sprouts (*Brassica oleracea* L. var 'italica') (Robertson et al. 1988), celery (*Apium graviolens* L.) (Nadel et al. 1990) and sugarbeet (*Beta vulgaris* L.) (Saunders and Doley 1986). Regenerated plants appear to have survived a genetic or epigenetic selection in culture which imparted improved embryogenic capacity to explant tissues.

Results of this study do not agree with the report by Broda (1984) which concluded that regeneration in red clover was controlled by three recessive genes. In nearly all cases, progeny ratios could be explained by a model of two complementary genes. These results also differ from the quantitative analysis of heritability of somatic embryogenesis in red clover by Keyes et al. (1980). The authors concluded that additive variance was significant, while reciprocal and dominance variances were not significant for somatic embryogenesis. An assumption for diallel analyses is absence of epistasis (Baker 1978; Kempthorne 1956). The current study suggests that somatic embryogenesis in the genotypes under study was controlled by complementary gene action, a type of epistasis. If the same was true for the material studied by Keyes et al. (1980) then their conclusions would be invalidated. We attempted to study somatic embryogenesis as a quantitative trait, however, in most cases either all or none of the explants from individual plants regenerated . Furthermore, the frequencies of embryogenic progeny were not normally distributed, ruling out an analysis of variance test. The data appeared to be better suited to treatment as a qualitative trait. No details were provided concerning actual embryogenic frequencies in the paper by Keyes et al. (1980). It is difficult to imagine completely different mechanisms controlling somatic embryogenesis in different red cover germplasms, particularly since studies from many different species all appear to come to the conclusion of complementary gene action involving two or three loci (e.g. alfalfa, Reisch and Bingham 1980; Wan et al. 1988; Hernandez-Fernandez and Christie 1989; Kielly and Bowley 1992; tomato, Koornneef et al. 1987; sorghum, Ma et al. 1987, cucumber, Nodolska-Orcyzk and Malepszy 1989, cotton, Gawel and Robacker 1990).

On the basis of the present study we concluded that somatic embryogenesis in a red clover selection was controlled by two complementary loci. We propose that the genes for somatic embryogenesis be designated *Rn1* and *Rn2*. A regenerative genotype is therefore *Rn1-Rn2-*. The trait is highly heritable, and it is anticipated that regeneration could be easily incorporated into other red clover germplasm. The regenerative germplasm has also been used successfully in suspension culture selection and genetic transformation using *Agrobacterium tumefaciens* (unpublished).

Table 2 Contingency test of independence[a] between abnormal leaf development and regenerative ability in progeny from $F_1 \times NRC$ backcrosses

Regenerative ability	Leaf morphology		Total
	Abnormal	Normal	
Regenerative	68	38	106
Non-regenerative	4	344	348
Total	72	382	454

[a] $\chi^2 = 241.68$; $P < 0.001$

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