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A GENETIC STUDY OF SOMATIC EMBRYOGENESIS IN RED CLOVER (*TRIFOLIUM PRATENSE* L.)

by

Nancy L. McLean

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
December, 1996

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "A Genetic Study of Somatic Embryogenesis in Red Clover (Trifolium Pratense L.)"

by Nancy Lynn McLean

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Red clover genotypes capable of regenerating plantlets *in vitro* from non-meristem-derived callus are rare. A previous study identified a clone comprised of a group of plantlets regenerated from a hypocotyl-derived callus culture and another group of plants derived 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. The present study had three objectives: (1) to determine the inheritance of the regenerative trait, (2) to examine meiotic pairing configurations in regenerants, and (3) to compare isozyme profiles of regenerative and nonregenerative plants from the clone. Results from F_1, F_2 and BC populations are compatible with genetic control of regeneration by two complementary genes. Structural chromosomal abnormalities were not visible in pollen mother cells during meiosis. No differences were found between regenerative and nonregenerative plants for alcohol dehydrogenase, glutamate dehydrogenase or esterase on starch gels. Isoelectric focusing of callus cultures from regenerative and nonregenerative plants revealed that regeneration was accompanied by a reduction in staining intensity and numbers of peroxidase bands compared to nonregenerative cultures. A unique peroxidase band was associated with nonregenerative cultures.

LIST OF ABBREVIATIONS

3A9EC	3-amino-9-ethylcarbazole
BA	6-benzylaminopurine
BC	backcross
°C	degree Celsius
cv.	cultivar
2,4-D	2,4-dichlorophenoxyacetic acid
DMF	dimethylformamide
DNA	deoxyribosenucleic acid
DTT	dithiothreitol
F_1	first filial generation, in this study it generally refers to progeny from crosses between F49R and nonregenerative cv. Florex genotypes
F_2	second filial generation, in this study it generally refers to crosses among F_1's
F49M	nonregenerative individuals from clone F49, derived from epicotyl tissue on hf-L2
F49R	regenerative individuals from clone F49, derived from hypocotyl callus
GA_3	gibberellic acid
g	gram
h^2	narrow sense heritability
H_2O_2	hydrogen peroxide
hf-L2	hormone-free L2 medium
IEF	isoelectric focusing
kDa	kiloDalton
L	litre

L2	callus medium (Collins and Phillips 1982)
μL	microlitre
μmol m^{-2} s^{-1}	micromole per square metre per second
mA	milliampere
mL	millilitre
mm	millimetre
mM	millimolar
min	minute
M	molar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	normal
NAD+	nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium
NR	nonregenerative
NRC	nonregenerative cv. Florex plants
%	percent
pI	isoelectric point
PAGE	polyacrylamide gel electrophoresis
PMS	phenazine methosulfate
PVP	polyvinylpyrrolidone
P	probability of a greater statistic
R	regenerative
rpm	revolutions per minute
S_1	first selfed generation
SEL	somatic embryogenesis induction medium (Collins and Phillips 1982)

SPL	shoot development medium (Collins and Phillips 1982)
V	volt
Vh	volthour
W	watt
wk	week
yr	year

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GENERAL INTRODUCTION

Red clover (*Trifolium pratense* L.) is a productive, widely adapted forage legume which is grown either alone or in mixtures with one or more grasses and sometimes other legumes for hay, silage, pasture or green manure. The major shortfall of red clover as a crop is poor persistence. Conventional breeding has led to only limited improvement in persistence therefore biotechnological techniques have been investigated.

Regeneration of plantlets from cultured cells or tissues is a prerequisite for practical plant breeding applications of many biotechnological techniques. The ability to control differentiation *in vitro* also has made and will continue to make important contributions to basic studies in plant physiology. Some plant species can be readily induced to undergo somatic embryogenesis under a variety of conditions while others refuse to regenerate despite concerted efforts and a myriad of conditions. In most cases regeneration in a species is genotype-dependent. Percentages of regenerative genotypes within species range from very low to very high.

Numerous reports state that the capacity to undergo somatic embryogenesis is a heritable trait. In some crops highly regenerative populations have resulted from selective breeding of regenerative genotypes e.g., Bingham et al. 1975 - alfalfa; Quesenberry and Smith (1993) - red clover.

Regeneration of plants from cultured protoplasts, cells or tissues sometimes produces plants which differ in some way from the donor plants. This phenomenon was termed somaclonal variation by Larkin and Scowcroft (1981). Somaclonal variants have been found to differ from donor plants in morphology, protein profiles, isozyme patterns, DNA restriction fragment lengths, and chromosome number.

Peroxidase is the enzyme most commonly implicated in somatic embryogenesis. Levels of various isozymes of peroxidase in explants, tissue cultures and/or media have been "linked" to regenerative ability in many studies.

In 1986 a search was initiated to find red clover genotypes from productive cultivars which were capable of somatic embryogenesis from non-meristematic tissue. Details of this study are provided in a paper by MacLean and Nowak (1989). 354 seedlings were each screened for regeneration on the two-step protocol of Beach and Smith (1979) and the three-step protocol of Phillips and Collins (1979). An additional 288 seedlings were screened on a combination of media from the two recommended protocols. The epicotyl of each seedling was maintained on hormone-free medium. Plantlets were regenerated from callus tissue of three genotypes. Only one of the three regenerative cultures was reliably capable of recurrent somatic embryogenesis when petiole tissue from regenerated plantlets was subjected to regeneration protocols. This genotype was designated "F49R".

"F" refers to cv. Florex, "49" refers to the fact that it was the 49th cv. Florex seedling evaluated and "R" refers to regenerated plantlets. "F49M" designates plants derived from the epicotyl of the same seedling (mother plant) which supplied the hypocotyl tissue which callused and regenerated F49R plants. The F49M epicotyl and plants derived from it were maintained on hormone-free medium. Petiole tissue from F49M plants did not regenerate on protocols which produced prolific regeneration from F49R petiole tissue. In fact, despite numerous attempts, I was unable to repeat the process of *in vitro* regeneration from callus tissue of F49M. F49R is capable of regeneration from cell suspension as well as from callus on semi-solid medium. The difference in regenerative ability between epicotyl-derived plants (F49M) and plants regenerated from callus (F49R) indicated that somaclonal variation might be responsible for granting the observed regenerative ability. Progeny analysis showed that this trait was heritable (MacLean and Nowak 1989).

There were three objectives of the present study: (1) to determine the nature of inheritance of *in vitro* regeneration in a selected red clover genotype, (2) to determine whether regenerants were associated with any gross chromosomal abnormalities evident during homologous chromosome pairing in pollen mother cells, and (3) to compare isozyme profiles of regenerative and nonregenerative plants of the same clone. The first objective was

specifically to determine how many genes controlled regeneration and what type of gene action was operating. The second and third objectives were both aimed at determining whether somaclonal variation existed between F49M and F49R for traits other than regeneration ability and, if so, could these traits be used as genetic markers for regenerative ability. The isozyme study concentrated mainly on peroxidase. This decision was based both on initial results and on available literature.

Chapter 1

INHERITANCE OF SOMATIC EMBRYOGENESIS IN A SELECTED RED CLOVER GENOTYPE

INTRODUCTION

In Vitro Regeneration

In vitro plant regeneration results either from shoot organogenesis (caulogenesis) or from somatic embryogenesis. The type of regeneration which occurs is believed to be due to auxin and cytokinin types, concentrations and ratios (Rupert and Collins 1985). Caulogenesis results from *de novo* production of and development from a structure resembling a shoot meristem. Somatic embryogenesis is the formation of a bipolar structure similar in appearance to a zygotic embryo but produced from one or more somatic cells rather than from the union of gametes.

Regeneration (caulogenesis or somatic embryogenesis) may occur either directly from cells of the explanted tissue used to initiate a culture or from callus or suspension cells which proliferate from an explant. The red clover used in this study regenerated indirectly from callus cells on the surface of hypocotyl segments or petiole segments. Direct embryogenesis then proceeded on the surface of the earlier produced somatic embryos (unpublished).

Development of plant organs from cell suspension cultures was first reported in 1958 by two groups working

with carrot (Reinert 1958; Steward et al. 1958). Many investigations of somatic embryogenesis have been published since 1958 but most are of a descriptive nature. Several reviews of somatic embryogenesis literature are available including Nomura and Komamine (1986) and Williams and Maheswaran (1986). Although it is possible to describe what is happening during cell division and differentiation from a single somatic cell to a cotyledonary stage somatic embryo, no one has yet published a mechanism describing how or why this process occurs.

In general, surface-sterilized plant tissue is placed on a synthetic medium containing salts of macro- and micro-nutrients, a carbohydrate source (usually sucrose), an auxin, and possibly a cytokinin and vitamins. If the tissue explants are capable of regeneration under the given conditions, then shoots or somatic embryos will form following subculture to a second medium in which the auxin is absent or at a reduced concentration.

In vitro regeneration is relatively easily achieved in some plant species (e.g., carrot and tobacco) but very difficult or as yet unachieved in others. Within species with limited regenerative capacity, genotype is probably the most important factor controlling regeneration. Highly regenerative genotypes have been shown to regenerate under various media and environmental conditions while other genotypes refuse to regenerate under all test conditions.

Like all traits, *in vitro* regeneration is controlled by environment as well as genetics. The environmental factors (e.g., media components, donor plant conditions, explant source, etc.) are often capable of masking capacity for regeneration. Some researchers have also reported large genotype X environment interactions (e.g., Seitz Kris and Bingham 1988; Frankenberger et al. 1981; Wheeler et al. 1985; Kurtz and Lineberger 1983).

Explant source can have a tremendous influence on whether or not regeneration can be accomplished. Many species, including soybean, which were first labelled as recalcitrant with regard to regeneration, have undergone successful regeneration from immature embryo- or shoot tip- or cotyledonary node-derived callus (Kameya & Widholm 1981; Cheng et al. 1980; Delzer et al. 1990). These species have yet to regenerate from tissues of non-meristematic origin such as hypocotyls, cotyledons, or leaves. Unfortunately somatic embryogenesis from meristematic tissue is often not compatible with procedures for genetic transformation or protoplast fusion which require differentiation from single cells. Maturity of explants may also be important with younger tissues being more responsive than older tissues (Rines and McCoy 1981; Webb et al. 1987; Trolinder and Goodwin 1988).

Red Clover Regeneration

In vitro regeneration in red clover was first reported in 1979 by two groups working independently (Beach and Smith 1979; Phillips and Collins 1979). Phillips and Collins (1979) extensively tested various media components and developed a unique salt formulation which was also well suited to alfalfa and soybean. The protocol consisted of callus induction on L2 medium, somatic embryo induction on SEL medium and plantlet development on SPL medium. Beach and Smith (1979) successfully regenerated red clover using Gamborg's B5 salts (Gamborg et al. 1968) supplemented with various combinations and concentrations of auxins, cytokinins and vitamins. The Beach and Smith (1979) protocol consisted of a callus induction medium, a regeneration medium and a rooting medium. Both groups used seedling hypocotyls as explants for callus production. Beach and Smith (1979) additionally explanted ovaries while Phillips and Collins (1979) also explanted radicle meristems, epicotyls, cotyledons, young leaves and shoot apical meristems. As expected, frequencies of regenerative cultures were much higher in callus derived from meristematic explants (30 - 80% of genotypes) than from callus from non-meristem derived callus (about 1% of genotypes) (Phillips and Collins 1979). Very low frequencies of regeneration from nonmeristematic explants were also reported by Broda (1984) and MacLean and Nowak (1989). Similar differences in

regeneration response between types of explants were found by Webb et al. (1987) in a study of 72 *Trifolium* species. Repkova (1989) was unable to obtain somatic embryogenesis from red clover hypocotyl, stem, petiole or leaf explants and was successful only with torpedo-stage zygotic embryo explants on the EC6 medium of Maheswaran and Williams (1984). In this case, direct somatic embryogenesis occurred from the hypocotyl region of immature zygotic embryos. Wang and Holl (1988) tested a combination of the regeneration protocols of Beach and Smith (1979) and Phillips and Collins (1979) using hypocotyl segments as explants. Callus was induced on L2 medium and was subcultured to either L2-based or B5-based regeneration media. Failure to obtain any regeneration led to testing of 70 different media compositions and some regeneration was eventually obtained on L2-based media. Unfortunately, no details were provided on the 70 media tested.

In 1980, Phillips and Collins (1980) published the first report of regeneration from cell suspensions of red clover. Seedling hypocotyl and epicotyl segments placed in an L2-based liquid medium were subcultured biweekly and suspension aliquots were plated on agar-solidified L2-based medium after 6 mos to 1 yr where somatic embryos developed. Crosses among regenerated plants failed to produce seed leading the authors to propose that all fertile regenerated plants were derived from the same genotype.

There are two reports of successful regeneration of red clover plantlets from protoplasts (Myers et al. 1989; Radionenko et al. 1994). Radionenko et al. (1994) observed direct embryogenesis from protoplasts followed by secondary embryogenesis from the primary embryoids with or without callus. Both papers emphasized the necessity of using a genotype previously selected for high regenerative capacity. A third reference to regeneration via somatic embryogenesis from red clover protoplasts is given in a review paper on protoplast culture and plant regeneration by Davey and Power (1988). The authors referred to an unpublished study which found different growth regulator requirements for regeneration from different cultivars and GA_3 was required for embryo development. The particular requirements noted by Davey and Power (1988) may reflect a lower regenerative potential of their material than genotypes used in other studies.

McGee et al. (1989) developed a modification of the Phillips and Collins (1979) three-step protocol whereby petiole segments from red clover plantlets grown *in vitro* on SPL medium were placed on a L2-based medium (L2A) and somatic embryos formed directly from explant cells with very little callus production. This protocol required only one culture medium and somatic embryos were produced in less than 1 mo versus 2-3 mos in the three-step protocol. Efforts

to reproduce this protocol in our laboratory using highly regenerative genotypes have failed (unpublished).

Inheritance of regeneration studies in red clover have produced contradictory results. Five reports are summarized below.

Keyes et al. (1980) conducted a quantitative genetic study of embryogenic response in red clover. Twenty-four randomly selected genotypes from cv. Arlington, were divided into three sets of eight and crossed in 4X4 design II matings. All genotypes were evaluated on two media protocols: one proposed by Phillips & Collins (1979) and the other proposed by Beach & Smith (1979). Hypocotyl segments were used as explants. An analysis of variance determined highly significant ($P<0.01$) additive variance on one protocol and significant additive variance ($P<0.05$) on the other protocol. Dominance and reciprocal effects were not significant on either protocol. From the estimated error mean squares the narrow sense heritabilities were calculated as $h^2=0.54$ on one protocol and $h^2=0.25$ on the other protocol. Both were statistically significant.

Broda (1984) approached regeneration in red clover as a qualitative trait and concluded that regeneration occurred as the result of three recessive genes. If regeneration in red clover depended on homozygous recessive genes at each of three loci then crosses among regenerative plants should yield nearly 100% regenerative progeny. Broda (1984) did not

state whether or not he made crosses among regenerative genotypes. Quesenberry and Smith (1993), however, conducted recurrent selection for *in vitro* regeneration from petiole explants using the Beach and Smith (1979) protocol. They did not find 100% regenerative progeny from crossing regenerative genotypes. Five selection cycles were required to increase the regeneration frequency from 4% to 72%. The narrow sense heritability, calculated from half-sib family data, was 40 to 50%. The authors concluded that the trait was highly heritable and they stated that the data suggested *in vitro* regeneration was controlled by relatively few genes.

MacLean and Nowak (1989) found that crosses between a highly regenerative genotype and non-regenerative plants yielded 29% regenerative progeny suggesting that the trait was dominant and highly heritable. Similarly, Myers et al. (1989) stated that ability to regenerate from red clover hypocotyl callus appeared to be controlled by one or more dominant factors based on the observation that one parent passed this trait on to many of its F_1 progeny.

There appears to be a difference in the highly regenerative genotypes reported by MacLean and Nowak (1989) and Myers et al. (1989) and the genotypes selected by Quesenberry and Smith (1993) since the F_1 population in the study by MacLean and Nowak (1989) was comprised of a higher frequency of regenerative genotypes than three cycles of

recurrent selection in the Quesenberry and Smith (1993) study. Results from MacLean and Nowak (1989) also conflict with Broda's model of three recessive genes since no regenerative genotypes would be expected in the F_1.

It is unfortunate that Keyes et al. (1980) did not include any actual embryogenesis data in their paper. Based on work by Phillips & Collins (1979), which was conducted in the same institution, the frequency of embryogenesis from nonmeristematic explants in red clover is only about 1%. Since Keyes et al. (1980) state that the diallel parents were random samples, it is difficult to imagine that the authors obtained sufficient embryogenesis data for quantitative analysis.

Perhaps the most valuable contribution of the Keyes et al. (1980) publication is the finding that genotype has a greater effect on somatic embryogenesis than does media protocol. MacLean and Nowak (1989) also found that a highly regenerative genotype responded to both the B5- and L2-based protocols. Rupert and Collins (1985) reported that there appears to be plasticity within regenerative genotypes of red clover for response to various growth regulator combinations. Similarly, Bhojwani et al. (1984) proposed that white clover (*Trifolium repens* L.) genotypes with high regenerative capacity should also be capable of regeneration on a wide range of media.

Stability of regeneration response over different environments may be related to the magnitude of the response. Chen et al. (1987) screened 50 genotypes of each of three cvs of alfalfa on three media protocols. The study found that highly regenerative genotypes regenerated on all three of the protocols and from both seedling cotyledons and leaf strips from 2-mos-old plants. Genotypes producing fewer embryos per explant often regenerated on only one or two protocols and from cotyledons and not leaves or vice versa. Similarly, Matheson et al. (1990) found that alfalfa genotypes with high or moderate regeneration could regenerate on two different protocols while other genotypes were media specific.

Inheritance of Regeneration in Other Species

Inheritance of regeneration has been investigated using both quantitative and qualitative genetics. Most of the quantitative studies deal with species in which regeneration is confined to regeneration from immature embryos or meristems whereas qualitative inheritance studies deal with embryogenesis from nonmeristematic explants. There are suggestions that these are two different traits and in all likelihood the former is a quantitatively controlled trait and the latter is qualitatively controlled by only a few genes. Since the present study deals with regeneration from nonmeristematic tissue and since the data appeared unfit for

quantitative analysis, the literature review is limited to qualitative studies.

The species in which inheritance of regenerative ability has been studied most intensively is alfalfa (*Medicago sativa* L.). Unlike red clover, all alfalfa researchers came to a similar conclusion: namely that embryogenesis in alfalfa is controlled by two complementary genes. In other words, dominance is required at two independent gene loci in order for embryogenesis to be expressed. This conclusion was first reached by Reisch and Bingham (1980) who looked at segregation ratios in F_1, F_2 and backcross generations of diploid alfalfa. Unlike later papers, they suggested that a low level of embryogenesis was obtained from genotypes with only one dominant gene, however regeneration from at least 75% of explants required dominance at two loci. The genetic model was not rejected in all tested populations except where inbreeding was a factor. Wan et al. (1988) studied tetraploid alfalfa using 10 F_1 and three S_1 populations derived from four regenerative and three nonregenerative genotypes. They also concluded that regeneration was dependent on dominant genes at each of two loci. They found no regeneration when the proposed genotypes contained dominance at only one locus. Wan et al. (1988) did not observe deviation from expected ratios in selfed populations. Differences between the findings of Reisch and Bingham (1980) and Wan et al. (1988) may be partially

attributable to the fact that the first paper used diploid germplasm while the second paper used tetraploid plants. Hernandez-Fernandez and Christie (1989) produced F_1 and S_1 generations from one regenerative and two nonregenerative tetraploid alfalfa plants. They proposed that the minimum gene requirement for regeneration from their plant material was *AaaaBBbb* or *AAAaBbbb*. Kielly and Bowley (1992) similarly found that regeneration in tetraploid alfalfa was controlled by two complementary genes. They, however, proposed that only one dominant allele for each gene (i.e., *AaaaBbbb*) was required for regeneration.

Crea et al. (1995) produced crosses between two embryogenic and five nonembryogenic tetraploid alfalfa plants. They tested their germplasm using the gene models proposed by Wan et al. (1988) and Hernandez-Fernandez and Christie (1989). Crea et al. (1995) agreed with the earlier reports that regeneration was dependent on dominance at two loci, however, it is difficult to understand how the authors came to that conclusion based on their results. First they assumed that the two regenerative parents had "low" regeneration even though they stated that 90% of calli regenerated. Second, crosses between regenerative F_1 and S_1 plants produced only 2% regeneration, which is incompatible with the proposed models.

Koornneef et al. (1987) found that regeneration from leaf disc callus introduced into *Lycopersicon esculentum*

from *L. peruvianum* was also controlled by two dominant genes. Similarly, regeneration from cucumber (*Cucumis sativus* L.) leaf explants was reported to be dependent on dominant alleles at two loci (Nadolska-Orczyk and Malepszy 1989). The cucumber study proposed that dominance at a third locus determined whether genotypes regenerated "frequently" rather than "intermediately". Dominant control of embryogenesis involving at least two gene loci was also suggested for sorghum (*Sorghum bicolor* (L.) Moench) (Ma et al. 1987) and cotton (*Gossypium hirsutum* L.) (Gawel and Robacker 1990).

MATERIALS AND METHODS

Red clover is a diploid, cross-pollinating species which is normally unable to produce seed through selfing due to gametophytic self-incompatibility (Taylor and Smith 1979). It was therefore necessary to cross F49R and F49M plants to other red clover genotypes in order to obtain progeny for an inheritance study. Table 1.1 includes descriptions of the plants used to produce F_1 populations.

Crosses were performed by hand. Pollen was collected on a small square of fine sandpaper glued to the end of a wooden toothpick. The sandpaper was then lightly brushed over the stigma of the recipient female parent. All crosses were made in reciprocal. Seedheads were collected

approximately 6 wk later. Seeds were extracted by hand from dry seed heads.

Table 1.1 Descriptions of plants used to produce F_1 populations

Symbol	Description
F49M	epicotyl-derived plants originating from the same cv. Florex seedling (F49) which produced a highly regenerative hypocotyl callus culture
F49R	plants regenerated from F49 hypocotyl callus
NRC's	non-regenerative cv. Florex plants

Reciprocal crosses between F49R and F49M plants failed to produce seed.

The progeny populations which were produced and evaluated are summarized in Table 1.2.

Table 1.2. Summary of crosses made and progeny evaluated in inheritance of somatic embryogenesis study

Generation	Description	# Progeny
	Crosses involving F49R	
F_1	F49R X NRC's	184
	(5 RF_1 and 6 NR F_1)	
F_2	RF_1 X RF_1	141
F_2	RF_1 X NRF_1	224
F_2	NRF_1 X NRF_1	136
	Backcrosses	
BC_1	F_1 X NRC's	403
BC_1	F_1 X F49R	266
	22 Regen BC's X NRC's	589
	Crosses involving F49M	
F_1 X F49M		105

Seeds from all crosses were evaluated for regeneration according to the following protocol. Seeds from each cross were scarified lightly with sandpaper and placed in small plastic Omnisette® (Fisher Scientific, P.O. Box 9200 Terminal, Ottawa, Ontario, Canada, K1G 4A9) cassettes. They were then surface sterilized in the cassettes by a rinse in 2% Liquinox detergent; followed by a 30 min wash in running tap water; a brief rinse in 70% ethanol; a 15 min treatment with 33% Javex solution (final concentration of 2% sodium hypochlorite); and three rinses with autoclaved distilled water. Surface-sterilized seeds were plated on hormone-free, sucrose-free B5 medium solidified with 7 gL^{-1} agar in sterile

disposable 96 cell well plates (Microtest III® tissue culture plate, Becton Dickinson Labware, Lincoln Park, New Jersey), one seed per well. Each well contained three drops of media from a 9-inch long Pasteur pipette. Seedlings were subcultured approximately 1 wk later to 25 X 150 mm culture tubes containing 10 mL hormone-free L2 medium. After 4 wk in the culture tubes, three 8-mm-long petiole segments were explanted from each seedling. The segments were placed in individual wells on L2 medium in sterile disposable 24 cell well plates (Corning Glass Works, Corning, New York) containing 2 mL medium per well. Four-wk-old callus tissue was subcultured to SEL medium in 24 cell well plates. After 4 wk on SEL, the tissue was subcultured to SPL medium in 24 cell well plates. Regeneration was evaluated after 4 wk on SPL medium.

Tissue culture researchers appear reluctant to discuss contamination by fungi and bacteria. Exceptions include Ray and Bingham (1989) and Shahin (1985). Experience in our laboratory has shown that slow-growing systemic bacteria make detection of regeneration difficult in some cultures. Suspect cultures were discarded and no data were recorded from them.

Ratios of regenerative to non-regenerative progeny were tested by Chi-square goodness-of-fit tests (Gomez and Gomez 1984).

Media components are given in Table 1.3.

Table 1.3. Tissue culture media components

Component	L2[z] (mgL^{-1})	SEL[z] (mgL^{-1})	SPL[z] (mgL^{-1})	B5[y] (mgL^{-1})
NH_4NO_3	1000	1000	1000	-
KNO_3	2100	2100	2100	2500
$CaCl_2 \cdot 2H_2O$	600	600	600	150
$MgSO_4 \cdot 7H_2O$	435	435	435	250
KH_2PO_4	325	325	325	-
$(NH_4)_2SO_4$	-	-	-	134
$NaH_2PO_4 \cdot H_2O$	85	85	85	150
KI	1	1	1	0.75
H_3BO_3	5	5	5	3
$MnSO_4 \cdot H_2O$	15	15	15	10
$ZnSO_4 \cdot 7H_2O$	5	5	5	2
$Na_2MoO_4 \cdot 2H_2O$	0.4	0.4	0.4	0.25
$CuSO_4 \cdot 5H_2O$	0.1	0.1	0.1	0.025
$CoCl_2 \cdot 6H_2O$	0.1	0.1	0.1	0.025
NaFeEDTA	25	25	25	43
inositol	250	250	250	100
nicotinic acid	-	-	-	1
pyridoxine ·HCl	0.5	0.5	0.5	1
thiamine·HCl	2	2	2	10
adenine	-	2	-	-
BA	0.1	-	-	-
2,4-D	-	0.01	-	-
picloram	0.06	-	0.002	-
sucrose	25000	25000	25000	20000[x]
pH	5.8	5.8	5.8	5.5

[z] Collins and Phillips (1982)
[y] Gamborg and Shyluk (1978)
[x] Sucrose was omitted from the seed germination medium

RESULTS

Examples of regenerative and nonregenerative genotypes at the end of each 4 wk passage of the 3-step regeneration protocol are presented in Fig. 1.1. Fig. 1.2 shows red clover somatic embryos at various stages of development.

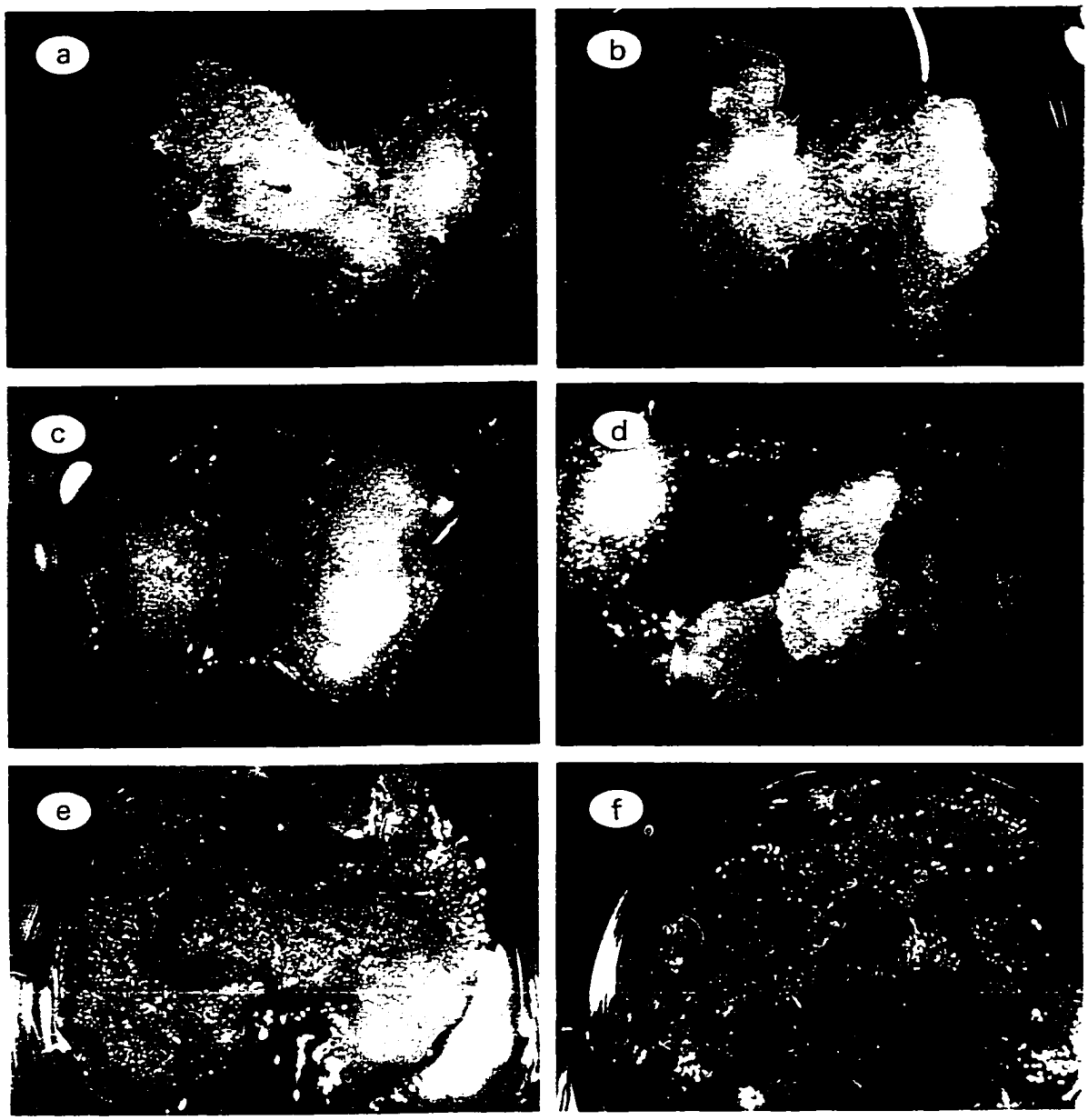

Fig. 1.1. Petiole callus at the end of each four wk passage in the three-step regeneration protocol: (a), (c), (e) nonregenerative genotypes on L2, SEL, and SPL media, respectively; (b), (d), (f) regenerative genotypes on L2, SEL, and SPL media, respectively.

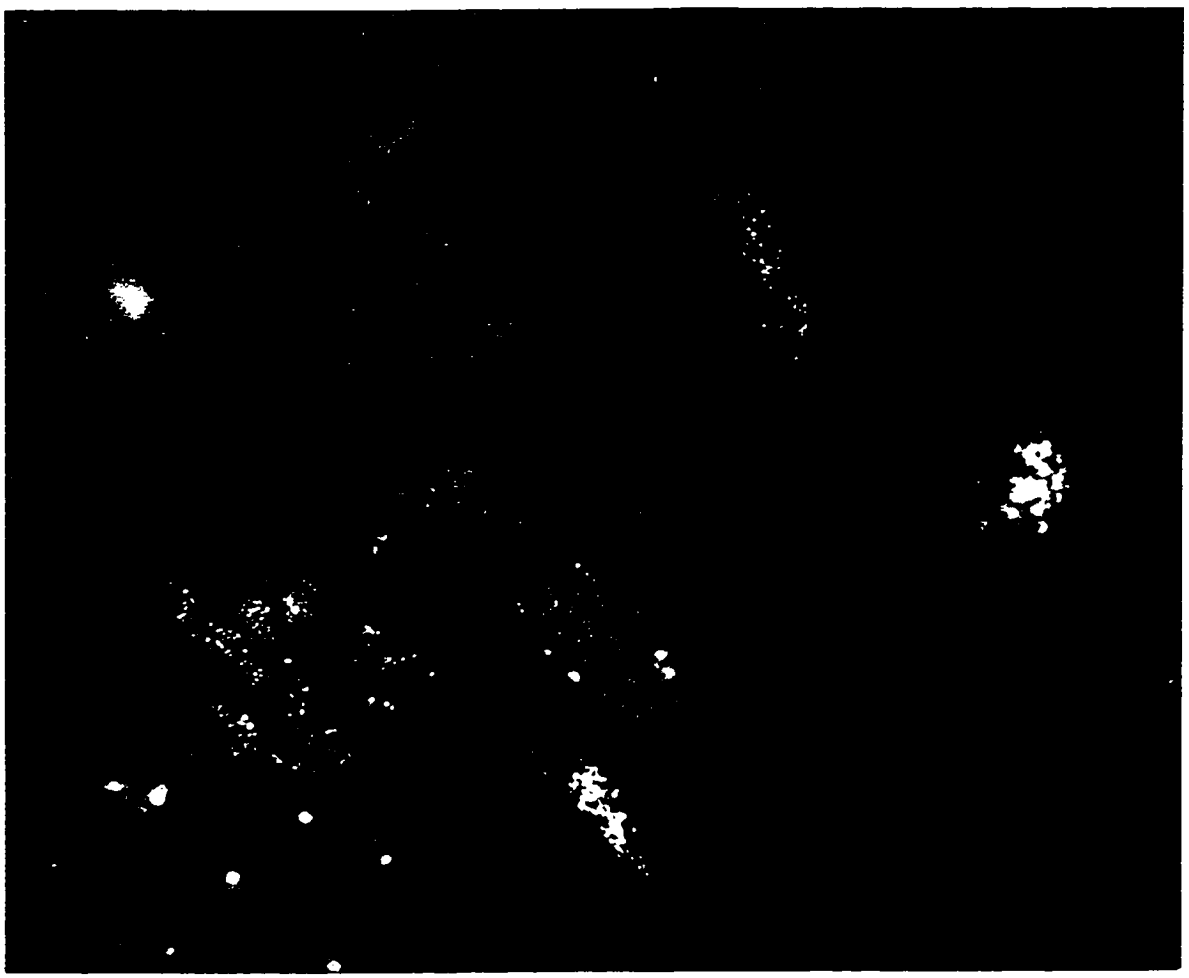

Fig. 1.2. Red clover somatic embryos.

F_1 generation

Results from reciprocal crosses between F49R plants and a number of nonregenerative control plants are presented in Table 1.4. The proportion of regenerative F_1's was somewhat higher when F49R was used as the female parent. However, regenerative offspring were obtained from crosses in which F49R was the pollen parent as well as crosses in which it was the ovule parent. In fact, the contingency test for reciprocal crosses between F49R and NRC's (Table 1.4) showed independence between the regeneration trait and whether F49R was the pollen or ovule parent. It was therefore decided to combine data over reciprocals in the F_1 generation and all subsequent generations.

Table 1.4. Contingency test for independence between regenerative ability and crossing direction of parents for F_1 progeny from reciprocal crosses between F49R and nonregenerative control (NRC) plants

F49R contribution	R	NR	Total
pollen	26	52	78
ovule	40	66	106
Total	66	118	184

H_0: Crossing direction of parents and regenerative ability of progeny are independent. $\chi^2 = 0.379$; $0.70>P>0.50$; therefore H_0 is not rejected.

A number of different F49R plants and different NRC plants were used to produce the F_1 population which was evaluated for regeneration. Chi-square tests of homogeneity were performed to determine whether data could be combined

over F49R plants and/or over NRC plants. Table 1.5 shows homogeneity among F49R plants when crossed to various NRC's. Likewise, Table 1.6 shows homogeneity among the various NRC parents when crossed to different F49R plants. Chi-square's were calculated for individual plant ratios and for the totals. The difference between the sum of the individual χ^2's and the χ^2 based on totals is the homogeneity χ^2 (Gomez and Gomez 1984). Since calculated χ^2's were less than tabular χ^2's at $\alpha = 0.05$, it was therefore acceptable to combine data over different F49R parents as well as over the different NRC parents. Total plant numbers in the homogeneity tables are different from each other and from the reciprocal test table because crosses were only included in the homogeneity tables when progeny per half-sib family totalled at least 12.

Table 1.5. Observed ratios of regenerative to nonregenerative progeny from crosses between F49R and nonregenerative control (NRC) plants totalled over NRC plants

F49R	Total	Observed		Expected (3:5)		χ^2 value
		R	NR	R	NR	
F49-1	42	13	29	15.75	26.25	0.768
F49-2	23	10	13	8.63	14.37	0.351
F49-4	19	7	12	7.13	11.87	0.004
F49-5	18	8	10	6.75	11.25	0.370
F49-6	17	6	11	6.37	10.63	0.035
F49-13	14	6	8	5.25	8.75	0.171
Total	133	50	83	49.87	83.13	0.003
Sum						1.702

Homogeneity χ^2 = 1.702 - 0.003 = 1.699 with 5 degrees of freedom. $0.90>P>0.75$.

Table 1.6. Observed ratios of regenerative to nonregenerative progeny from crosses between F49R and nonregenerative control (NRC) plants totalled over F49R plants

NRC	Total	Observed		Expected (3:5)		χ^2 value
		R	NR	R	NR	
F2	24	9	15	9	15	0.00
F7	17	8	9	6.37	10.63	0.68
F86	17	7	10	6.37	10.63	0.10
Fs7	41	12	29	15.37	25.63	1.19
Fs8	16	6	10	6	10	0.00
Fs11	13	2	11	4.87	8.13	2.71
Total	148	56	92	55.5	92.5	0.01
Sum						4.69

Homogeneity χ^2 = 4.69-0.007 = 4.68 with 5 degrees of freedom. $0.50>P>0.30$

Table 1.7 is a Punnett square showing F_1 genotypes which would result from crossing F49R and NRC's assuming that F49R had the genotype *AaBb* and that nonregenerative control plants were homozygous recessive for the first regeneration gene and were, on average, heterozygous for the second gene (i.e.*aaBb*). The ratio of regenerative to nonregenerative F_1 progeny from this cross would be 3:5.

Table 1.7. Punnett square showing the possible F_1 genotypes if F49R = *AaBb* and nonregenerative controls (NRC's) = *aaBb*.

Gametes F49R / NRC	*AB*	*Ab*	*aB*	*ab*
aB	*AaBB* (R)	*AaBb* (R)	*aaBB* (NR)	*aaBb* (NR)
ab	*AaBb* (R)	*Aabb* (NR)	*aaBb* (NR)	*aabb* (NR)

The goodness-of-fit χ^2's in Tables 1.5 and 1.6 show that the null hypotheses that the data fit a 3:5 ratio are not rejected. A 3:5 ratio requires F49R to have the genotype *AaBb* while NRC's have the genotype *aaBb*. It should not be assumed, however, that all NRC's are required to have the genotype of *aaBb*. While this possiblity exists it is also impossible to rule out other possible genotypes for some of the NRC plants. Alternate genotypes for NRC's and resulting phenotypic ratios are presented in Table 1.8. Table 1.9 shows individual χ^2 values for goodness-of-fit to 1:3 and

1:1 as well as to 3:5. The theoretical ratios are based on crosses in which F49R has the genotype *AaBb* and for the ratio 1:3 NRC = *aabb*; for the ratio 3:5 NRC = *aaBb*; and for the ratio 1:1, NRC = *aaBB*. Progeny ratios from all NRC's fit the 3:5 ratio but a few have a better fit to the 1:3 ratio (Fs7 and Fs11) and one has a better fit to the 1:1 ratio (F7).

Table 1.8. Theoretical ratios of regenerative to nonregenerative F_1 progeny from crosses between the proposed genotypes of F49R and nonregenerative control (NRC) plants.

Proposed NRC genotypes	Proposed F49R genotype	Expected ratios of F_1 progeny (R:NR)
aabb	*AaBb*	1 : 3
aaBb	*AaBb*	3 : 5
aaBB	*AaBb*	1 : 1

Table 1.9. Observed ratios of regenerative to nonregenerative F_1 progeny from crosses between F49R and nonregenerative control (NRC) plants.

NRC	R	NR	χ^2		
			1:3	3:5	1:1
F2	9	15	14.00**	0.00NS	1.50NS
F7	8	9	4.41*	0.68NS	0.06NS
F86	7	10	2.37NS	0.10NS	0.53NS
Fs7	12	29	0.40NS	1.19NS	7.05**
Fs8	6	10	1.33NS	0.00NS	1.00NS
Fs11	2	11	0.64NS	2.71NS	6.23*

*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

When all progeny are considered, including crosses in which less than 12 offspring were evaluated, the overall ratio fits the 3:5 ratio of regenerative to nonregenerative offspring (Table 1.10).

Table 1.10. Goodness-of-fit test for combined F_1 data assuming F49R = *AaBb* and nonregenerative controls = *aaBb*

	Observed	Expected	χ^2	$P>\chi^2$
R	66	69	0.130	
NR	118	115	0.078	
Total	184	184	0.216	$0.7>P>0.5$

F_2's (F_1's X F_1's)

Theoretical ratios of regenerative to nonregenerative F_2 progeny based on F_1 genotypes from the Punnett square in Table 1.7 are presented in Table 1.11. Observed ratios of regenerative to nonregenerative F_2 progeny are given in Table 1.12. Blanks in Table 1.12 indicate repeated failure to obtain seeds from some crosses (at least five crosses in both directions). In general, crosses among nonregenerative F_1 plants yielded practically no regenerative progeny except when one of the nonregenerative F_1 parents was plant "I". Progeny resulting from crosses between nonregenerative F_1 plant "I" and other nonregenerative F_1's, however, included about 21% (18/86) regenerative phenotypes. Crosses between regenerative and nonregenerative F_1's yielded slightly higher than 50% regenerative offspring (125/226). Crosses

among regenerative F_1's yielded almost 70% regenerative offspring (98/141).

Table 1.11. Theoretical ratios of regenerative to nonregenerative F_2 progeny based on proposed genotypes for crosses among nonregenerative F_1's, between nonregenerative and regenerative F_1's and among regenerative F_1's

Proposed genotypes	NR F_1's				R F_1's	
	aabb	*aaBb*	*aaBB*	*Aabb*	*AaBb*	*AaBB*
NR F_1's						
aabb	0:n					
aaBb	0:n	0:n				
aaBB	0:n	0:n	0:n			
Aabb	0:n	1:3	1:1	0:n		
R F_1's						
AaBb	1:3	3:5	1:1	3:5	9:7	
AaBB	1:1	1:1	1:1	3:1	3:1	3:1

Table 1.12. Summary of observed ratios of regenerative to nonregenerative F_2 progeny

F_1	NR F_1's					
	A	B	E	G	H	I
	NR F_1's X NR F_1's					
B	0:16					
E	0:11	0:6				
G	0:19	1:16	0:1			
H	0:5	1:9	0:1	0:7		
I	13:31	2:10	4:23	8:17	---	
	R F_1's X NR F_1's					
C	---	12:19	4:3	11:2	1:5	13:13
D	3:7	4:4	2:6	4:8	1:1	9:0
F	1:10	3:10	0:5	1:5	4:4	4:5
J	0:1	3:2	---	---	---	3:1
K	1:1	3:1	1:3	4:3	6:7	13:14

Table 1.12 continued.

F_1	R F_1's			
	C	D	F	J
	R F_1's X R F_1's			
D	1:4			
F	11:2	7:4		
J	4:2	7:3	10:2	
K	16:9	12:6	16:5	14:6

Observed ratios of regenerative to nonregenerative F_2 progeny were tested for goodness-of-fit to theoretical ratios based on genotypes and phenotypes from Table 1.7.

Chi-square values are given in Table 1.13 for crosses from which at least 12 F_2 progeny were evaluated.

Table 1.13 Chi-square goodness-of-fit tests for F_2 data with a minimum of 12 progeny per cross

Parents	Progeny ratio		χ^2			
	R	NR				
NR F_1 X NR F_1			*0 : n*	*1 : 3*	*1 : 1*	
A X B	0	16	---	5.33 *	16.00 **	
A X G	0	19	---	6.33 *	19.00 **	
A X I	13	31	---	0.48 NS	7.36 **	
B X G	1	16	---	3.31 NS	13.24 **	
B X I	2	10	---	0.44 NS	5.33 *	
E X I	4	23	---	1.49 NS	13.37 **	
G X I	8	17	---	0.65 NS	3.24 NS	
NR F_1 X R F_1			*1 : 3*	*3 : 5*	*1 : 1*	*3 : 1*
B X C	12	19	3.11 NS	0.02 NS	1.58 NS	21.77 **
B X F	3	10	0.26 NS	1.15 NS	3.77 NS	18.69 **
C X G	11	2	24.64 **	12.31 **	6.23 *	0.64 NS
D X G	4	8	0.44 NS	0.09 NS	1.33 NS	11.11 **
H X K	6	7	3.10 NS	0.42 NS	0.08 NS	5.77 *
C X I	13	13	8.67 **	1.73 NS	0.00 NS	8.67 **
I X K	13	14	7.72 **	1.31 NS	0.04 NS	10.38 **

Table 1.13 cont'd

	R F_1	*X R* F_1	*9 : 7*	*3 : 1*
C X F	11	2	4.25 *	0.64 NS
C X K	16	9	0.61 NS	1.61 NS
D X K	12	6	0.79 NS	0.67 NS
F X J	10	2	3.58 NS	0.44 NS
F X K	16	5	3.39 NS	0.02 NS
J X K	14	6	1.54 NS	0.27 NS

*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

A two complementary gene model can explain why crosses between some nonregenerative F_1 plants produced a proportion of regenerative progeny. For instance crosses between the nonregenerative F_1 plant "I" and other nonregenerative F_1 plants produced 20.9% regenerative progeny. If the genotype *Aabb* is assigned to "I" and the genotype *aaBb* is assigned to other nonregenerative F_1 plants, the expected ratio of regenerative to nonregenerative progeny is 1:3. This ratio is compatible with the observed 20.9% regenerative progeny ($\chi^2 = 0.76$, $0.5>P>0.3$). Likewise, using the theoretical Punnett square genotypes, regenerative F_1's crossed to nonregenerative F_1's would yield regenerative to nonregenerative ratios ranging from 3:1 (*AaBB* X *Aabb*) to 1:1 (*AaBB* X *aaB-* or *aabb*) to 3:5 (*AaBb* X *Aabb* or *aaB-*) to 1:3 (*AaBb* X *aabb*). The observed ratios fall within this wide

range with an average of 55% regenerative offspring (126/226). Regenerative by regenerative F_1's from the Punnett square would produce two different regenerative to nonregenerative ratios: 3:1 (*AaBB* X *AaBB* or *AaBb*) or 9:7 (*AaBb* X *AaBb*). The combined data from crosses between regenerative F_1's appear closer to the 3:1 ratio (70% regenerative offspring 98/141).

Backcrosses to nonregenerative controls (F_1's X NRC's)

Theoretical ratios of regenerative to nonregenerative backcross progeny based on proposed NRC and F_1 genotypes are presented in Table 1.14. Observed ratios of regenerative to nonregenerative progeny resulting from these backcrosses to NRC's are given in Table 1.15. There are some similarities between the backcross data and the F_2 data. For instance, crosses between nonregenerative F_1's and NRC's usually failed to produce regenerative progeny except when the nonregenerative F_1 parent was "I"; just as when nonregenerative F_1's were intermated. It should be noted that one NRC (plant Fs7) failed to produce regenerative progeny when crossed with plant "I". For this reason data from crosses with Fs7 were excluded from the totals. Crosses between nonregenerative F_1 plant "I" and NRC's excluding Fs7 yielded 13% regenerative progeny (10/78). Data from crosses between regenerative F_1's and NRC's yielded 43% regenerative

progeny when Fs7 was excluded. Crosses between regenerative F_1's and Fs7, meanwhile yielded 31% regenerative progeny.

Table 1.14. Theoretical ratios of regenerative to nonregenerative progeny from backcrosses of F_1's to NRC's based on proposed genotypes

Proposed F_1 genotypes	Proposed NRC genotypes		
	aabb	*aaBb*	*aaBB*
NR F_1's			
aabb	0:n	0:n	0:n
aaBb	0:n	0:n	0:n
aaBB	0:n	0:n	0:n
Aabb	0:n	1:3	1:1
R F_1's			
AaBb	1:3	3:5	1:1
AaBB	1:1	1:1	1:1

Table 1.15. Observed ratios of regenerative to nonregenerative progeny from backcrosses of F_1's to NRC's

	F2	F7	F86	FS8	FS11	Total	Fs7
NR F_1's							
A	0:1	0:8				0:9	0:17
B		0:9		0:23	0:4	0:36	0:13
G		0:3			0:9	0:12	
H		1:20	0:12			1:32	0:2
I	2:10	5:17	2:38		1:3	10:68	0:35
R F_1's							
C		4:2	8:12	5:19	19:19	36:52	9:26
D	6:7		7:15		15:11	28:33	3:11
F		2:2			1:4	3:6	1:6
J	2:1	7:4	1:2	1:2	1:0	12:9	12:13
K		2:6	0:1		5:6	7:13	2:3

Observed ratios of regenerative to nonregenerative F_1 X NRC progeny were tested for goodness-of-fit to theoretical ratios from Table 1.14. Chi-square values are given in Table 1.16.

Table 1.16. Chi-square values from goodness-of-fit tests of ratios of regenerative to nonregenerative progeny from backcrosses of F_1's to NRC's

F_1	Totals over NRC's except Fs7	χ^2			Crosses to Fs7	χ^2	
NR F_1's							
		0:n	*1:3*	*1:1*		*0:n*	
A	0:9	---	---	---	0:17	---	
B	0:36	---	12.00 **	36.00 **	0:13	---	
G	0:12	---	4.00 *	12.00 **	---	---	
H	1:32	---	8.49 **	29.12 **	0:2	---	
I	10:68	---	6.17 *	43.13 **	0:35	---	
R F_1's							
		1:3	*3:5*	*1:1*		*1:3*	*1:1*
C	36:52	11.88 **	0.44 NS	2.91 NS	9:26	0.01 NS	8.26 **
D	28:33	14.21 **	1.84 NS	0.41 NS	3:11	0.10 NS	4.57 *
F	3:6	---	---	---	1:6	---	---
J	12:9	11.57 **	3.46 NS	0.43 NS	12:13	7.05 **	0.04 NS
K	7:13	1.07 NS	0.05 NS	1.80 NS	2:3	---	---

*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

By comparing Table 1.14 and Table 1.16 it is possible to assign genotypes to some of the F_1 and NRC parents. Specifically, among the nonregenerative F_1's only one plant ("I") produced any regenerative progeny when crossed to NRC's. According to Table 1.14, the only possible genotype for this plant is *Aabb*. Only one NRC (Fs7) failed to produce regenerative progeny when crossed to plant "I". From Table 1.14, Fs7 would have the genotype *aabb*. All other NRC's produced regenerative offspring in crosses with "I". The ratios of regenerative to nonregenerative offspring were closer to 1:3 than to 1:1 suggesting that they had the genotype *aaBb*. A χ^2 goodness-of-fit test was rejected at the 5% level but not at the 1% level for the 1:3 ratio and was rejected for the 1:1 ratio. Among the regenerative F_1's, crosses to Fs7 indicated that plants "C" and "D" had the genotype *AaBb*, and plant "J" appeared to have the genotype *AaBB*. Crosses between regenerative F_1's and the other NRC's are in keeping with these genotypes. Insufficient data were available to assign genotypes to F_1 plants "F" and "K".

Backcrosses to F49R (F_1's X F49R)

Table 1.17 presents theoretical ratios of regenerative to nonregenerative progeny from backcrosses of F_1's to F49R based on genotypes presented in Table 1.7.

Table 1.17. Theoretical ratios of regenerative to nonregenerative BC progeny from backcrosses of proposed F_1 genotypes to the proposed F49R genotype

Theoretical F_1 genotypes	Proposed F49R genotype	Expected ratios of R:NR progeny from crosses between F_1's and F49R
NR F_1's		
aabb	AaBb	1 : 3
aaBb	"	3 : 5
aaBB	"	1 : 1
Aabb	"	3 : 5
R F_1's		
AaBb	"	9 : 7
AaBB	"	3 : 1

Observed ratios and χ^2 goodness-of-fit tests to the theoretical ratios are presented in Table 1.18. Regenerative to nonregenerative ratios for progeny from backcrosses of regenerative F_1's to F49R are compatible with proposed genotypes of *AaBb* and *AaBB* for the regenerative F_1's, just as was seen in the backcrosses to NRC's. Backcrosses of nonregenerative plants "A", "G", and "H" produced progeny ratios compatible with the proposed genotypes *aaBb* or *aaBB*. However, results from backcrosses of nonregenerative F_1 plants "B", "E" and "I" are difficult to explain. Backcrosses of "B" and "E" to F49R produced ratios of 3:1; much higher than expected for nonregenerative F_1's. A backcross of nonregenerative F_1 plant "I" to F49R also produced a higher than expected ratio of regenerative to

nonregenerative progeny. The expected ratio of 3:5, assuming that "I" had the genotype *Aabb*, was rejected at the 5% level but not at the 1% level. Observed ratios from "B", "E" and "I" backcrossed to F49R are not compatible with genotypes proposed for these plants based on F_2 and BC to NRC results.

Table 1.18. Observed ratios of regenerative to nonregenerative BC progeny from backcrosses of F_1's to F49R and χ^2's from goodness-of-fit tests to theoretical ratios

F_1	R Prog	N Prog	χ^2			
NR F_1's			*1:3*	*3:5*	*1:1*	*3:1*
A	9	8	7.08 **	1.73 NS	0.06 NS	4.41 *
B	14	4	26.74 **	12.46 **	5.56 *	0.07 NS
E	14	3	29.82 **	14.59 **	7.12 **	0.49 NS
G	9	14	2.45 NS	0.03 NS	1.09 NS	15.78 **
H	15	15	10.00 **	2.00 NS	0.00 NS	10.00 NS
I	14	8	17.52 **	6.41 *	1.64 NS	1.52 NS
R F_1's			*9:7*	*3:1*		
C	25	16	0.37 NS	4.30 *		
D	23	16	0.12 NS	5.34 *		
J	20	4	7.15 **	0.89 NS		
K	24	11	2.16 NS	0.77 NS		

$\chi^2_{1\ df\ \alpha=0.05} = 3.841$

*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

Regenerative BC's (F_1's X F49R) X NRC's

There are four possible genotypes for regenerative progeny from backcrosses between F_1's and F49R. These genotypes as well as the three proposed genotypes for nonregenerative control plants and theoretical ratios from crossing these two groups are presented in Table 1.19.

Table 1.19. Theoretical ratios of regenerative to nonregenerative progeny from crosses between regenerative backcrosses and NRC's [(F_1 X F49R) X NRC] based on proposed genotypes

Proposed regenerative backcross genotypes	Proposed NRC genotypes		
	aabb	*aaBb*	*aaBB*
AaBb	1:3	3:5	1:1
AABb	1:1	3:1	n:0
AaBB	1:1	1:1	1:1
AABB	n:0	n:0	n:0

Table 1.20 contains observed ratios of regenerative to nonregenerative progeny from the (F_1 X F49R) X NRC crosses. Totals were calculated over the various NRC parents except Fs7 based on earlier findings that this genotype behaved differently than other NRC's.

Table 1.20. Observed ratios of regenerative to nonregenerative progeny from crosses between regenerative backcross progeny and nonregenerative controls [(F_1 X F49R) X NRC's]

NRC BC[z]	F7	FS8	F2	FS11	Total w/o Fs7	Fs7
16Aa	5:6	7:6			12:12	
16Ab		3:3			3:3	
16Da	8:13	5:6	0:1		13:20	
16Db	9:14	8:11	3:4	4:2	24:31	5:13
33Bd		4:2	4:3		8:5	
3Ka	3:5	1:0	6:1	4:9	14:15	
3Kc	0:2	0:4	6:9		6:15	5:15
4Hd	2:0		7:2	0:4	9:6	
D1f	5:4		9:6	12:15	26:25	6:18
D3e	8:11	4:7		5:6	17:24	
E3d	6:1				6:1	
I6c	1:6	2:3			3:9	1:5
J11d	2:5	9:4	1:4	2:3	14:16	
J11e	10:8	7:10	2:5	1:2	20:25	1:6
K15e		5:5	1:3	2:4	8:12	5:13
16Dc		3:1			3:1	
J33a	2:7	9:7			11:14	
I11h	2:4	1:0			3:4	
J2b	1:10				1:10	
J6b	9:11	3:3			12:14	1:9
J6c		3:6			3:6	
K5j	1:1				1:1	
Totals					217:269	24:79

[z]In BC parents, the upper case letter identifies the F_1 plant, the number identifies the individual F49R plant and the lower case letters denote individual full sibs (e.g., 16Aa and 16Ab are full sibs from a cross between F49R16 and the F_1 plant "A".

Table 1.21 provides goodness-of-fit test results based on the genotypes proposed for F_1 X F49R progeny and on ratios resulting from crossing these genotypes to NRC's (as given in Table 1.19). Most crosses between regenerative BC's and NRC's produced regenerative to nonregenerative progeny ratios of 3:5 or 1:1. Crosses between regenerative BC's and Fs7, however, resulted in a ratio of 1:3 regenerative to nonregenerative progeny. The results suggest that most of the regenerative backcross (F_1 X F49R) parents have the genotype *AaBb* or *AaBB*. None of the regenerative BC parents produced progeny ratios consistent with the genotype *AABB* when crossed to NRC's. The chance of obtaining the *AABB* genotype would be 1/16 with F_1 = *AaBb* X F49R = *AaBb*; 3/16 with F_1 = *AaBB* X F49R = *AaBB*; and less if nonregenerative F1's were used. It was therefore not surprising that none of the 15 crosses between regenerative backcross progeny and NRC's, for which greater than 12 progeny were evaluated, yielded progeny ratios indicative of a homozygous dominant condition at both genes in the BC parent (i.e., 100% regenerative progeny).

Table 1.21. Chi-square values from goodness-of-fit tests to theoretical ratios of regenerative to nonregenerative progeny from crosses between regenerative backcross progeny and nonregenerative controls [(F_1 X F49R) X NRC's]

NRC	Total w/o Fs7	χ^{2y}			Fs7	χ^{2y}	
BC[z]	Obs'd	3:5	1:1	3:1	Obs'd	1:3	1:1
16Aa	12:12	1.60 NS	0.00 NS	9.72 **			
16Ab	3:3						
16Da	13:20	0.03 NS	1.48 NS	22.48 **			
16Db	24:31	0.88 NS	0.89 NS	28.85 **	5:13	0.07 NS	3.56 NS
33Bd	8:5	3.21 NS	0.69 NS	1.26 NS			
3Ka	14:15	1.44 NS	0.03 NS	11.04 **			
3Kc	6:15	0.71 NS	3.86 *	24.15 **	5:15	0.00 NS	5.00 *
4Hd	9:6	3.24 NS	0.60 NS	1.80 NS			
D1f	26:25	3.95 *	0.02 NS	15.69 **	6:18	0.00 NS	6.00 *
D3e	17:24	0.27 NS	1.20 NS	24.59 **			
E3d	6:1						
I6c	3:9	0.80 NS	3.00 NS	16.00 **	1:5		
J11d	14:16	1.08 NS	0.13 NS	12.84 **			
J11e	20:25	0.93 NS	0.56 NS	22.41 **	1:6		
K15e	8:12	0.15 NS	0.80 NS	13.07 **	5:13	0.07 NS	3.56 NS
16Dc	3:1						

Table 1.21 cont'd

J33a	11:14	0.45 NS	0.36 NS	12.81 **	
I11h	3:4				
J2b	1:10				
J6b	12:14	0.83 NS	0.15 NS	11.53 **	1:9
J6c	3:6				
K5j	1:1				
Totals	217:269				24:79

[z] In BC parents, the upper case letter identifies the F_1 plant, the number identifies the individual F49R plant and the lower case letters denote individual full sibs (e.g., 16Aa and 16Ab are full sibs from a cross between F49R16 and the F_1 plant "A".
[y] χ^2 goodness-of-fit tests were done only when at least 12 full sib progeny were evaluated.
*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

F49M X (F49R X NRC's)

Table 1.22 contains theoretical ratios of regenerative to nonregenerative progeny from crosses between F_1's (F49R X NRC's) and F49M plants based on the assumption that F49M has a genotype of *aaBb*.

Table 1.23 presents observed ratios of regenerative to nonregenerative progeny from crosses between F49M and F_1's (F49R X NRC's) and χ^2's from goodness-of-fit tests to the theoretical ratios in Table 1.22. As expected, there were no regenerative progeny from crosses with nonregenerative F_1's except for plant "I". The observed ratio of 4 R: 12 NR is compatible with the proposed genotype of *Aabb* for "I". Only two crosses between F49M and regenerative F1's yielded

sufficient progeny for χ_2 tests. F49M X plant "C" produced a progeny ratio not significantly differentfrom 3:5 at the 1% significance level and F49M X F_1 plant "J" also produced a progeny ratio not significantly different from 3:5. Although only a limited number of F49M progeny were evaluated for regenerative ability, the ratios of regenerative to nonregenerative progeny support the proposed genotype of *aaBb* for F49M.

Table 1.22. Theoretical ratios of regenerative to nonregenerative progeny from crosses between proposed F_1 genotypes and the proposed F49M genotype

Proposed F_1 genotypes	Proposed F49M genotype	Expected ratios of R:N progeny from crosses between F_1's and F49R
NR F_1's		
aabb	*aaBb*	0 : n
aaBb	*aaBb*	0 : n
aaBB	*aaBb*	0 : n
Aabb	*aaBb*	1 : 3
R F_1's		
AaBb	*aaBb*	3 : 5
AaBB	*aaBb*	1 : 1

Table 1.23. Observed ratios of regenerative to nonregenerative progeny from crosses between F_1's and F49M and χ^2's from goodness-of-fit tests to theoretical ratios

F_1 parent	R progeny	NR progeny	χ^2	
NR F_1's			*0 : n*	*1 : 3*
B	0	12	---	4.00*
H	0	12	---	4.00*
I	4	12	---	0.00NS
R F_1's			*3 : 5*	*1 : 1*
C	1	11	4.36*	8.33**
F	0	5		
J	2	13	3.74NS	8.07**
K	3	7		

*, ** = $P<0.05$ and $P<0.01$, respectively; NS, not significant

Repeatability of regeneration response in F_1 X F49R backcrosses

Progeny evaluations of regenerative ability were routinely based on at least one regenerating callus out of a total of three explants. Regeneration is often evaluated using larger numbers of explants. Accuracy of the evaluation was tested by repeating evaluations of a number of backcross progeny (F_1 X F49R). Three petiole segment explants were taken from 5-wk-old plantlets. Another three petiole segment explants were taken from the same *in vitro* plantlets when they were approximately 8-wk-old. Results are presented in Table 1.24. Evaluations were repeated on a total of 86 plantlets. Numbers of regenerating explants were recorded.

Sixty-seven of the 86 plantlets showed the same numbers of regenerating explants in both evaluations. Four of the 86 plantlets (4.7%) were evaluated as regenerative in one evaluation and nonregenerative in the other evaluation. In each case the genotypes were classified as nonregenerative 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. Twenty-nine of the plantlets produced 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 plantlets (71%). Intermediate classes were relatively rare.

Table 1.24. Repeatability of regeneration response in progeny from backcrosses of F_1's to F49R

Regenerative explants out of 3		F_1 X F49R progeny in each class (n)
Evaluation of cultures from 5-wk-old plantlets	Evaluation of cultures from 8-wk-old plantlets	
0	0	32
0	1	1
0	2	2
0	3	1
1	0	0
1	1	3
1	2	2
1	3	0
2	0	0
2	1	0
2	2	3
2	3	5
3	0	0
3	1	3
3	2	5
3	3	29
total		86

Regenerative ability and abnormal leaf morphology

F49R plants transplanted to soil in the greenhouse or field always exhibited at least a few leaves with abnormal leaf morphology. Some plants had no normal leaves and were grossly abnormal while others had only a few abnormal leaves at the crown (Fig. 1.3). This characteristic persisted even

after cuttings were taken from apparently "normal" shoots on a plant. When these shoots were rooted, repotted and left to grow in the greenhouse, abnormal leaves were invariably detected at the base of the plants. These leaves, which had extended petiolules, almost appeared to be unifoliate and were very similar in appearance to leaves of white clover plantlets regenerated from cotyledon callus (Pelletier and Pelletier 1971). F49M plants had no abnormal leaves. A minority of the seedlings grown for regeneration evaluations also exhibited some leaves with this characteristic. Progeny of F_1 X NRC's were evaluated for the abnormal leaf characteristic as well as regenerative ability. Results based on regenerative ability of the F_1 parent are presented in Table 1.25. There appears to be a higher incidence of abnormal leaves from crosses where the F_1 parent was regenerative.

Table 1.26 shows the results of testing for independence between regenerative ability and leaf morphology. It was clear that the two traits were not independent ($P<0.001$).

Fig. 1.3. F49R plant with some leaves showing abnormal morphology.

Table 1.25. Frequencies of progeny from different F1 parents with some degree of abnormal leaf morphology in *in vitro* seedlings from F_1 X NRC backcrosses

F_1 parent	Abnormal	Normal	Frequency abnormal
NR F_1's			
A	0	26	0.00
B	0	49	0.00
G	0	12	0.00
H	0	49	0.00
I	3	63	0.05
R F_1's			
C	31	80	0.28
D	15	59	0.20
F	0	16	0.00
J	12	23	0.34
K	5	7	0.42

Table 1.26. Contingency test of independence between abnormal leaf development and regenerative ability in progeny from F_1 X NRC backcrosses

Regenerative ability	Leaf morphology		Total
	Abnormal	Normal	
R	68	38	106
NR	4	344	348
Total	72	382	454

$\chi^2 = 241.68$ $P<0.001$

DISCUSSION

It is tempting to speculate that a mix-up in labelling caused F49M (epicotyl-derived) and F49R (regenerated from hypocotyl callus) to have different regeneration capacities.

Perhaps these two are not after all derived from the same seedling. Crosses between the two, however, have failed to produce seed. Chances are slim for a coincidence of events whereby one of a pair of cultures was mislabelled and was given a label matching another plant to which it could not be crossed. Gametophytic self-incompatibility was assumed although this was not proven microscopically. Further evidence that F49M and F49R were indeed derived from the same seedling was derived from 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. Another experiment involved taking explants from greenhouse-grown F49M and F49R plants subjected to varying nutrient stress. All efforts to date to repeat regeneration from F49M have been unsuccessful in the Truro laboratory. In one case, regeneration of abnormal leaf-like structures occurred following a high 2,4-D treatment in the dark, however, no plants have been regenerated from these cultures although they have been subcultured at monthly intervals for three years.

Maternal inheritance of the regeneration trait must be ruled out in this material because regeneration was achieved in many cases when F49R was the pollen parent. Furthermore,

a χ^2 test of independence showed that crossing direction was not significantly related to ratios of regenerative to nonregenerative F_1 progeny (Table 1.4) It was therefore concluded that genes controlling somatic embryogenesis in this plant material were located in the nucleus and not the cytoplasm.

The first logical hypothesis to test when dealing with qualitative traits, after ruling out maternal inheritance, is whether the trait is controlled by only one gene. It was assumed that a gene affecting regenerative ability was altered in culture since F49M tissue (derived from the epicotyl of seedling F49) is nonregenerative while F49R tissue (derived from hypocotyl callus of the same seedling) is 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 NRC's included a large proportion (66/184 = 35.9%) of regenerative plants (Table 1.10). A further assumption was that only one gene controlling regeneration was affected in culture; therefore the genotype assigned to F49R was *Aa*. Nonregenerative plants were assigned the genotype *aa*. A cross between the two genotypes would yield a 1:1 ratio of regenerative to nonregenerative plants. The observed ratio was almost a perfect fit to a 3:5 ratio which inferred that perhaps two complementary genes

were responsible. However, if penetrance of the phenotype is less than 100%, then a one gene hypothesis cannot be ruled out. In fact, if penetrance of the dominant phenotype averages 75% then the resulting phenotypic progeny ratio from a cross between the genotypes *Aa* and *aa* would be 3:5 (1/2 *Aa* X 3/4 penetrance = 3/8 regenerative progeny). Incomplete penetrance would also explain why none of the crosses between regenerative backcrosses (F_1 X F49R) and NRC's yielded 100% regenerative progeny.

The one gene, incomplete penetrance model breaks down however, when the F_2 data are examined (Table 1.12). For example, three possible outcomes would be expected: nonregenerative F_1 X nonregenerative F_1 would yield 0 *A-* = 0 regenerative progeny; regenerative F_1 X nonregenerative F_1 would yield 1/2 *Aa* X 3/4 penetrance = 3/8 regenerative progeny; and regenerative F_1 X regenerative F_1 would yield 3/4 *A-* X 3/4 penetrance = 9/16 regenerative progeny. Crosses among nonregenerative F_1's excluding "I" did in fact fail to produce regenerative offspring (1:49) but crosses between regenerative and nonregenerative F_1's produced a progeny ratio very close to 1:1 (89:92) when crosses with "I" were excluded and crosses among regenerative F_1's actually yielded a ratio very close to 3:1 (98:43).

If, as hypothesized above, regeneration in F49R and F49R progeny was due to a mutation in culture, then it is possible that the mutation is lethal in the homozygous

condition. The main problem with this theory is that often the frequencies of regenerative progeny were higher than expected. If lethality was involved, the proportions of regenerative progeny would have been lower in the F_2 generation (e.g., *Aa* X *Aa* would yield a phenotypic progeny ratio of 2:1 regenerative to nonregenerative if *AA* was lethal). The actual ratio was often closer to 3:1. Errors in phenotyping would favour the nonregenerative class.

A single gene model, even with consideration of penetrance or lethality was inadequate to explain the progeny regeneration data. The F_1 results strongly suggested a regeneration ratio of 3:5. This ratio is compatible with two complementary genes. In this model gene "A" would remain as stated above with F49R having the genotype *Aa* and NRC's having the genotype *aa*. A second gene would also be involved. In order to obtain the 3:5 ratio, both F49R and the NRC's require the genotype *Bb*. A Punnett square showing the resulting F_1 genotypes and phenotypes from this cross is presented in Table 1.7.

Selfing in red clover is rare due to a gametophytic self-incompatibility mechanism controlled by *S* alleles. In the present study it was unknown whether any genes affecting regenerative ability were linked to self-incompatibility alleles. If so, segregation ratios would be distorted. For example, if F49R had an *S* allele genotype of S_1S_2 and an NRC had the genotype S_3S_4 four types of progeny would result as

presented in Table 1.27. Furthermore, if the *S* locus was linked to one of the regeneration loci (e.g., *a*), the genotypes and phenotypes as presented in Table 1.28 would result.

Table 1.27. Theoretical progeny genotypes resulting from crossing F49R with an assumed *S* allele genotype S_1S_2 to an NRC with an assumed *S* allele genotype S_3S_4

gametes F49R	NRC	S_3	S_4
S_1		S_1S_3	S_1S_4
S_2		S_2S_3	S_2S_4

Table 1.28. Theoretical progeny genotypes resulting from crossing F49R with an assumed genotype S_1S_2Aa to an NRC with an assumed genotype S_3S_4aa

gametes F49R	NRC	S_3a	S_4a
S_1A		S_1S_3Aa R	S_1S_4Aa R
S_2a		S_2S_3aa NR	S_2S_4aa NR

Segregation ratios could be distorted if these F_1's were backcrossed to F49R. For instance crosses between plants with the genotypes *Aa* X *Aa* would produce progeny ratios of 1:1 (Table 1.29) rather than the usual 3:1 monohybrid F_2 ratio. Also, if a nonregenerative F_1 with the genotype S_2S_4aa was backcrossed to F49R (S_1S_2Aa) there would be two different results depending on the direction of the cross (Table 1.30). Continuing along this same line of

thinking, the homozygous dominant genotype for the regeneration gene linked to an *S* allele would be extremely rare (depending on the map distance between the two loci).

Table 1.29. Theoretical backcross progeny genotypes and phenotypes resulting from reciprocal crosses between F_1's and F49R assuming F_1 = S_1S_4Aa and F49R = S_1S_2Aa

		Ovule parent			
Pollen parent		R F_1		F49R	
	gametes	S_1A	S_4a	S_1A	S_2a
F49R	S_1A	inviable	inviable		
	S_2a	S_1S_2Aa R	S_2S_4aa NR		
R F_1	S_1A			inviable	inviable
	S_4a			S_1S_4Aa R	S_2S_4aa NR

Table 1.30. Theoretical backcross progeny genotypes and phenotypes resulting from reciprocal crosses between F_1's and F49R assuming F_1's = S_2S_4aa and F49R = S_1S_2Aa

		Ovule parent			
Pollen parent		NR F_1		F49R	
	gametes	S_2a	S_4a	S_1A	S_2a
F49R	S_1A	S_1S_2Aa R	S_1S_4Aa R		
	S_2a	inviable	inviable		
NR F_1	S_2a			inviable	inviable
	S_4a			S_1S_4Aa R	S_2S_4aa NR

It is apparent from Tables 1.29 and 1.30 that linkage between a regeneration gene and the *S* locus (self-incompatibility gene) would produce confusing segregation

ratios in cases of breeding between relatives. Only one regeneration gene is considered in order to simplify the explanation. It does, however, show that it is prudent to look at crosses where inbreeding is minimal when attempting to assign genotypes to the red clover plants used in this study. In the present study, F_1 plants were backcrossed to nonregenerative control cv. Florex plants and to F49R plants. Goodness-of-fit tests to theoretical ratios, based on proposed genotypes, produced expected results for backcrosses to NRC's but results for three F_1 parents backcrossed to F49R could not be explained by the proposed model. In each of the three cases, the observed ratios of regenerative to nonregenerative progeny were higher than expected. It must be noted that the NRC's used to produce backcross progeny were not the same plants used to produce F_1 progeny. The F_1's used in these crosses were obtained from seeds produced on F49R plants in the field. The NRC's to which the F_1's were subsequently crossed were different from the field plants and were maintained in a greenhouse. Therefore, the NRC backcrosses involved no inbreeding, while the F49R backcrosses involved a degree of inbreeding. The lack of fit may possibly be attributable to inbreeding effects and may or may not involve linkage of a regeneration gene to an *S* allele.

The only crosses with no inbreeding in the present study are the crosses between F49R and nonregenerative

control plants (NRC's) and backcrosses between F_1's and NRC's. Results from these crosses as well as the majority of crosses from the other tested generations are compatible with complementary gene action involving two independent gene loci. Crosses between F49M and F_1's provide additional evidence that F49M has the genotype *aaBb* (Tables 1.22 and 1.23) compared to a genotype of *AaBb* for F49R.

This is not the first report where enhanced regeneration was observed from explants of plants previously regenerated from culture. This phenomenon was observed in Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* D.C.) (Clare and Collin 1974), broccoli (*Brassica oleracea* L. var. *italica*) (Robertson et al. 1988), celery (*Apium graveolens* 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) who 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. I attempted to study somatic embryogenesis as a quantitative trait, however, in most cases either all or none of the explants from individual plants regenerated (e.g., Table 1.24). Furthermore, 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 are 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 clover 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; *Lycopersicon* - Koornneef et al. 1987; cucumber - Nadolska-Orcyzk and Malepszy 1989; cotton - Gawel and Robacker 1990; and sorghum - Ma et al. 1987).

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 10 calli had a shoot bud. Examination of data from the experiment in which seedlings were evaluated twice (explants taken at 5 wk and again at 8 wk), however, did not justify increasing the number of explants. The fact that less than 5% were incorrectly classified as nonregenerative 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 leads to the conclusion that increasing the number of explants is 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 nonregenerative. The reverse case is unlikely. Therefore ratios of regenerative to nonregenerative progeny may be

slightly lower than the actual genotypes they represent.

It was tempting to speculate that 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). This phenotype appeared to have variable expressivity whereby the numbers of affected leaves varied from very few to many. Abnormal leaves in F_1 X NRC progeny were observed in seedlings on hormone-free medium suggesting that the phenotype was the result of genetic rather than epigenetic changes. It is interesting to note that abnormal leaf morphology was reported among red clover regenerants in two previous studies (Phillips and Collins 1980; Wang and Holl 1988). Wang and Holl (1988) also reported that not all leaves on a plant were affected.

The mutation which granted regenerative ability to F49R appeared to be associated with abnormal leaf morphology. Thirty-eight of the 106 regenerative F1 X NRC progeny had no abnormal leaves as 5- or 8-wk-old seedlings (Table 1.26). Some of these plants would possibly develop the abnormal leaf trait later. Likewise, four of the 348 nonregenerative F1 X NRC progeny expressed abnormal leaves but it is possible that these four plants were misclassified as

nonregenerative. The abnormal leaf phenotype appears to be a good candidate for a marker of regenerative ability in this germplasm.

A better understanding of somatic embryogenesis would perhaps result from looking at the information available on zygotic embryogenesis. A great deal of knowledge has accumulated concerning transcriptional control of genes by hormone-receptor complexes, by homeobox gene products, by methylation of cytosine bases, or by repetitive DNA sequences.

Homeobox genes are believed to control embryo development in *Drosophila* and some vertebrate species. Base sequences are highly preserved within homeobox genes even among different species (Weaver and Hedrick 1995). In *Drosophila* three groups of homeobox genes are active at successive stages during embryo development (Weaver and Hedrick 1995). These genes are believed to work by controlling transcription from other genes (Weaver and Hedrick 1995).

A similar system in plants would explain why somatic embryogenesis is so often controlled by two or three complementary genes. There are of course many more genes involved in the complex process of somatic embryogenesis but perhaps transcription from groups of these genes is controlled by only a few "master" genes. It is logical to assume that these "master" genes are present in all

genotypes which are capable of forming embryos and germinating from seeds with zygotic embryos but perhaps transcription of these master genes is usually impossible in somatic cells. It is possible that the genes which distinguish regenerative from non-regenerative genotypes are those which control transcription from the hypothesized "master" genes in somatic cells. In theory, however, if we could mimic conditions of the zygotic embryos it should be possible to achieve somatic embryogenesis in most if not all "normal" genotypes.

It has long been known that steroid hormones in animals act by binding to hormone receptor proteins. The hormone-receptor complex is then able to bind to transcriptional control regions of several genes thereby allowing transcription to proceed simultaneously from these genes (Weaver and Hedrick 1995). It is conceivable that similar mechanisms work for other types of hormones. In the present scenario of somatic embryogenesis from red clover, genes responsible for transcription from the hormone-receptor gene(s) would ultimately control the fate of somatic cells.

Differentiation in eukaryotes depends on a hierarchy of genes. A hierarchy implies a very small number of genes at the top of the pyramid and a very large number of genes at the base. It is proposed that the two or three genes which appear to control somatic embryogenesis in the red clover used in this study are among those close to the top of the

pyramid. Furthermore, these genes may act by controlling transcription (expression) from genes on lower levels of the pyramid. The transcriptional control may be similar either to homeobox genes or to hormone-receptor complexes, both of which are involved with differentiation in animals. The ultimate result of the action of the proposed genes is production of somatic embryos and subsequent plantlet regeneration from single somatic cells of red clover. As in animal studies, how genes bring about differentiation remains a mystery.

It is also tempting to propose a role for methylation in somatic embryogenesis. Methylation status is known to be affected by tissue culture conditions. Phillips et al. (1990) suggested that all somaclonal variation was "directly or indirectly related to ... DNA hypo/hypermethylation". There is evidence that methylation negatively affects gene expression (Bezděk et al. 1991; Smulders et al. 1995). Methylation of genes which have a role in somatic embryogenesis would prevent expression from those genes and thus terminate the process of embryogenesis. Methylation may also help to explain why somatic embryogenesis can usually be achieved when meristems are included with explants, particularly young meristems in the form of mature or immature zygotic embryos. It is possible that the DNA in these rapidly dividing tissues has little or no methylation. Methylation is an epigenetic process in that it is

reversible, however, unlike other epigenetic events, methylation status is sometimes, or "partly", inherited (Smulders et al. 1995; Karp 1995). It may be that F49M callus tissue produced a cell with reduced methylation of cytosine bases which in turn allowed expression of somatic embryogenesis. Resulting embryos developed into plants which may have retained reduced methylation. Capacity for regeneration appears to have declined over time (10 years). This may be due to a progressive increase in methylation. Similar reduction in or loss of regenerative ability has been documented in other red clover germplasms (Myers et al. 1989; Taylor et al. 1989). It would be interesting to quantify methylation in F49R and F49M and among F49R plants with different regenerative capacities.

Recently it has been suggested that repetitive DNA sequences may play a role in gene regulation. There may be a link between effects of methylation and repetitive sequences on gene expression since repetitive sequences are often highly methylated (Bezděk et al. 1991). The composition of repetitive DNA in a plant genome varies quantitatively and qualitatively throughout development (Altamura et al. 1987). Loss of repetitive sequences upstream of a gene may have the same effect as reduced methylation (i.e., permit transcription). Arnholdt-Schmitt (1993) reported a reduction in some repetitive DNA sequences during induction of somatic embryogenesis in carrot cultures.

The F49M and F49R plants provide valuable material for investigating genetic regulation of somatic embryogenesis. The present study concluded that F49M and F49R differ in just one dominant allele. It remains to be determined what this allele actually does.

Chapter 2

SOMACLONAL VARIATION IN A RED CLOVER CLONE: MEIOTIC CHROMOSOME PAIRING AND ISOZYME ANALYSIS

INTRODUCTION

Plantlet regeneration from tissue culture was initially seen as a means of quickly generating large numbers of genetically identical individuals. Later developments included screening for desirable variants often following a mutagenic treatments (e.g., X-rays or EMS). It was soon apparent, however, that control cultures often produced as much variation as mutagenized cultures (Street 1975).

Somaclonal variation was the term coined by Larkin and Scowcroft (1981) to describe any variation observed among plants regenerated from callus, cell suspension or protoplast culture. The variation could be either genetic or epigenetic. Confirmation of genetic variation involved evaluation of the particular trait in sexual progeny of the regenerants (Evans 1988). Genetic somaclonal variation has ranged from the level of the entire genome (e.g., polyploidy) down to single point mutations (Scowcroft et al. 1987). Epigenetic changes generally are not expressed in the progeny and have often been attributed to plant growth regulator effects (Karp 1995).

Variation expressed in regenerants originates either in explant cells or during periods of culture in a highly undifferentiated state (Bayliss 1980; Lee and Phillips

1988). Callus and cell suspension cultures often contain large proportions of karyotypically abnormal cells. These cells may or may not be at a selective disadvantage compared to cells with the normal karyotype. It is generally accepted however, that when culture conditions are not limiting to growth, karyotypically normal cells proliferate at a greater rate than abnormal cells (Bayliss 1980). It is generally agreed that variation accumulates over time spent in a relatively undifferentiated state (Lörz and Scowcroft 1983; Lee and Phillips 1988; Fluminhan and Kameya 1996). It has therefore been advised that periods of undifferentiated cell growth be minimised or preferably avoided when the objective is clonal propagation (Bayliss 1980).

Several factors have been shown to increase somaclonal variation. These include frequency of subculture and type and concentration of growth regulators in the medium. Long subculture intervals result in a higher degree of variation than short intervals (Binarová and Doležel 1988). The effect of growth regulators on somaclonal variation is much less clear. Many reports claim that auxins, particularly 2,4-D, are associated with genetic changes in culture. However, other reports failed to find a relationship between karyological stability and 2,4-D (eg., Johnson et al. 1984; Fish and Karp 1986; Binarová and Doležel 1988).

The vast majority of plants regenerated from callus or suspension cultures are karyotypically normal plants, even

though the source cultures may have contained large proportions of karyotypically abnormal cells. Regeneration appears to provide a sieve whereby only those cells with relatively normal chromosome complements are capable of undergoing the complex process of somatic embryogenesis or organogenesis (Chand and Roy 1981; Berlyn 1982; Lorz and Scowcroft 1983; Evans et al. 1984). The most common deviations among regenerants are polyploidy and chromosomal rearrangements (Evans et al. 1984; Lee and Phillips 1988). Aneuploidy is common in plants which are normally polyploid but very rare in diploids (Evans et al. 1984). True diploids apparently are not buffered against the ill effects of aneuploidy to the same extent as polypoids (Evans et al. 1984).

Chromosome structural changes can be recorded microscopically either through observing banding patterns on root tip chromosomes or, preferably, through observing homologous pairing and separation in prophase, metaphase and anaphase of meiosis I in pollen mother cells (Bayliss 1980). Chromosomal rearrangements follow chromosome breakage. Rearrangements frequently encountered in somaclonal variants include amplifications, deletions, translocations and centric fusions (Skirvin 1978; Scowcroft et al. 1987; Lee and Phillips 1988). Laggards and micronuclei are also documented (Bayliss 1980). Detection of such abnormalities requires observation of chromosomes in cells undergoing

meiosis and is difficult in many species due to small chromosome size and/or genomes with indistinguishable chromosomes. Technical ability and experience on the part of the researcher are also extremely important and even the best cytological technique detects only a fraction of the chromosomal rearrangements (Lee and Phillips 1988).

Recent techniques allow more sensitive detection of chromosomal changes. RFLP (restriction fragment length polymorphisms) and RAPD (random amplified polymorphic DNA) methods, for example, have been used to detect differences at the DNA level which would be invisible by microscopy (e.g., Muller et al. 1990). Analysis of changes in lengths of repetitive DNA sequences has been used for evaluating somaclonal variation. Zheng et al. (1987) found different levels of amplification (up to 75-fold) of highly repeated seqences in cultured rice cells. Copy number of repetitive seqences in exponentially growing carrot cultures, however, were reduced (Arnholdt-Schmitt 1993).

It is important to clarify the definition of "clone". The term clone is used here to describe a line of individuals derived from the same initial seedling. This is in accordance with the definition of clone as "a population of cells derived from a single cell by mitoses" (Schaeffer et al. 1984). Individuals within a clone are not necessarily identical in phenotype or genotype. Somaclonal variation describes differences observed among individuals of a clone

which have undergone a passage of tissue, callus, cell or protoplast culture. The term clone must not be confused with clonal propagation which refers to " asexual reproduction of plants that are considered to be genetically uniform ..." (Schaeffer et al. 1984). The term somaclone refers to plants regenerated from tissue cultures originating from somatic cells (Evans et al. 1984). In other words, clone F49 is comprised of many somaclones.

Somaclonal Variation in Red Clover

The first two reports of *in vitro* regeneration from callus tissue in red clover both reported that regenerated plants displayed normal morphology and contained the normal diploid chromosome complement (Beach and Smith 1979; Phillips and Collins 1979). Similarly, MacLean and Nowak (1989) found that all plants regenerated from a single regenerative genotype contained the normal diploid chromosome complement although pollen viability, as estimated by staining, varied significantly among regenerants.

The first report of regeneration from cell suspension cultures of red clover, however, described abnormal morphology and/or chromosome numbers in eight of 22 regenerants (Phillips and Collins 1980). Morphological abnormalities included fasciated stems, abnormal flower

heads and abnormal leaves. Four plants had low to normal pollen viabilities and two plants were tetraploid.

Wang and Holl (1988) found that length of time spent in callus culture affected chromosome complements in red clover regenerants. Regeneration from hypocotyl callus after 3 mos produced normal karyotypes while regeneration after nearly 1 yr as callus produced 23% plants with abnormal karyotypes: either tetraploids (23 of 119 plants) or diploid/tetraploid mosaics (4 plants). This finding is in accordance with a study of diploid alfalfa by McCoy and Bingham (1977) where both regenerative ability and frequency of cells with normal diploid karyotypes decreased in proportion to length of time in culture prior to regeneration.

Wang and Holl (1988) also found variation in leaflet number following regeneration. The authors, however, proposed that the observation may have been due to environmental effects since not all leaves on a plant were affected.

The findings with red clover are in agreement with studies in other species which found that degree of somaclonal variation was related to length of time in culture and that regeneration from cell suspensions is more prone to somaclonal variation than regeneration from callus, assuming all other factors are equal.

Isozymes

The term "isozymes" was coined by Markert and Moller (1959) to describe "the different molecular forms in which proteins may exist with the same enzymatic specificity". The differences in "molecular types" may be due either to different genes or to "modifications of a single gene product" (Markert and Moller 1959). Isozymes of a particular enzyme can be separated by electrophoresis and visualized with specific stains.

Isozyme analysis is a valuable tool in evaluating genotypic variation because variations in isozyme banding patterns can be detected when morphological phenotypes are identical. One useful characteristic of isozyme patterns is that heterozygotes can be identified in the F_1 generation. Isozyme genes are also located throughout the genome so that linkage to other traits is common. In comparison to DNA-based markers, isozymes require less time, cost and expertise (Douches and Ludlum 1991).

A drawback of isozymes is the fact that banding patterns can vary with the developmental stage of plant growth. It cannot be assumed that the shifts in observed band patterns and/or intensities are due to differential gene action, however, but rather to expression of the genetic information through differential transcription, translation, activation, degradation, inhibition, accumulation, and organelle localization (Scandalios 1974).

It is important therefore to extract from comparable tissues when comparing different plants.

Isozymes have found a variety of uses especially by plant breeders. Isozymes are often used as aids in cultivar identification (e.g., Weeden 1984 - white seeded snap beans *Phaseolus vulgaris*; Douches and Ludlum 1991 - potato cultivars).

A relatively recent application of isozymes is to use this technique to reveal somaclonal variation. As in cultivar identification, differences in banding patterns may be evident while morphology is seemingly identical. Three examples are given below. It must be noted that there are also many reports which failed to find any variation in isozyme banding patterns following *in vitro* regeneration (e.g., Rubluo et al. 1984; Maheswaran and Williams 1987; Griga et al. 1995).

Noh and Minocha (1990) applied shoot tissue extracts from 119 visibly similar plants which regenerated as multiple shoots from a single seedling of aspen (*Populus tremuloides* Michx.) to starch gels. After electrophoresis, gels were sliced and stained for shikimic dehydrogenase (SKD), isocitric dehydrogenase (IDH), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), and 6-phosphogluconate dehydrogenase (6-PGD). Of the 119 plants, 30 showed variation in SKD, 41 varied in IDH, and some variation was also observed in MDH and PGI. No variation was

observed in 6-PGD. The observed "changes were stable through micropropagation over more than 1 yr, indicating that these variations were not simply related to growth conditions."

Two sugarcane clones were used in a study to compare isozyme banding patterns in parental clones with those in plants regenerated from 1- to 3-mo old callus cultures (Heinz and Mee 1971). Four enzymes were employed: esterase, peroxidase, amylase and transaminase. The two parental clones were derived from a karyotypically stable population (clone A) and a chromosomal mosaic population (clone B). Twenty-nine regenerants were tested in clone A and none of these differed from the parental clone. In clone B, 55 of the 68 regenerants examined were different from the parent. No differences (additions or deletions of bands) occurred for esterase. Isoperoxidases produced the largest number of variant regenerants. None of the regenerants varied in chromosome number. The 68 regenerants from clone B included 32 which differed morphologically from the parent. Percentage of regenerants differing from parental clone B in at least one of the four enzyme systems was 87.5% (28/32) for plants with morphological differences and 75% (27/36) for plants with no detectable morphological differences.

Glutamate oxalacetate transaminase (GOT), alcohol dehydrogenase (ADH) and esterase (EST) banding patterns were compared between potato cv. Spunta and several somaclones regenerated from leaf callus (Allicchio et al. 1987). A

number of somaclones lacked a cathodal GOT band, some lacked an anodal ADH band and some somaclones showed one or two additional EST bands.

There are no reports to date of somaclonal variation in isozyme banding patterns of red clover. Wang and Holl (1988) found that culture passage did not affect banding patterns of phosphogluconase isomerase, phosphoglucomutase, malate dehydrogenase and shikimate dehydrogenase and 6-phosphogluconate dehydrogenase.

Attempts have been made to find linkage between isozymes and genes controlling somatic embryogenesis. The enzyme most commonly implicated in somatic embryogenesis is peroxidase.

Peroxidase

"Plant peroxidases are heme-containing monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidize a wide variety of molecules" (Lagrimini et al. 1990). Purification of a cationic peroxidase from peanut cell suspension medium revealed a molecular weight of 44 kD in a single peptide with 16% glycoprotein, a protoheme group and 2 moles of Ca per mole of enzyme (van Huystee and Chibbar 1987). Some of the processes which depend on peroxidase include "auxin metabolism, lignin biosynthesis, polymerization of cell wall proteins and oxidation of pigments" (van Huystee and Chibbar 1987). Attempts have been

made to associate cationic peroxidases with particular functions and anionic peroxidases with other functions, however, Chibbar and van Huystee (1984), as part of the team in unquestionably the most thorough investigation of the role of peroxidases in tissue cultures ever conducted, failed to find a difference in the activities of two purified enzymes; one anionic and one cationic secreted by peanut cells in suspension. The two enzymes even had the same pH optimum.

Isozymes of peroxidase (or isoperoxidases) are often numerous in any given plant tissue and are easily detected by a variety of staining techniques. For this reason peroxidases have been investigated more than any other plant enzyme system (Scandalios 1974). This does not mean that peroxidases are well understood. Peroxidase along with esterase and phosphatase, which also produce many bands on zymograms, are believed to be families of enzymes "the members of which have characteristic but overlapping substrate specificities" (Markert and Moller 1959). Researchers have been tempted to use peroxidases as genetic markers due to their abundance. However, the complexity of the peroxidase enzyme system has limited its use in genetic studies (Scandalios 1969).

Studies of peroxidases are further complicated by the fact that bands which stain for peroxidase activity often also produce a positive reaction for IAA oxidase activity

(e.g. Lee 1971). It is generally accepted that the major pathway of auxin degradation is enzymatic oxidation catalyzed by IAA oxidase. It is also widely believed that IAA oxidase may actually be one or more peroxidases. In fact, Gove and Hoyle (1975) determined that all peroxidase isozymes had IAA oxidase activity. Similarly, Shinshi and Noguchi (1975) concluded that IAA oxidase activity in tobacco cell suspensions was due to peroxidases. Homogenates of tomato tissues were found to have similar zymograms when stained separately for IAA or peroxidase (Palmieri et al. 1978). The homogenates also oxidized IAA at a rate which was proportional to their peroxidase activity. Other reports claim that they can successfully separate IAA oxidases from peroxidase (e.g. Beffa et al. 1990). Siegel and Galston (1967) were surprised to observe IAA oxidase activity from the apoprotein (nonheme portion) of peroxidase as long as Mn2+ and dichlorophenol were present. Guaiacol peroxidation, however, required the entire peroxidase molecule (Siegel and Galston 1967).

Peroxidases have multiple roles in secondary cell wall formation. Tobacco cell wall isoperoxidases were found to catalyze NADH oxidation resulting in H_2O_2 formation in the presence of Mn^{2+} and phenol as cofactors (Mäder and Amberg-Fischer 1982). Coniferyl alcohol and ferulic acid are also known to be primary building blocks of lignin. The polymerization of cinnamyl alcohols is catalyzed by

peroxidase in the presence of H_2O_2 (Mäder and Fussl 1982). Mäder and Fussl (1982) concluded that coniferyl alcohol has two functions in cell wall formation. First it acts as a cofactor in peroxidase-catalyzed H_2O_2 production through NADH oxidation; then it acts as a substrate for peroxidase-catalyzed lignification (Mäder and Fussl 1982).

Aside from the involvement of peroxidases in lignification of cell walls, peroxidases are also implicated in the formation of extensin. Extensin is an insoluble hydroxyproline-rich glycoprotein found in primary cell walls (McNeil et al. 1984). Everdeen et al (1988) determined that an extensin peroxidase with a pH optimum of 5.5-6.5 was responsible for intermolecular cross-linking of extensin precursors.

Tightening of plant cell walls is now generally agreed to be at least partly due to peroxidase-catalysed coupling of phenolic residues on polysaccharides and glycoproteins. There must also be a mechanism for loosening of cell walls in order for growth to occur. Fry (1989) proposed that "the principal effect of auxin in all land plants" is to cause a loosening of cell walls by increasing cellulase activity.

Some recent evidence points to the importance of the cell wall in determining somatic embryogenetic capacity. Indirect somatic embryogenesis has been found to proceed from proembryogenic cell masses which differ from non-embryogenic cells in their restricted cell size. Thus there

appears to be a requirement for reduced cell expansion which is controlled by the cell wall. Additionally, extracellular enzymes associated with the cell wall and oligosaccharide derivatives released from the cell wall have been associated with somatic embryo production and development. The cell wall therefore appears to have both a mechanical and a regulatory role in somatic embryogenesis (Fry 1990).

Peroxidases have also been shown to play a role in chorophyll degradation (Abeles et al. 1988).

A role of peroxidase which is often ignored in plant studies is in production of antibiotics for the purpose of defense against microbial organisms. This process is well documented in fungi and mammals (Morrison and Schonbaum 1976) however, I found no references to this process in plants.

Many studies have examined the effects of auxins, particularly 2,4-D, on peroxidase levels in cultured plant tissues or cells. The results are generally confusing. In some cases, 2,4-D treatment increased some peroxidases and decreased others (e.g. Stuber and Levings 1969). In other cases whether 2,4-D inhibited or promoted particular isozymes was dependent on the level of 2,4-D supplied (e.g. Lee 1972). The results were interpreted as showing that at least some of the peroxidases were responsible for regulating endogenous auxin levels (Stuber and Levings 1967; Lee 1972) or that peroxidase was partly responsible for cell

wall expansion required for auxin-induced growth (O'Neill and Scott 1987).

Arnison and Boll (1976a) stained peroxidases in bush bean (*Phaseolus vulgaris*) suspension culture cells grown with or without 2,4-D and kinetin. Cells grown without the growth regulators were more intensely stained. In contrast to cells grown with growth regulators, cultures grown without growth regulators produced "very small but actively dividing cells" as well as some "elongated, twisted cells with thickened cell walls." Differences existed between peroxidase zymograms from the two treatments, however, most of the differences were quantitative (Arnison and Boll (1976b).

Lee (1972) studied the combined effects of auxin, cytokinin and gibberellin on isoperoxidases in tobacco callus tissue. He found a positive association between fast-migrating isoperoxidases in polyacrylamide gel and conditions (10 μM 2,4-D, 0.2 μM kinetin and 2 μM GA) which promoted rapid growth in tobacco callus tissue. It was noted that the Rf values for these fast-migrating isoperoxidases were the same as for two fast-migrating IAA oxidases. Cells in this callus were large and loosely aggregated. Higher 2,4-D concentrations (greater than 10 uM) inhibited the fast migrating isoperoxidases but significantly increased intensities of slow migrating bands and produced a high level of total peroxidase activity. It was concluded in this

study that "it is the relative level of isoenzymes rather than the level of total peroxidase that is related to plant growth."

McCown et al. (1970) looked at isoperoxidases in two cvs. of *Dianthus caryophyllus*. They found three peroxidase isozymes in callus tissue that were not observed in stem tissue and some isozymes in the stem were not observed in the callus. It was suggested that high intensities of some bands in callus tissue may be due to IAA or 2,4-D induction.

Peroxidase banding patterns have been compared between embryogenic and nonembryogenic cultures. Several studies report differences in peroxidase activity and banding patterns in embryogenic versus non-embryogenic callus tissue however the results are often confounded with media growth regulator effects. A few exceptions exist which employed embryogenic and nonembryogenic tissue grown under the same culture conditions. These studies are summarized below.

Isoperoxidase zymograms resulting from isoelectric focusing were compared in three lines of carrot cell suspensions (Joersbo et al. 1989). A non-embryogenic line had been developed from an embryogenic line following selection for tolerance to S-trans-aminoethoxyglycine. The two lines thus originated from the same original callus. Both lines had a similar growth pattern and peroxidase zymogram in the presence of 2,4-D. When 2,4-D was absent however, the embryogenic line produced somatic embryos and

an isoperoxidase with a pI of pH 7.0. This isozyme was absent or just barely visible in the non-embryogenic line and in an habituated line grown in the absence of 2,4-D. Furthermore, detection of the isozyme correlated well with the period of early embryogenesis. The authors stated that this peroxidase (designated P 7.0) "could probably be used as a biochemical marker of somatic embryogenesis".

Peroxidase banding patterns on starch gels as well as total peroxidase activity were compared between citrus ovular callus lines which differed only in growth patterns (Kochba et al. 1977). Both callus lines grew in the absence of plant growth regulators but one of the lines was embryogenic under these conditions. Peroxidase activity peaked during the appearance of somatic embryos. A total of four cathodic and two anodic bands were observed. One cathodic band was present only in the embryogenic line and its appearance was associated with embryoid development.

Bajaj et al. (1973) looked at peroxidase activity and banding patterns from polyacrylamide column electrophoresis in differentiating and undifferentiated callus tissue of *Sinapis alba*. The differentiating (roots and shoots) and undifferentiated callus were obtained from the same culture growing on the same medium. Peroxidase activity in undifferentiated callus was significantly greater than in differentiating callus and both were significantly greater than in *in vivo* plant tissue. The same three bands were

present in the differentiating and undifferentiated callus but the slowest moving band was very faint in the undifferentiated callus and later disappeared.

Verma and van Huystee (1970) warned against using heterogeneous cutures for biochemical studies. They sieved cell suspension cultures initiated from peanut cotyledons and found that peroxidases and catalases varied depending on the size of cell aggregates. Although no morphological signs of differentiation were evident in cell aggregates of 0.15-0.5 mm diameter, a difference was found in protein synthesis and levels of peroxidase and catalase activities. Quantitative as well as qualitative differences were observed in regards to peroxidase isozymes. In general, peroxidase activity increased as cell aggregate size increased. Also, a new peroxidase isozyme was detected in the 0.5-2 mm diameter fraction while still another appeared in the 2-4 mm diameter fraction.

Enzymes in Spent Culture Medium

A very interesting feature of peroxidases is their ability to accumulate in culture medium (Perrey et al. 1991; McCown et al. 1970; Arnison and Boll 1975). These isozymes are very stable (McCown et al. 1970). Saunders et al. (1964) suggested that peroxidases were perhaps the most thermostable of all enzymes. Arnison and Boll (1975) studied eight enzymes in bean (*Phaseolus vulgaris*) callus and

suspension cultures. Peroxidase was the only one which accumulated in the medium. According to van Huystee (1987), media in which peanut cell suspension cultures have grown is an "enriched source of peroxidase". Peroxidase activity in media from bush bean cotyledon cell suspension cultures was higher when 2,4-D and kinetin were present (Arnison and Boll 1976b). These cultures also had faster rates of growth. De Jong et al. (1968) found different peroxidases in tobacco suspension culture media depending on the temperature at which the cultures were grown. Highest peroxidase activity was found from media of cultures grown at the coldest temperature (13 versus 25 or 35°C).

In a study of carrot cell mutant ts11, cultures were treated with tunicamycin, a compound which "prevents the attachment of high-mannose oligosaccharides to asparagine residues of proteins passing through the endoplasmic reticulum, and results in reduced glycosylation of secreted proteins" (van Engelen and De Vries 1992). Tunicamycin was seen to cause expansion of small cells in embryogenic clusters (van Engelen and De Vries 1992) and prevented embryogenesis but this effect could be overcome by adding conditioned medium. In this case the responsible chemical was a 38 kDa cationic peroxidase (Cordewener et al. 1991). Only one or possibly two of the four different 38 kDa cationic peroxidases present in the medium were effective in restoring embryogenesis. However, the effect of this enzyme

was mimiced by a preparation of cationic horseradish peroxidase. It was suggested that the carrot and horseradish peroxidases were identical or very similar since both had a pI of 7.6. Total peroxidase activity in the culture medium was not noticeably affected by tunicamycin treatment as only the isoperoxidase of pI 7.6 was absent. Microscopic analysis revealed that the effect of the complementing isoperoxidases was to prevent expansion and vacuolation of the surface cells of proembryogenic masses. It was suggested that the role of the peroxidase was to maintain small cell size (van Engelen and De Vries, 1992). As stated previously, peroxidase is known to catalyze secondary cell wall formation through the formation of cross links between cell wall polymers which leads to reduced cell wall extensibility (Fry 1986).

The objectives of the present study were to determine whether regenerants were associated with any gross chromosomal abnormalities evident during homologous chromosome pairing in pollen mother cells and to compare isozyme profiles of regenerative and nonregenerative plants of the same clone.

MATERIALS AND METHODS

Pollen Mother Cell Cytology

Immature flower buds, approximately 7 mm long, were fixed directly in ethanol: chloroform: glacial acetic acid

(6:3:1 v/v/v) and stored for at least 24 h in a refrigerator. The fixed buds were rinsed three times with 70% ethanol with each rinse lasting at least 30 min. The buds were then stored in 70% ethanol in a refrigerator.

Three different stains were initially tested for chromosome staining: (1) Snow's alcoholic hydrochloric acid-carmine (Snow 1963) followed by squashing in 1% acetocarmine; (2) modified carbol fuchsin (Kao 1982); and (3) altered carbol fuchsin - a mixture of standard carbol fuchsin and modified carbol fuchsin (1:1 v/v) (Martens and Reisch 1988). Modified carbol fuchsin gave better chromosome staining and less background staining than Snow's with acetocarmine or altered carbol fuchsin.

Slides were prepared by squashing anthers from four to six flowers in two drops of modified carbol fuchsin (Kao 1982) on a slide under a coverslip. Coverslips were ringed with hot Paraplast® prior to viewing with an Olympus® CH-2 trinocular microscope with SPlan objectives at 40X x 10X and oil immersion 100X x 10X. Suitable squashes were photographed using Kodak® TMAX 100 ASA black and white print film in an Olympus® OM-2S Program camera on an OM-Mount Photomicro Adapter. An Olympus® IF 550 green filter was placed over the light source to increase contrast.

Pollen Staining

Pollen viability was estimated by staining pollen grains with a solution of 2% acetocarmine: glycerol (1:1 v/v). Pollen grains which were plump and stained red were considered viable while those which were shrivelled and pale were considered non-viable. Slides were prepared from anthers of three flowers from an inflorescence. Two samples of 100 pollen grains were counted per slide. Three replications were employed using different inflorescences for each replication.

Starch Gel Electrophoresis

Starch gel electrophoresis procedures generally followed the methods of Vallejos (1983). Gels were composed of 12.5% electrophoresis grade modified potato starch (Select Starch, Diamond Research Products, Raleigh, N.C.) and 5% sucrose. The gel and extraction buffer contained 0.015 M Tris and 0.004 M citric acid·H_2O and was adjusted to pH 7.8 with NaOH. 400 mL buffer and 20 g sucrose were combined. 100 mL of this solution was removed and mixed with 50 g starch. The remaining 300 mL was heated to boiling then added to the starch mixture in a vacuum flask and vigourously swirled. The entire mixture was aspirated, poured onto a plexiglass frame which produced a gel slab 180 mm X 114 mm X 10 mm. Gels were covered immediately with a

sheet of heavy plexiglass, cooled at room temperature, then covered with plastic wrap and refrigerated over night.

Samples were prepared from immature leaf tissue except in the case of peroxidase where callus tissue was also used. Samples were prepared by grinding 300 mg leaf tissue with 300 μL gel buffer, 10 mg DTT (dithiothreitol, Bio-Rad Laboratories, Richmond, CA) and 50 mg PVP-40 (polyvinylpyrrolidone, Sigma Chemical Co., St. Louis, MO) using a mortar and pestle in a tray of ice. In the case of callus cultures, fresh weights of 0.6, 0.8 and 1.2 g and a buffer volume of 600 μL were employed. Samples were placed in 1.5 mL microcentrifuge tubes and centrifuged for 10 min at 12000 rpm in an Eppendorf® Microcentrifuge Model 5414 (Brinkman Instruments, Inc., Cantiague Rd., N.Y. 11590) in a 4°C coldroom. The supernatants were drawn onto 5 mm X 10 mm filter paper wicks (Whatman® No. 3 qualitative) which were then inserted into slots in a refrigerated gel halfway between the electrodes. The samples were run at 40 mA in a 4°C cold room for 5 h. Layers of filter paper were inserted as a spacer at the cathodal end when the gel began to shrink. The tray buffer consisted of 0.3 M boric acid adjusted to pH 7.8 with NaOH. Prior to staining, gels were sliced into 1.5 mm thick layers using a thin violin string attached to a metal support frame. The top and bottom layers were discarded.

Greenhouse leaf extracts were also prepared without PVP-40 or DTT or both in order to determine the effects of these chemicals on banding patterns.

Gels were stained for alcohol dehydrogenase, glutamate dehydrogenase, esterase or peroxidase as described by (Vallejos 1983). Composition of the staining solutions are given in Table 2.1.

Isoelectric Focusing

Greenhouse petiole segments and callus which had been initiated from greenhouse petiole segments were ground on ice with extraction buffer in microcentrifuge tubes using hand-held teflon-tipped pestles. The extraction buffer contained 10 mM $NaH_2PO_4 \cdot H_2O$ and 1% PVP-40 (polyvinylpyrrolidone avg. mol. wt. 40,000, Sigma Chemical Co., P.O. Box 14508 St. Louis, MO) and was adjusted to pH 6.0 with 1 N NaOH (Lagrimini and Rothstein 1987). The ratio of tissue to extraction buffer was 2:1 (eg. 0.2g + 100μL) for callus tissue and 1:1 for greenhouse petiole tissue. Ground samples were spun for 15 min at 4°C in an Eppendorf® Microcentrifuge (rpm = 12000). The supernatants were transferred to fresh microcentrifuge tubes and stored at -20°C.

Table 2.1. Composition of isozyme staining solutions for starch gel electrophoresis

Components	Volume or Mass
Alcohol dehydrogenase	
Tris 0.1M, pH 7.5	100 mL
NAD+	30 mg
MTT	20 mg
PMS	4 mg
Ethanol	6 mL
Glutamate dehydrogenase	
Tris 0.1 M, pH 7.5	100 mL
$CaCl_2$ 10 mM	0.2 mL
Na glutamate	800 mg
NAD+	30 mg
NBT	20 mg
PMS	4 mg
Esterase	
Na phosphate 0.1 M, pH 6.2	100 mL
α-naphthyl butyrate 1%	3 mL in acetone
fast blue RR salt	100 mg
Peroxidase	
Na acetate 50 mM, pH 5.5	100 mL
N,N dimethyl formamide	10 mL
3-amino-9-ethylcarbazole	40 mg
H_2O_2 3%	0.667 mL

Prepared samples were thawed and applied to Phastgel® IEF 3-9 gels. Separation was according to PhastSystem® (Pharmacia, Laboratory Separation Division, S-751 82 Uppsala, Sweden) as follows. Two gels were run

simultaneously. Gels were prefocused (2000 V, 2.5 mA, 3.5 W, 15°C, 75 Vh). Samples were applied along the center using Sample Applicator 8/1 (8 wells of 1 μL each). The separation bed was set at 200 V, 2.5 mA, 3.5 W, 15°C, 410 Vh. The entire procedure lasted about 30 min. Gels were immediately developed for either peroxidase staining or silver staining of proteins. Gels stained with silver included 2-3 lanes with calibration proteins (Pharmacia® Broad pI Calibration Kit - pH 3-10). Each vial was diluted with 800 μL distilled water. One μL of diluted calibration protein was applied per lane. Table 2.2 contains the silver staining development method. IEF gels containing calibration proteins were also stained with Coomassie blue in order to compare positions of bands on the gels with photographs included with the IEF pI standards kit.

Peroxidases were stained according to the method in Table 2.3. Reagents included DMF (N,N-dimethylformamide, ACS reagent, Sigma Chemical Co., P.O. Box 14508 St. Louis, MO 63178 USA) and 3A9EC (3-amino-9-ethylycarbazole approx. 90% Sigma Chemical Co.). All gels were air-dried overnight then stored in transparent plastic sheets with pockets (Print File® Archival Preservers) which are made for storing photographic slides.

Table 2.2. Silver staining procedure

Step #	Solution	Time (min)	Temp (°C)
1	20% trichloroacetic acid	5	20
2	50% ethanol, 10% acetic acid	2	50
3	10% ethanol, 5% acetic acid	2	50
4	10% ethanol, 5% acetic acid	4	50
5	5% glutaraldehyde	6	50
6	10% ethanol, 5% acetic acid	3	50
7	10% ethanol, 5% acetic acid	5	50
8	water	2	50
9	water	2	50
10	0.4% silver nitrate	10	40
11	water	0.5	30
12	water	0.5	30
13	0.0125% formaldehyde in 2.5% Na_2CO_3	0.5	30
14	0.0125% formaldehyde in 2.5% Na_2CO_3	4	30
15	5% acetic acid	5	50

Table 2.3. 3-Amino-9-ethylcarbazole staining procedure for peroxidase on isoelectric focusing gels

Step #	Solution	Time (min)	Temp (°C)
1	3-amino-99-ethylcarbazole staining solution[z]	30	20
2	water	1	20
3	water	1	20
4	water	1	20
5	water	1	20

[z] 0.4 mL 3-amino-9-ethylcarbazole stock solution (40 mg 3A9EC per mL DMF) was added to 3.6 mL dimethylformamide, which was then mixed with 76 mL 0.05 M sodium acetate buffer pH 5.5. 0.267 mL 3% H_2O_2 was added just prior to use.

A regression line was calculated using average distance of calibration proteins from the most cathodic band in three silver-stained gels. Distances were measured to the closest 0.1 mm directly on the gels. The slope and intercept of the regression line were determined using SAS (SAS Institute, Inc. 1985). Peroxidase pI values were calculated by inserting averages for measured distances from the most cathodic band stained with 3A9EC into the regression equation.

RESULTS

Cytology

Anthers squashed in modified carbol fuchsin resulted in better chromosome staining and less background staining than anthers squashed in altered carbol fuchsin or buds stained with Snow's alcoholic hydrochloric acid-carmine followed by squashing in 1% acetocarmine. This result differs from the findings of Martens and Reisch (1988) who concluded that altered carbol fuchsin gave better results than modified carbol fuchsin in root tip squashes of grape.

Well-spread squashes of chromosomes in diakinesis or metaphase I of meiosis were obtained and photographed from a total of 28 cells from eight F49R plants and from a total of 13 cells from two F49M plants. Representative photographs of meiosis in pollen mother cells from three different F49R plants are presented in Fig. 2.1. Characteristic

configurations such as loops, rings, fragments or anaphase bridges were absent.

F_1 plants, produced by crossing F49R plants to non-regenerative cv. Florex plants, were analyzed for pollen stainability to see if the trend of reduced pollen viability of regenerative material was maintained. Results are presented in Table 2.4. It is evident that the three regenerative F_1 plants do not rank lower than the three non-regenerative plants.

Table 2.4. Pollen stainabilities of six F_1 plants (F49R X NRC) plants

F_1 Genotype	Regeneration Status	Pollen Stainability (%)
A	NR	40.17 ± 6.99
G	NR	86.98 ± 3.13
I	NR	59.63 ± 11.01
C	R	86.76 ± 3.88
F	R	69.84 ± 12.56
K	R	70.99 ± 13.33

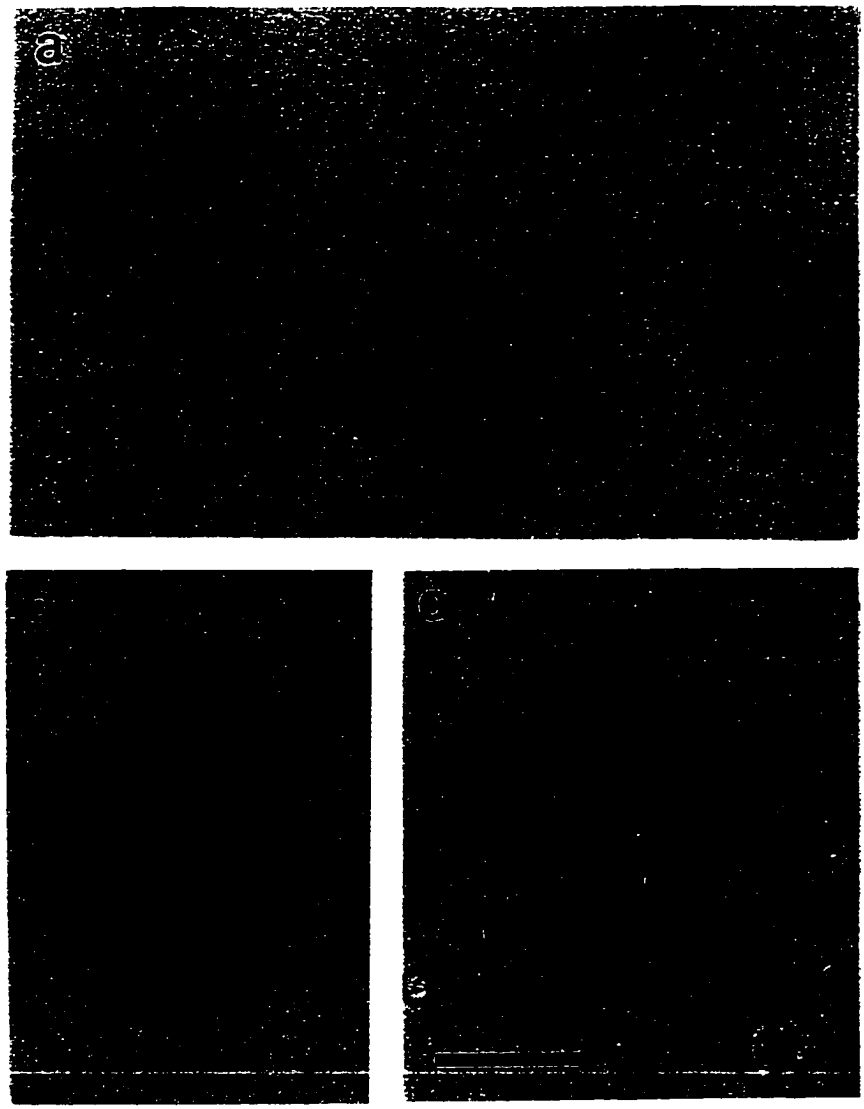

Fig. 2.1. Normal meioses in F49R pollen mother cells: (a) diakinesis in F49R4; (b) metaphase I in F49R14; (c) anaphase I in F49R6. (scale bar = 10μm)

Starch Gel Electrophoresis

Preliminary electrophoresis was conducted with starch gels. Zymograms for alcohol dehydrogenase, glutamate dehydrogenase and esterase showed no variation between F49M and F49R petiole tissue from greenhouse-grown plants. Peroxidase zymograms were the only ones which showed any possible variation between F49M and F49R. Banding for alcohol dehydrogenase and glutamate dehydrogenase was identical and it was assumed that the same isozymes were stained by both methods. Starch gel results for peroxidase staining are presented in Table 2.5.

Table 2.5. Numbers of peroxidase bands in each of four banding groups on starch gels at various stages of the regeneration protocol for F49M and F49R cultures.

Banding group	fresh explants	L2 medium			
		weeks in culture			
	0	1	2	3	4
fast-migrating cathodic	0-1	2	2	2	1-2
slow-migrating cathodic	2-4	4-5	3	2-3	3
slow-migrating anodic	1-4	5	4	4	3-4
fast-migrating anodic	-	1	2	2	2

Table 2.5. continued

Banding group	SEL medium				SPL medium			
	weeks in culture							
	5	6	7	8	9	10	11	12
fast-migrating cathodic	1	-	-	-	-	-	-	-
slow-migrating cathodic	5	4	2	3-4	0-1	1	0	2-3
slow-migrating anodic	5	5	3-4	5	4	4	4	4
fast-migrating anodic	1	2	1	2	1	1	1	1

Extracts from petiole explants on L2 medium produced the greatest number of bands and more intensely stained bands than extracts of intact petioles from greenhouse plants or from petiole callus which had progressed to SEL or SPL medium (Table 2.5). In some cases there was an apparent difference in banding pattern of slow-migrating cathodic bands between F49R and F49M extracts, however, this difference was not consistent (data not shown).

Extracts from petiole callus which had completed a 4 wk passage on L2 medium and was subsequently transferred to SEL medium showed a similar banding pattern to L2 cultures except for less intense staining, particularly of cathodic bands. Also, a fast-migrating cathodic band disappeared between 1 and 2 weeks after subculture to SEL medium (Table 2.5).

Cultures on SPL medium had previously been subjected to 4 wk on L2 followed by 4 wk on SEL medium. The slow-migrating cathodic bands were weak or nonexistent when these extracts were electrophoresed and stained. The slow-migrating anodic bands were similar to those from L2 or SEL extracts. Only one fast-migrating anodic band was evident and it was weaker than corresponding bands from extracts of cultures on L2 medium or up to 3 wk on SEL medium (Table 2.5). F49R lanes were sometimes fainter than F49M lanes (especially near the end of the 4 wk passage on SPL medium).

Extracts were prepared from greenhouse petioles from a number of individual F49R and F49M plants. Variation was often seen among plants for bands in the slow-migrating cathodic group, however, there was no evident pattern to this variation and variation was as great within groups of F49R plants or groups of F49M plants as it was between them. There was no variation between or within F49R and F49M plants within runs for the group of slow-migrating anodic bands but there were 1-4 bands depending on the particular run/gel. A fast-migrating cathodic band was sometimes faintly visible. Fast-migrating anodic bands were never observed in greenhouse petiole extracts.

Removal of DTT from the extraction procedure for greenhouse petiole tissue resulted in an increase in the number of bands. Removal of PVP from the extraction procedure, however, produced fewer bands.

Isoelectric Focusing

Unlike the starch gels, IEF gels provided very consistent results for peroxidase bands. It must be noted that extracts were prepared for IEF only after a 4 wk passage on one of the three media whereas extracts were prepared for starch gels at 2-3 day intervals throughout the 12 wk culture protocol. Ten bands stained with 3A9EC (Table 2.6). Determination of pI values was not entirely straightforward. In the present report the following regression equation was calculated: $pI = -0.154(x) + 9.235$, where x = distance from the most cathodic band (r^2 = 0.9952).

Unlike the starch gel results, IEF gels showed consistent distict differences in staining intensities between F49M and F49R lanes at the end of a 4 wk passage on SEL and SPL media (Table 2.6). With starch gels, F49M lanes were sometimes darker than F49R lanes but only at the end of the passage on SPL medium. With IEF gels, bands at pI 7.9, 8.1 and 9.1 were darker in lanes from F49M extracts than corresponding bands in lanes from F49R extracts. The difference was greatest between a band at pI 7.9 from extracts of callus on SPL for 4 wk. In addition, a band at pI 7.6 was only evident in extracts from F49M callus tissue on SEL and SPL medium (see arrow in Fig. 2.2).

Both starch and IEF gels produced similar results with regard to band staining intensities. Both types of gels

revealed the same order of staining intensities for F49M's and F49R's: L2 > SEL > SPL > greenhouse petioles.

Buffer pH for starch gel electrophoresis was 7.8. Therefore, isozymes with a pI less that 7.8 should have migrated to the anode while isozymes with a pI greater than 7.8 should have migrated to the cathode. IEF results show three bands with pI's greater than 7.8. The numbers of cathodic bands on starch gels ranges from zero to seven. There was a reduction in cathodic bands in starch gels as the culture protocol progressed. No corresponding reduction was seen in IEF results. Numbers of anodic bands ranged from five to seven in starch gels. This corresponds to six to seven IEF bands with pI's less than 7.8. The numbers of anodic bands remained quite steady throughout the culture period for both starch and IEF gels.

IEF gels were also stained with $AgNO_3$ in order to compare peroxidase banding patterns with protein bands. The most obvious difference was that most of the peroxidase staining occurred at the cathodic end of the gels while most of the protein staining was toward the anode (Fig. 2.2). The order of protein staining from darkest to faintest was L2≥G>SEL>SPL (data not shown for greenhouse tissue). F49M lanes were less intensely stained than F49R lanes when extracts were taken from cultures on SEL or SPL medium. Banding patterns did not differ greatly among media or between F49M and F49R.

Table 2.6. Peroxidase banding patterns and intensities on isoelectric focusing gels from greenhouse petiole extracts and from petiole callus cultures at the end of each 4 wk passage in the regeneration protocol.

	Greenhouse[z]		L2		SEL		SPL	
pI	M[y]	R[y]	M	R	M	R	M	R
9.2	**	**	***	***	*	*	*	*
8.1			*	*	**	*	**	*
7.9	**	**	****	****	****	***	****	**
7.5					*		*	
7.4			**	**	*	*	*	*
7.2			**	**	*	*	**	*
6.9			**	**	*	*	*	*
6.7			**	**	*	*	*	*
5.8	*	*	**	**	**	*	**	*
3.7	**	**	****	****	***	***	***	***

[z] Greenhouse petiole tissue extracts were only half as concentrated as culture extracts.
[y] M=F49M and R=F49R

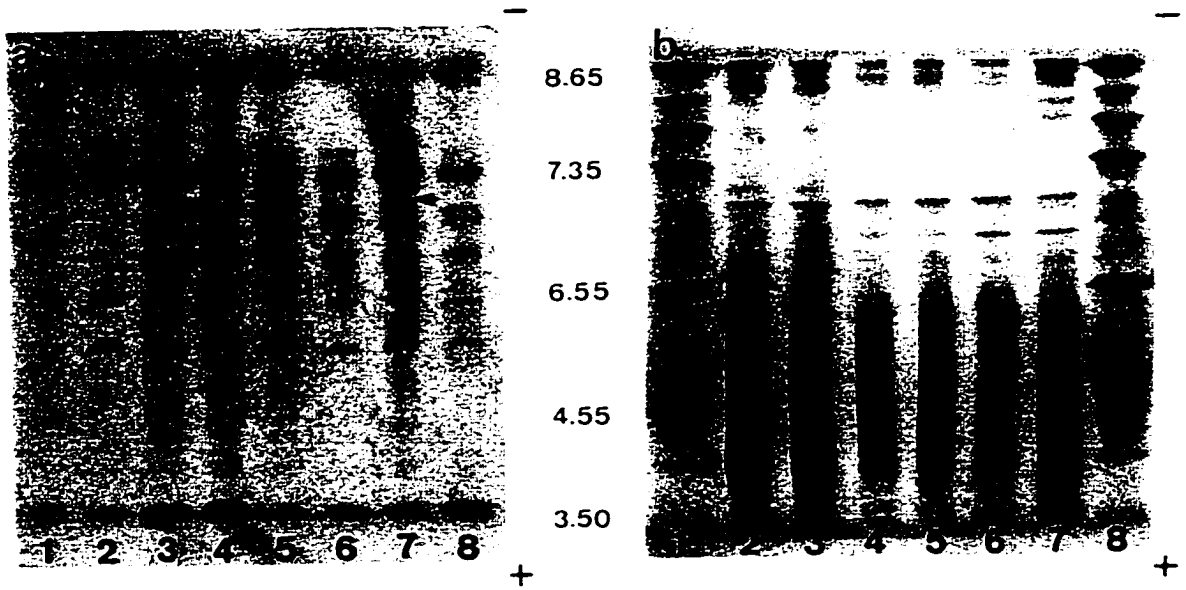

Fig. 2.2. Peroxidase and protein profiles on isoelectric focusing gels from F49M and F49R greenhouse petiole extracts (peroxidase only) and petiole callus cultures at the end of each passage in the three-step regeneration protocol: (a) stained for peroxidase with 3-amino-9-ethylcarbazole, lanes 1, 3, 5, 7: F49M petioles, L2, SEL and SPL cultures, respectively,; lanes 2, 4, 6, 8: F49R petioles, L2, SEL and SPL cultures, respectively; arrow in lane 7 indicates a band unique to F49M cultures (b) stained for proteins with silver nitrate, lanes 1 and 8: pI standards; lanes 2, 4, 6: F49M on L2, SEL and SPL, respectively; lanes 3, 5, 7: F49R on L2, SEL and SPL, respectively.

DISCUSSION

Cytology

Callus, by definition, is "an unorganized, proliferative mass of differentiated plant cells" (Schaeffer et al. 1984). Callus tissue cells are known to be prone to somaclonal variation. The hypothesis that the observed regeneration in F49R and progeny of F49R was due to some variation in the genome which occurred during culture is supported by the observation that regeneration was not obtained from F49M or F_1 progeny of F49M (data not shown). Also, originally only one of five F49 hypocotyl callus cultures subjected to the Beach and Smith (1979) or Phillips and Collins (1979) protocols was regenerative and regeneration proceeded from a small area on the regenerative culture. In contrast, later attempts to regenerate plantlets from explants of F49R resulted in regeneration from large proportions of explants and regeneration usually proceeded from the entire surface of cultures (Fig. 1.1). The fact that many progeny of F49R also had somatic embryogenesis capability suggests that whatever happened to the original regenerative F49 culture was genetic in nature.

None of the F49 regenerants were found to have abnormal chromosome numbers. Aneuploidy at the diploid level was not expected due to the fact that red clover is a true diploid ($2n = 2x = 14$) and aneuploidy is usually associated only with naturally polyploid species. Polyploidy would have been

much less surprising especially since tetraploid red clover cultivars exist. Nevertheless, no changes in chromosome number were observed among regenerants either through counting chromosomes in mitotic root tip cells (MacLean and Nowak 1989) or in meiotic pollen mother cells.

The objective of this exercise was to determine whether any microscopic changes occurred prior to regeneration of F49R plantlets from the F49M callus tissue. Changes including deletions, amplifications, translocations and inversions, if large enough, can be detected by microscopic observation of homologue pairing during meiosis I. Characteristic configurations such as loops, rings, fragments or anaphase bridges were absent. It was therefore concluded that if a mutation did occur in F49M callus prior to regeneration of F49R plantlets, the change was not detectable by the microscopy procedures used in this study.

Pollen stainability with acetocarmine was used as a measure of pollen viability. Reduced pollen stainability may indicate chromosomal aberrations. Earlier research showed a higher and more stable level of pollen stainability in a group of three F49M plants (91.23% ± 0.78%) than in a group of 11 F49R plants (65.09% ± 30.25%) (MacLean and Nowak 1989). Variability within the F49R group was similar to that within a group of control cv. Florex plants (74.34% ± 25.93%) (MacLean and Nowak 1989). It was concluded that the difference in pollen stainability between F49R and F49M,

although statistically insignificant, shows that some degree of somaclonal variation with respect to pollen stainability exists between F49M and F49R as well as within the group of F49R plants.

F_1 plants, produced by crossing F49R plants to non-regenerative cv. Florex plants, were analyzed for pollen stainability to see if the trend of reduced pollen viability of regenerative material was maintained in F49R progeny. Results are presented in Table 2.4. The three regenerative F_1 plants did not rank lower than the three non-regenerative plants. It was concluded that the regeneration trait does not necessarily reduce pollen stainability. This finding is in keeping with the results from observing meiotic chromosome pairing in which no abnormalities indicative of microscopic chromosomal changes were observed. If F49R plants regenerated as the result of a mutation then the change occurred at the submicroscopic level and did not adversely affect pollen stainability.

Isozymes

Starch gel electrophoresis of petiole tissue extracts and subsequent staining for alcohol dehydrogenase, glutamate dehydrogenase and esterase failed to reveal any differences between F49M and F49R. The enzymes investigated in this study were chosen based on previous reports that they had been used to detect somaclonal variation or differences in

regenerative ability in other species (eg., alcohol dehydrogenase - Allicchio et al. 1987; Eizenga and Cornelius 1991; glutamate dehydrogenase - Everett et al. 1985; Fransz et al. 1989; esterase - Allicchio et al. 1987; Everett et al. 1985). The primary aims of the present isozyme study were to detect somaclonal variation among greenhouse plant material and to find a marker for embryogenic ability which did not require tissue culture evaluations. Alcohol dehydrogenase, glutamate dehydrogenase and esterase starch gel zymograms failed to meet either criterion.

Peroxidase banding patterns on starch gels sometimes varied between F49M and F49R but the results were not reproducible (data not shown). Inconsistency of peroxidase results on starch gels led to a search for improved methodology to study peroxidase isozymes in F49M and F49R tissues.

It is difficult to compare the many different reports dealing with peroxidase in plant tissues. First, the number of papers dealing with the subject is overwhelming. Second, nearly every one of these papers uses a different method of evaluating peroxidase activity. The fact that peroxidase is relatively nonspecific is reflected in the abundance of different stains/substrates which can be used to visualize its presence. Common stains include benzidine, guaiacol, orthodianisidine and amino-ethyl carbazole. Another complication is the variety of electrophoretic techniques

for separating isozymes. Gels may be composed of starch, agar, agarose or polyacrylamide. The latter may be in the form of discs, or horizontal or vertical slabs. In addition, horizontal polyacrylamide gels may or may not contain ampholytes which separate isozymes according to isoelectric points. Isoelectric focusing is often preferred due to reproducibility and because of the information provided on isoelectric points. In addition to the choices for stain and gel is the extraction procedure. In some cases tissues are simply squeezed and resulting "sap" is applied directly to a gel whereas other procedures employ sequential extractions with different buffers and may or may not include reducing agents and/or polyvinylpyrrolidone.

The stain used in this study was 3-amino-9-ethylcarbazole (3A9EC). This method was developed by Graham et al. (1965) as an alternative to benzidine which faded and had a "tendency to form large crystals". The authors found that 3-amino-9-ethylcarbazole did not fade but was less sensitive than benzidine. Arnison and Boll (1974) also found that 3A9EC and orthodianisidine stained bush bean peroxidases less intensely than benzidine or guaiacol. In the present study we also attempted to stain with 4-chloro-1-naphthol according to the procedure of Lagrimini and Rothstein (1987), however, only a few barely visible bands were observed (data not shown)

In this study with red clover, IEF gels were much more reproducible than starch gels. Another advantage of the IEF system was the much shorter time required to run and develop gels. Two gels could be run and stained at once. Conditions during separation were much more controlled with the IEF system employed. Starch gels invariably suffered from heating and shrinking necessitating use of a spacer part way through the runs. Starch gels required slicing and the delicate art of separating slices prior to staining. Perhaps most important is the fact that IEF gels could be purchased for 10 dollars each and always produced good results whereas starch gels needed to be freshly prepared prior to use and produced poor to fair results. A final advantage of the IEF system was the fact that they could be simply air-dried overnight and stored indefinitely in slide protectors.

Some "isoperoxidases" may arise during extraction procedures or storage (Scandalios 1974). Extracts for starch gel electrophoresis were prepared with dithiothreitol and polyvinylpyrollidone. Presence and absence of these additives was tested using greenhouse petioles (data not shown). Absence of both additives resulted in less distinct bands, especially in the slow-migrating cathodic region and a loss of the fast-migrating anodic bands. Addition of PVP in the absence of DTT produced a greater number of bands than when both additives were present. DTT in the absence of PVP resulted in fewer bands than when both were present.

There are conflicting reports concerning the ability of compounds containing -SH groups (including dithiothreitol, 2,3-dimercaptopropanol, cysteine, glutathione, thioureas, thiamine and allyl mustard oil) to inhibit peroxidase activity (Saunders et al. 1964). Wendel and Weeden (1989) state that reducing agents (such as dithiotreitol) may inactivate some peroxidases. In the present study, it was decided to omit DTT from the extraction procedure for IEF based on the observation that it appeared to inhibit peroxidase activity. Wolter and Gordon (1975) looked at total peroxidase activity and PAGE peroxidase zymograms from aspen callus tissue samples prepared with or without PVP. Total activity was reduced and cathodic bands were absent in samples prepared without PVP. Their results for banding were similar to mine. It was decided, therefore, to include PVP in the extraction buffer since it appeared to enhance staining of peroxidase bands. The purpose of PVP is to improve enzyme stability by preventing phenolic substances from complexing with the enzyme (Wendel and Weeden 1989).

A problem in the present study was inconsistency of results. In some cases samples from a single extract, applied to different lanes of the same starch gel yielded slightly different band patterns. In contrast, Lavee and Galston (1968) reported consistent results when three replicates of *Pelargonium* pith cultures were repeated at least three times. In their case, starch gels were stained

with guaiacol to reveal peroxidase bands. However, only one band was evident. McCown et al. (1970) also report reproducibility within and between extractions, although the complexity of their extraction procedure (repeated centrifugations followed by dialysis and concentration) and a modified staining procedure employing a decreased level of H_2O_2 may help explain their success. DTT was employed in their extraction procedure.

Numbers of bands vary greatly among published reports. Despite the many variables, my starch gels closely resembled PAGE gels of Wolter and Gordon (1975) from aspen callus cultures and my IEF banding patterns were similar to those of Rucker and Radola (1971) from tobacco callus cultures with and without leaves.

Peroxidase activity for both F49M and F49R tissues, as evident through numbers of bands and band intensities on both starch and IEF gels, was greatest in callus cultures on L2 (callus induction) medium (Fig. 2.2). This was the period of most rapid growth. As the callus was subcultured to SEL (somatic embryo induction) medium, then SPL (shoot production) medium, there was a simultaneous reduction in numbers of bands, staining intensities and culture growth. Therefore there is an apparent relation between level of peroxidase activity and rate of culture growth as well as the level of differentiation. Wolter and Gordon (1975) found

that peroxidase activity and growth rate of aspen callus cultures were highly and positively correlated ($r = 0.96$).

There seems to be concensus whenever peroxidase levels in fresh explants are compared to peroxidase levels in callus tissue derived from those explants. Peroxidase is always reported to be more concentrated in the callus (Bajaj et al. 1973; del Grosso and Alicchio 1981; Haddon and Northcote (1976); Lagrimini and Rothstein 1987).

Comparisons between peroxidase levels in "undifferentiated" callus tissue and regenerating tissues were less concordant. In some cases higher peroxidase levels and/or greater band numbers were associated with the less differentiated state (e.g., Bajaj et al. 1973 - *Sinapis alba*), as in the present study. In other cases the opposite was reported (e.g., Kochba et al. 1977). Still other reports found that some bands increased in intensity while others decreased as regeneration proceeded (Rucker and Radola 1971; Fransz et al. 1989). In some cases where peroxidase increased with differentiation, the level of differentiation was controlled by media rather than genotype. For instance, Thorpe and Gaspar (1978) related higher peroxidase levels to increased levels of differentiation. The shoot-forming and callus-forming cultures they compared were grown on different media. Similarly, the embryogenic "line" referred to by Zhou et al. (1992) was grown on a medium with BA and

NAA while the nonembryogenic "line" grew on a medium with 2,4-D as the sole growth regulator.

The present red clover investigation is one of the few to compare peroxidase banding patterns in genotypes originating from the same seed yet differing in regenerative ability. The data presented here do not support previous findings which linked the presence of a given peroxidase isozyme band(s) to the ability of a culture to regenerate (e.g., Kochba et al. 1977; Fransz et al. 1989; Joersbo et al. 1989). However differences were found between lanes from F49M (nonregenerative) and F49R (regenerative) plants after 4 wk on SEL and SPL media. In general, peroxidase bands from cultures on SEL or SPL in F49M lanes were more intensly stained than in F49R lanes. Also, F49M lanes from cultures on SEL or SPL exhibited a unique band at pI=7.6. The more intense staining and presence of an extra band in F49M cultures on SEL and SPL as compared to F49R cultures which received the same treatment, corresponds to the absence of somatic embryo formation in these cultures compared to F49R cultures. It is possible that the extra band in nonembryogenic cultures was associated with senescence. Kochba et al. (1977) found two anodic bands in citrus callus cultures after 15 wk which they believed were related to tissue senescence.

A study similar to this red clover investigation was conducted using embryogenic and non-embryogenic carrot

suspension cultures derived originally from the same callus (Joersbo et al 1989). The embryogenic line produced embryoids in the absence of 2,4-D while the non-embryogenic line did not. The greatest difference in agarose IEF electrophoretograms between the two types of cultures was the presence of a band at pI 7.0 under embryogenic conditions. This band was "virtually absent" in the non-embryogenic cultures. It must be noted that observations made by the authors appear to be quite subjective. In fact the stated band at pI 7.0 was present in nonembryogenic culture extracts 5-6 days after subculture in 2,4-D-free medium. This does contrast with appearance of the band on days 2-6 in embryogenic cultures. However, since the band was also present in nonregenerating embryogenic cultures supplemented with 2,4-D on days 4-6, it is difficult to reach the same conclusion as the authors; namely that pI 7.0 "may be a useful marker in research on early events in somatic embryogenesis."

It is impossible with this data to determine what caused the high levels of peroxidase in tissue on the callus induction medium or what caused the apparent reduction in peroxidase activity in embryogenic as compared to nonembryogenic cultures. Eligible candidates include increased growth rate, degree of dedifferentiation, or response to high endogenous and/or exogenous auxin levels. These three factors are of course interrelated.

Additionally, conditions on culture media and *in vitro* may be more conducive to post-translational modifications of peroxidases leading to more bands. The objective of the present investigation was to determine whether F49M and F49R differed in peroxidase isozyme profiles. The results shown here failed to find a distinguishing band, particularly one which could be linked to the embryogenesis response. The only band found exclusively in one or the other was a band peculiar to F49M (nonregenerative) cultures on SEL and SPL media. It would be interesting to measure endogenous active and metabolized auxin levels as well as antioxidant levels in the cultures and compare these to peroxidase levels in order to have a broader view of events at the cellular level in these callus cultures.

In addition to all of its other presumed roles, peroxidase is also capable of chlorophyll catabolism (Matile 1980; Abeles et al. 1988). This alone could explain the increase in peroxidase staining from callus extracts which no longer require chorophyll-dependent photosynthesis and the decrease in peroxidase which coincided with appearance of regenerated green embryoids and plantlets. Kossatz and van Huystee (1976) found that ALA (aminolevulinic acid) activity which is normally active in chlorophyll synthesis, was as high in nonchlorophyllous peanut suspension cultures as in green tissue. They proposed that this enzyme may be involved in a process where the heme which is no longer

required for chlorophyll synthesis is instead shunted to peroxidase synthesis.

The results of the present investigation showed no direct relationship between protein staining and peroxidase staining (Fig. 2.2). In fact, silver-stained gels revealed protein levels in lanes from greenhouse petiole extracts were as darkly stained as those from callus cultures even though the callus cultures were twice as concentrated (data not shown). Also protein staining was primarily toward the anode while peroxidase staining was primarily towards the cathode. These results are in agreement with a report by van Huystee and Chibbar (1987) who raised an antibody against purified peroxidase and found that the particular peroxidase accounted for 2% of the total protein produced in peanut cell suspension cultures versus only 0.2% of the total protein produced in mature leaves of a 6-8 wk-old plant.

CONCLUSIONS

The present study found that two complementary genes controlled somatic embryogenesis in a red clover population. Plants capable of somatic embryogenesis were often characterized by an abnormal leaf morphology. Embryogenically competent "variants" did not exhibit any gross structural chomosomal changes. Isozyme banding patterns on starch gels for alcohol dehydrogenase, glutamate dehydrogenase and esterase failed to detect somaclonal variation between nonregenerative F49M plants and regenerative F49R plants. Peroxidases have been implicated in somatic embryogenesis, however, they did not appear to be a limiting factor in somatic embryogenesis for this particular red clover germplasm. Peroxidase zymograms on starch and IEF gels from regenerative petiole callus cultures showed fainter staining than corresponding non-regenerative cultures on embryo induction and particularly on shoot proliferation media. Level of differentiation was negatively correlated with numbers and staining intensities of peroxidase bands detected with 3-amino-9-ethylcarbazole on IEF gels.

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IMAGE EVALUATION
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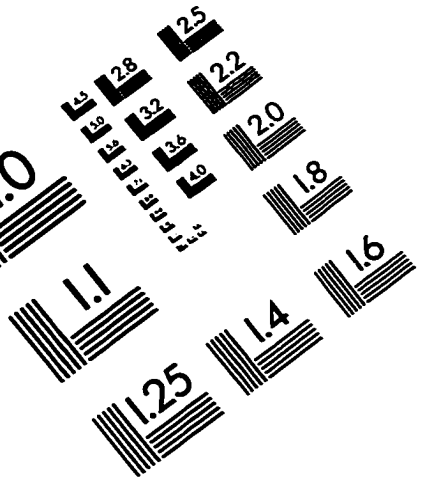

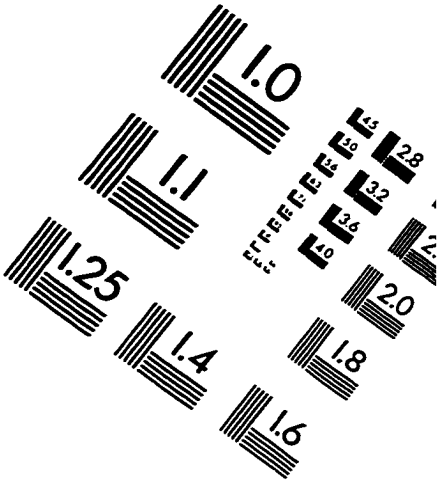

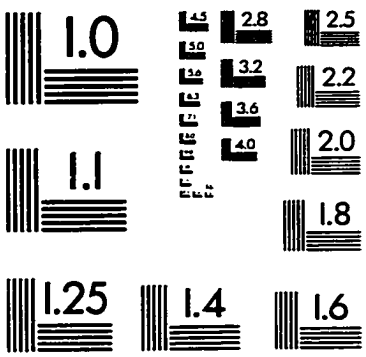

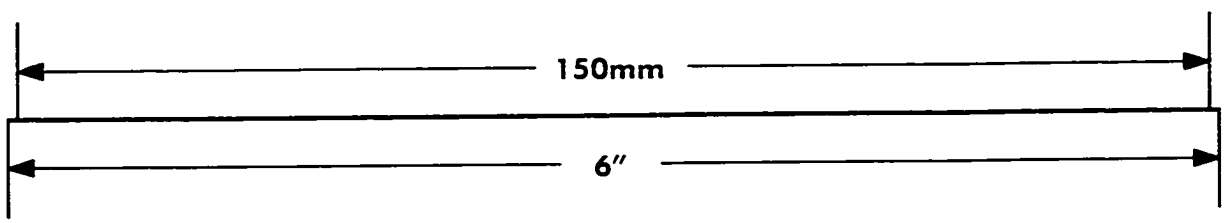

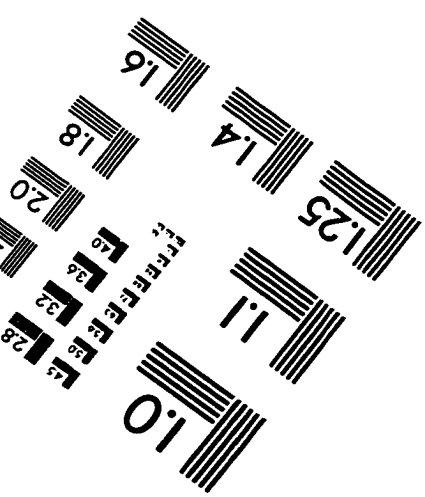

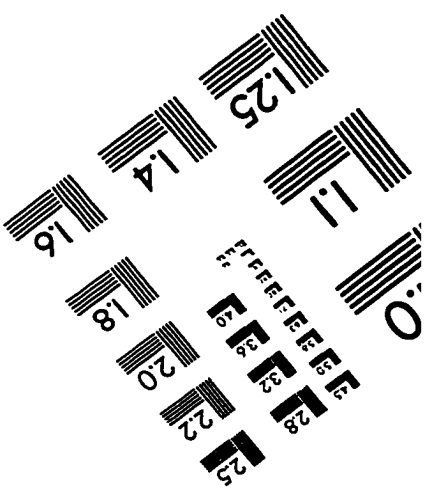

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