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IN VITRO SELECTION AND MOLECULAR
CHARACTERIZATION OF RED CLOVER
(*TRIFOLIUM PRATENSE* L.)
REGENERATIVE SOMACLONAL VARIANTS

by

Marek Nelke

Submitted in partial fulfillment of the requirements
for
the degree Doctor of Philosophy
at
Dalhousie University
Halifax, Nova Scotia
August, 1997

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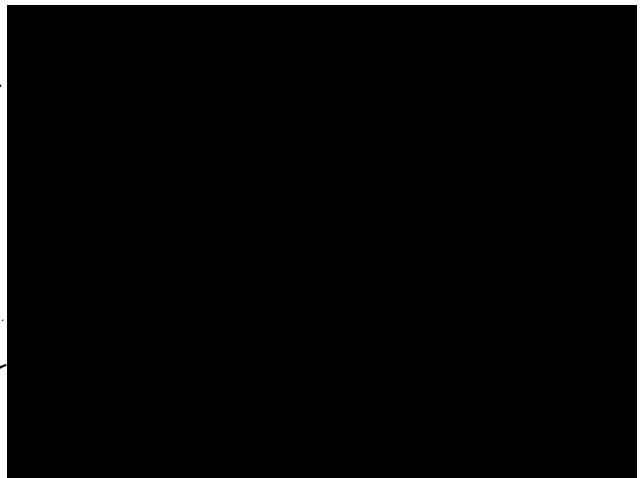
The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled “In Vitro Selection and Molecular Characterization of Red Clover (*Trifolium pratense* L.) Regenerative Somaclonal Variants”

by Marek Nelke

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

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DEDICATION

In memory of my mother, Helena who was too good to stay with us longer.

Teraz, gdy dni przypomnieć mogę -
te, które ciesza, te - co bola.
chetnie wspominam każda nogę...
i każda sucha pyrkę z solą.

e

Lech Konopinski

(Jest takie miasto, 1983)

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ABSTRACT

The expression of the regenerative trait in red clover is sporadic. A regenerative somaclonal variant, F49R capable of regeneration from somatic cells and transmission of this trait to progeny, was obtained from a diploid cultivar Florex ($2n=24$) (MacLean 1997). In this study DNA rearrangements in "the hot spot" of F49R genome were detected by DNA fingerprinting with Jeffreys' probe 33.6. These rearrangements seem to be linked to regeneration trait. This highly mutable DNA region was stable in non-regenerative callus of F49M, explant source for F49R. A common RFLP band linked to proline analogue (Azc) tolerance was also identified in this mutational "hot spot". The substantial changes in quantity of repetitive DNA associated with differentiation (amplifications) and de-differentiation (de-amplifications) of plant cell in tissue culture were detected by dot hybridization of genomic DNA using as probes repetitive DNA sequences isolated from a red clover lambda genomic library. These changes in repetitive DNAs during red clover regeneration took place independently of each other in different families of repetitive DNA sequences resulting in alternation in their relative proportion in the genome. This study showed that expression of multigene family, MsCOR A (Laberge et al. 1993), can vary in regenerative somaclonal variants, even though genomic organization of the gene is unchanged. Northern and Western analysis showed that cold-acclimated F49R and its regenerative progeny had induced expression (2-5 fold) of gene associated with improved cold-tolerance compared to cold-acclimated F49M and non-regenerative progeny of F49R. Genomic organization of the MsCOR A gene, revealed by Southern blotting, was the same for F49R and F49M.

LIST OF ABBREVIATIONS

Azc	L-azetidine-2-carboxylic acid
bp	nucleotide base pairs
cDNA	complementary deoxyribonucleic acid
Ci	Curie
Da	Daltons
dATP	deoxyadenosine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Hyp	trans-4-hydroxy-L-proline
kb (p)	kilobase (pairs)
M	molar
mRNA	messenger ribonucleic acid
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
pfu	plaque forming unit
RNA	ribonucleic acid
RFLPs	restriction fragment length polymorphisms
rRNA	ribosomal ribonucleic acid
SCE	sister chromatid exchange
SDS	sodium dodecylsulphate
VNTRs	variable number of tandem repeats
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

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PUBLICATIONS

Much of the research described in chapter 3 appeared in:

Nelke, M., Nowak, J., Wright, J.M. & MacLean, N.L. 1993. DNA fingerprinting of red clover (*Trifolium pratense* L.) with Jeffreys' probes: detection of somaclonal variation and other applications. *Plant Cell Rep.* 13: 121-125.

CHAPTER 1: INTRODUCTION

A Review of somaclonal variation

I. Introduction

The term "somaclonal variation" was introduced by Larkin and Scowcroft in 1981. It was defined as "a genetic variation displayed amongst tissue culture regenerants and their progeny". The genotype, explant source and culture conditions are factors contributing to plant regeneration in tissue culture and to the frequency of somaclonal variants (Peschke & Phillips 1992). Not all plant species, however, can regenerate from tissue culture (Chaleff 1983; MacLean & Nowak 1989), and even if they can, not all variations are stably inherited. Some traits may only be epigenetic. The concept of epigenetic change was proposed for expression of information that is present in the plant genome but that is normally not expressed in cultured cells. The epigenetic change is not transmitted through the gametes and its underlying mechanisms remain unknown (Meins 1989). The most convincing evidence of epigenetic changes *in vitro* is cytokinin habituation observed in calli derived from tobacco pith explants (Meins 1989). The calli lost their requirement for exogenous cytokinin for growth when the standard culture temperature was raised by 10°C. However, the regenerants from the cytokinin-habituated clones produced exclusively non-habituated pith cells under the standard tissue culture

regime. "Somaclonal" as opposed to the "epigenetic" is a regenerant from tissue culture with a heritable new trait(s).

The potential for utilization of somaclonal variation in plant breeding was recognized as early as 1981 (Larkin & Scowcroft). Successful selection for specific traits, such as resistance to diseases and amino-acids analogues, has been reported for many agronomically important species (e.g., improved leaf rust resistance in wheat, Oberthur et al. 1993; and in sugarcane, Screenivasan et al. 1987; mosaic virus resistant tomato, Smith et al. 1993; disease resistant trees, Ostry & Skilling 1988; *Fusarium* resistance and proline overproducers in alfalfa and red clover, Nowak et al. 1994). Some somaclonal variants are available on commercial markets, e.g., ultra-crisp celery, sweeter carrot, VineSweet tomato produced by "DNA Plant Technology" (Binwey 1993) and patents on valuable somaclonals have appeared (Whitaker 1991; Bird 1991).

There are limitations to the application of somaclonal variation for improved crop production. Firstly, the regeneration protocol has to be established as tissue cultures of different species and genotypes have different requirements for induction of either organogenesis or embryogenesis (e.g., amount and ratio of growth hormones in media). Secondly, the desirable genetic variant has to be monitored not only to ensure Mendelian inheritance of the acquired trait but also for stability of other important traits present in the donor genotype (e.g., synthetic seed

production, gene introduction via recombinant DNA technology). Most valuable variants to date appear to arise from single gene mutations (Miller 1985; Van den Bulk 1991). Unfortunately, in many plant species, *in vitro* systems for selection of quantitative characters have not yet been developed.

Gametic reproduction introduces stable adaptive genetic diversity into progeny by combining the genes of two parents. Somatic embryogenesis and callus organogenesis introduce genetic diversity into clonal progeny, but variability is gained by unpredictable mutational changes induced by tissue culture process. Somaclonal variation is being detected in constantly self-pollinated inbred rye lines maintained since 1926 (Puolimatka & Karp 1993).

A better understanding of molecular and genetic mechanisms that give rise to somaclonal variation may result in more controlled introduction of desirable genetic diversity into plants. It is difficult to distinguish between pre-programmed epigenetic/genetic changes, required for the de-differentiation process to occur, and developmentally independent mutation(s) imposed by the tissue culture process. Genetics of organogenesis and somatic embryogenesis is not a subject of this paper. For reviews of these topics see Hicks (1994) and Henry et al. (1994). This study concentrates on genetic mechanisms that may determine somaclonal variation in plant tissue culture. Contrary to the previous reviews dealing with genetics of somaclonal

variation (see for example Karp 1995, 1991; Peschke & Phillips 1992), the main emphasis of this review is placed on genomic changes at the DNA sequence level.

II. Biochemical changes

Changes in isozyme and seed protein banding patterns are the primary tests to detect biochemical modifications in plants derived from tissue culture. Isozymes are enzymes with different primary structures but which can catalyze the same reaction (Moore & Collins 1983). Loci which code for isozymes are codominant such that heterozygotes can be readily distinguished from homozygotes in F_1 or F_2 populations.

Isozymes and isomorphic protein analysis by gel electrophoresis have been used for screening somaclonal variation. In a study by Wang and Holl (1988), 60 red clover plants screened for 4 isozymes did not show changes related to tissue culture regeneration process. All the electrophoretic profiles detected were present in the original genotypes of seed-derived plants. Sabir (1991) analyzed 764 beet regenerants for variation using 7 isozyme markers and calculated from the observed band frequency of isozyme variants that approximately 50 genes in each regenerant could be affected and changed in tissue culture.

Seed proteins which have been monitored include prolamins (polymorphic among genotypes; Dahleen 1991-oat; Breiman 1987a- and Shimron-Abarbanell 1991;-barley) and

glutelins (variable subunits; Obukhova 1991-wheat). One avenin oat variant was identified by immunoblotting of isoelectrofocusing profiles with anti-avenin antibodies in 15% of the fifth generation progeny of karyotypically stable regenerants (Dahleen 1991). By morphological analysis of avenin variant lines, the presence of this prolamine isomorph was associated with occurrence of some agronomically important traits in oat, such as increased seed weight, seed number, grain yield and bundle weight.

The conclusions drawn from detected variations of the few cited representative studies should be viewed critically. Variation in proteins detected by gel electrophoresis (IEF and SDS PAGE) can result from: (i) post-translational modifications due to environmental or physiological factors; (ii) the fact that at a given time only a small proportion of genes is expressed; and (iii) variations between tissue type and stage of development, (Hayward 1987).

III. "Karyotypic" changes

Changes in chromosome number

In vitro chromosomal instability is a general phenomenon of plant cell culture. Many numerical variations are selected against during regeneration of plants, especially in diploids and may contribute to the loss of the morphogenetic capacity in long-term cultures (Winfield 1993). Changes in chromosome number, reflected in formation

of polyploids and aneuploids, may be a causative agent of somaclonal variation (Peschke & Phillips 1992). Association between the specific phenotype and the karyotypic change, however, can not be determined by cytological analysis alone (Meins, 1983). Bayliss (1980) reported culture-induced changes in chromosome number in 40 out of 55 plant species studied. D'Amato (1985) divided regenerants into four separate classes: (1) pollen-derived (androgenetic) plants; (2) regenerants from somatic cell and tissue cultures; (3) aneusomatic plants (presence of cells with many different aneuploid chromosome numbers in one regenerant); and (4) somatic hybrid plants. He cited for each class an impressive list of species in which changes in chromosome number, as a consequence of tissue culture, were detected. Changes in chromosome number are profoundly represented in long-term cultures. Leitch (1993) reported that long-term wheat line suspension cultures showed elimination of chromosomes and a wide range of differences in the number of metaphase chromosomes between cells. On average, the cells contained significantly reduced number of chromosomes (14-25) compared to that found in hexaploid wheat ($2N=6x=42$). A high frequency of tertiary chromosomes was found by Stelly (1989) in somaclonals regenerated from 18-month-old callus cultures of cotton. Higher ploidy levels in regenerants derived from long-lasting calli of diploid sugarbeet was also reported by Jacq (1992). Winfield (1993) observed instability in chromosome number in cell suspensions of diploid, tetraploid

and hexaploid wheat that increased with the age of culture. Protoplast-derived regenerants showed the highest frequency of deviation from the protoclones chromosome complement (Johnson 1984; Ward 1993). Regeneration from polyploids is associated with higher frequency of aneuploidy than regeneration from diploids or haploids, and the lower the ploidy level the greater the chance of polyploidization (Raja et al. 1992; Ward et al. 1993; Ashmore & Shapcott 1989).

A mechanism for altered chromosome number of culture-derived somaclonal variants has been proposed by Bayliss (1973). He found physically unorganized calli and cell suspensions of carrot possessed abnormal mitotic spindle formation that led to the occurrence of multipolar segregation and lagging of chromosomes (absence of the anaphase). The involvement of this process in ploidy reduction in cultured carrot cell lines and lily callus, has recently been reported by Nutti Ronchi (1992a) and Deumling & Clermont (1989), respectively. Amitosis, nuclear fragmentation and cellularization followed by mitosis, has been shown to be an important process in reduction in chromosome number and haploidization *in vitro*, mainly in endoreduplicated cells, in tobacco, potato, soybean, wheat and bean (D'Amato 1985) and pea (Natali & Cavallini 1987). The polyploidization of somatic cells can occur as a consequence of endoreduplication (chromosomes' duplication prior to mitosis), c-mitosis (absence of cytokinesis) and endomitosis (repeated nuclear divisions within a single cell)

(D'Amato 1985; Natali 1987). Thus, introduction into culture of an explant with some degree of polysomatism (polyploid and diploid cells) would result after callus mitotic divisions in cell lines with altered chromosome number. Plants which regenerate from such material would be chimeric (mosaics with two or more karyotypes-aneusomaty and mixoploidy). Chimeras can arise from 2 or more callus cells with different karyotypes, not necessary of explant origin, which may participate in generation of a new apical meristem or embryo (Pijnacker & Ramulu 1990). As such, cytological analysis of somaclonal variation should determine the presence or absence of chimeral plants among regenerants. Progeny analysis of F_1 and F_2 generations of self-pollinated callus-derived regenerants would indicate the presence of tissue culture-imposed chimeric plants. Chimeric regenerants have been reported in potato (Pijnacker & Ramulu 1990; Cardi 1993), maize (Lee 1987b; Zehr 1987) and oat (McCoy 1982). Progeny of some of the regenerants segregated for variant traits in the F_2 but not the F_1 generation.

Among the components of culture media, the growth regulators, especially auxins, are usually required for callus induction and proliferation. 2,4-dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin and its concentration has been implicated in the alternation of chromosome number in cultured cells of maize (Nagl 1988), wheat hybrids (Bai 1993) and barley (Ziauddin 1990). Indolyl-acetic acid (IAA), another auxin, induced endomitosis

in cultured haploid petunia, where 90% of the regenerants were diploid (Liscum & Hangarter 1993). Cytokinins, cell division promoting plant growth regulators, inhibit polyploidization in tissue culture (Ashmore & Shapcot 1989; Liscum & Hangarter 1993). *Haplopappus gracilis* (bristleweed) (Ashmore & Shapcot, 1989) exhibited a low incidence of polyploidization in regenerants when kinetin, in addition to 2,4-D, was included in the culture medium. In contrast, the regenerants grown in auxin only-supplemented medium displayed a significant increase in chromosome number. In haploid petunia (Liscum & Hangarter 1993), where explant leaf tissues were exposed to auxin or cytokinin treatments prior to growth on regeneration medium, benzyladenine had a stabilizing effect on chromosomes; 80% of regenerants were haploid. Growth regulator-influenced chromosomal changes are associated with the duration of cell cycle (Gould 1984). The mitotic cell cycle in eucaryotes is composed of 4 phases: G1, S, G2 and M, the duration of each phase being species- and cell- type specific. In carrot cell suspension culture, exogenous auxin application increased G1 and the overall length of the cell cycle (Bayliss 1975). According to the author, the G1 prolongation represents poor adaptation of cells to the tissue culture conditions. Other culture media components, such as sucrose concentration, may also trigger ploidy changes in tissue culture as observed in potato callus culture (Pijnacker & Ferweda 1990). Sucrose concentration-dependent changes in the normal mitotic cell cycle towards

endoreplication were detected in monohaploid but not in dihaploid and tetraploid potato. The authors concluded that sucrose requirements increase during the endoploidization of the monohaploids. As a result, the transcriptional capacity of cells increased sufficiently to perform accelerated by the sugar metabolic processes. Changes in chromosome number in tissue culture regenerants are commonly associated with reduced fertility and with altered genetic ratios in progeny of self-fertilized plants (D'Amato 1985).

Gross "chromosomal" rearrangements

Lee and Phillips (1988) suggested two mechanisms that may cause chromosomal rearrangements in tissue culture. The first is based on late replication of heterochromatin and the second on consequences of imbalance in the nucleotide pool. Any disturbance between DNA replication in S phase and cell division may cause chromosomal aberrations. Heterochromatic regions of chromosomes normally replicate later than euchromatic regions in S phase, and they are very sensitive to mitotic cycle fluctuations. Such fluctuations occur more frequently in tissue culture than *in vivo* (Gould 1984). The potential of late DNA replication as a mechanism of chromosomal rearrangements in tissue culture was first recognized by Sacristan's (1971) studies on callus culture of *Crepis capillaris* (hawk's beard). Later studies on oat regenerants showed that the most common cytogenetic alternations were chromosome breakages followed by loss of

chromosomal pieces and as a consequence, the production of heteromorphic pairs during diakinesis (McCoy 1982). The authors suggested that late replication of pericentromeric heterochromatic regions as a causative agent of observed bridges and breakages during anaphase. Aberrations in these regions and the confirmation of their late replication was demonstrated in oat root tip cells (Johnson, 1987). Li and Stelly (1989) showed frequent cytological aberrations in mitotic cells from callus derived from cell suspension culture of cotton. The presence of the anaphase bridges was the most profound structural characteristic. They proposed the breakage-fusion-bridges (BFB) theory to explain the high incidence of dicentric chromosomes in culture, and further suggested that imprecise replication of heterochromatic regions as a trigger for BFB cycles. Lee and Phillips (1987a) concluded from these studies on maize that simultaneous breakages in homologous chromosomes of tissue culture cells could produce duplications and deletions, whereas simultaneous breakages in non-homologous chromosomes would cause reciprocal, but not always exact exchanges. The type and frequency of rearrangements may depend on the distribution of heterochromatic blocks in chromosomes. These blocks of heterochromatin are known to be species specific. Oat chromosomes, for example, have heterochromatic pericentromeric blocks, whereas maize chromosomes have heterochromatic blocks in knobs, composed of different heterochromatid types (Lee & Phillips 1987a). The

differences in the distribution of heterochromatin may explain the predominance of telocentric chromosomes among oat regenerants, and the absence of such patterns in maize regenerants.

There is evidence that the intracellular pool of deoxyribonucleotides (dNTPs) affects the activity of several components of procaryotic and eucaryotic DNA metabolism (Lee & Phillips 1988). Imbalance in this pool can have serious genetic consequences. It affects mitotic recombinations, aneuploidy and structural aberrations that have been documented in procaryotes (Kunz 1982), yeast (Kunz & Haynes 1982), and some mammals (Weinberg 1981). Lee and Phillips (1988) suggested that *in vitro* cultured plant cells may also be sensitive to an imbalance of nucleotides, as they are periodically transferred from depleted to fresh culture media. Investigations on the effect of nutrient medium components at the end of the subculture intervals and fluctuation of the metabolic processes in plant tissue culture systems need to be conducted.

Many kinds of chromosomal rearrangements observed in tissue culture may be the outcome of 2 forms of mitotic recombination, mitotic crossing over and sister chromatid exchange. Involvement of these processes in generation of tissue culture variation has been suggested previously (Larkin & Scowcroft 1981; Lee & Phillips 1988). The frequency of mitotic crossing over in nature is low (Lorz & Scowcroft 1983). The phenomenon results from segment

exchanges between homologous chromosomes during mitosis and, in hybrid genotypes, which is detected as somatic mosaics showing recessive phenotypes. Due to the artificial environment in tissue culture, the frequency of somatic crossing-over may be increased (Larkin & Scowcroft 1981) in addition to the effect of some physical and chemical treatments. The multivalents observed in diakinesis of cytologically abnormal maize regenerants (Lee & Phillips 1987a) might reflect exclusive chromosomal interchanges without the involvement of the late replication breakages. Puolimatka and Karp (1993) analyzed metaphase I of donor plants and their regenerants for 6 different genotypes of rye and detected a high frequency of culture-induced translocations (the multivalent configurations) and *de novo* translocations, generally absent (bivalent forming) in donor plants. The authors observed changes in chiasmata distribution (visible manifestations of crossovers) in regenerants which are consistent with changes in meiotic recombination induced by tissue culture. Changes in repetitive sequences in culture may be involved and thus, meiotic crossing-over in regenerants affected. The formation of quadrivalents, composed of 2 ring bivalents connected by a single chromatin, in metaphase I, is an indication of pairing between homologous regions of nonhomologous chromosomes. These chromosomal structures were observed in pollen mother cells of crosses between tissue culture-derived, homozygous diploid tobacco and parental hybrids (Reed 1991). In tissue

culture of tomato, Evans (1989) observed somatic crossing-over in regenerated lines. Analyzing heterozygous alleles at five loci, he found that 19 out of 61 regenerants were products of mitotic crossing-over. Singsit (1989) observed higher cross-over frequencies in 50% of potato tissue culture regenerants. The frequencies were calculated from increases in genetic map distances between markers of regenerants and controls. Increases and decreases in recombination rates between sets of DNA markers were also detected in callus-derived tomato regenerants (Compton & Veilleux 1991). Moreover, Lee and Phillips (1988) reported that somatic crossing over may be asymmetric or occur between nonhomologous chromosomes, thereby enlarging the range of possible products from mitotic crossing-over. Similar to somatic crossing-over, tissue culture may induce asymmetric exchanges and permit the recovery of plants carrying the modified genotypes due to sister chromatid exchange (SCE).

SCE results from breakage in the DNA, followed by repair. Depending on the efficiency of the repair mechanisms, and if these breakages between the chromatids were symmetrical, no visible genetic consequences would be observed. If the repair mechanisms were ineffective, nonsymmetric exchanges could take place resulting in deletions and duplications. It has been shown on human chromosomes that DNA breakages are not generated at random, but occur in fragile regions of chromosomes often associated with oncogene insertion (Sutherland 1983). The frequency of

SCE can be detected by fluorescent staining of chromosomes after treatment of cells with 5-bromodeoxyuridine added to a thymidine-free medium. Using this method, Murata (1989) evaluated the effects of synthetic auxins (alpha-naphthaleneacetic acid, 2,4-dichloro- phenoxyacetic acid and 2,4,5-trichloro-phenoxyacetic acid) and one cytokinin (kinetin) on induction of SCE in wheat suspension cultures. He found a significant increase in the frequency of SCE when 2 mg/l of the auxin, 2,4,5-trichlorophenoxyacetic acid, was added to the medium. Dimitrov (1987) suggested that a delay in late replication increases SCE in tissue culture. The author's study on *Crepis capillaris* (hawk's beard) tissue culture regenerants observed SCE in the junction between early- and late- replicating regions and linked SCE to genetic instability of tissue culture systems.

Chromosomal, numerical and structural changes may occupy a central role in generating several forms of somaclonal variations because they can induce many changes to genome functions. However, chromosomal changes detected by cytological techniques, such as "staining", occur at the level of chromatin structure and therefore depend on a certain degree of chromatin condensation. Therefore, to obtain higher resolution of DNA changes and to understand the nature of genetic variation produced in tissue culture, karyotypic analysis should be performed in conjunction with genome analysis at the DNA sequence level. Advances in DNA-based technology has allowed a more thorough investigation of

genome structure and function, and makes us realize the intricacy and fluidity of the plant genome. Shapiro (1991) called the genome "the pre-programmed complex interactive information system".

IV. Molecular changes in the nuclear genome

Transposition

McClintock (1950) was the first to discover transposable elements in maize plants. Once activated by mutagen, these DNA-elements (0.4-17 kbp) can excise from one chromosomal site and re-integrate into a distinct site whereupon they may affect gene expression. Transposable elements were discovered in maize, snapdragon, soybean (see review by Doring & Starlinger 1986) and alfalfa (Groose & Bingham 1986a,b). It is interesting to note that in tomato, the most extensively mapped plant, transposable elements have not yet been detected. Transposable elements can exist in plants in two forms: autonomous elements which can self-transpose and non-autonomous elements, which can only be transposed in the presence of an autonomous element (Doring & Starlinger 1986). In many plant genomes, are also present genetic elements and they are called retrotransposons, and have structural features resembling those of retroviruses. Retrotransposons replicate by means of RNA intermediates. These RNA transcripts are copied into double-stranded DNA by a reverse transcriptase enzyme, which is encoded by the

retrotransposon. Integration at the new genome site requires specific inverted repeats at the ends and specific transposon-encoded enzymatic functions (Pouteau et al. 1991). Retrotransposons can occur at high copy number in plant genomes but are not included in the category of transposable elements, because they have been found in plants as non-mobile integrated units (VanderWiel et al. 1993). Thus far, only one mobile retrotransposon has been detected in plants (Grandbastien et al. 1989). It has not been yet demonstrated that other retrotransposons can induce mutant allele in new loci. Larkin and Scowcroft (1981) first proposed that the action of transposable elements may be a principal cause of somaclonal variation in plants. Tissue culture regimes may induce transposition of DNA sequences resulting in an effective form to increase the adaptation of regenerants to new environments. Activated transposons may be involved in the developmental process of de-differentiation of explant cells, as they potentially can initiate a range of DNA changes such as duplication, deletions, translocations (Lee & Phillips 1988). Staffort (1986) proposed that transposons activated in tissue culture act as controlling elements for reversion to a "wild-type" phenotype. Due to imprecise excision, mutations in the formerly repressed gene may lead to its expression. Repeated insertions and excisions could therefore cause somaclonal variation. Groose & Bingham (1986a,b) demonstrated a typical phenomenon of transposon activity on anthocyanin pigmentation amongst *in vitro*

regenerated alfalfa plants. They observed frequent reversions of unstable recessive alleles and recovery of new functional alleles at a locus. Revertants were stable *in vitro* and when re-cultured produced only pigmented regenerants. It has been also established that most of the non-revertant regenerants were stable in the recessive state. The activation of transposable elements in the Activator-Dissociation (Ac-Ds) system in tissue culture was observed in regenerants generated from maize explants with no active Ac elements (Peschke et al. 1987). The activity of Ac elements was demonstrated in progeny from crosses between the regenerated plants and tester plants carrying the non-autonomous element Ds, that transposes itself only in presence of Ac. The authors considered also that alternations in methylation patterns of inactive transposable elements could be responsible for their activation during tissue culture. The DNA analysis of Ac elements in an active and inactive state showed that when active, the Ac is nonmethylated in one or more sites, while these sites are methylated when Ac is inactive. There are several other reports on activation of transposable elements in plant tissue culture identified by their ability to induce mutant alleles at new loci (Peschke & Phillips 1991- activation of Suppressor-mutator (Spm) in maize; Planckaert & Walbot 1989- activation of Mutator system (Mu) in maize).

Repetitive DNA

A large portion of eucaryotes DNA does not code for protein. This DNA, on average 99% in the plant genome, is predominantly composed of repetitive sequences (Bassi 1991). These sequences are thought to represent mobile genetic elements (transposons) and sequences with no evidence of genetic mobility, such as the satellite DNA of centromeres and telomeres and most of retrotransposons. The repetitive sequences may be tandemly arranged and/or interspersed throughout the genome (see review by Smyth 1991). Transposons and retrotransposons represent the majority of interspersed repetitive DNAs of plant genomes. Transposons do not seem to be present above several hundred copies per genome but non-mobile retrotransposons are more abundant (the del 2 element, for example, constitutes 4% of the lilia genome - Smyth 1991) and universally present in plant species. Retrotransposons may be of viral origin, with long terminal repeats (LTR), generated from RNA polymerase transcript intermediates or non-viral origin, without LTR, derived from RNA transcripts from coding regions - retropseudogenes (see review by Smyth 1991). Tandemly arranged DNA includes repeated coding sequences such as histone and ribosomal genes and non-coding DNA repeats subdivided into satellites, minisatellites and microsatellites according to the size of the DNA monomer unit (see review by Wright 1993). Minisatellites and microsatellites often form clusters of monomer repeats

that are dispersed throughout the genome. Tandemly repeated sequences are generated during genome duplication by slippage of DNA synthesis, rolling circle replication and unequal crossing-over (Charlesworth et al. 1994). Recent studies on gene control in eukaryotes indicate that non-coding repetitive DNA might play vital roles in normal genome function (e.g., mutation in the minisattelite DNA near the Harvey ras gene may contribute to 10% of all cases of breast and bladder cancers, and acute leukemia - see review by Nowak 1994). Bassi (1991) hypothesizes that repetitive/non-coding DNA sequences in plants may, in addition to other functions, act as mediators between the environment and gene expression. This hypothesis is supported by several studies, cited below, that provide evidence for developmental consequences of gain or loss of repetitive DNA sequences, especially those occurring during plant regeneration in tissue culture. Quantitative variation of this DNA occurs *in vivo* and *in vitro* (see review by Bassi 1990). Stress conditions can make changes normally occurring at slow rate, more frequent. Heritable variations were found in flax varieties after growing for one generation in different specified environments (Cullis & Cleary 1986). From 9 flax repetitive DNA probes used in DNA slot blotting, 8 detected differences in copy numbers between some of the lines. More data on external factor-induced quantitative DNA variation in *in vivo* plants can be found in Bassi (1990). The most profound and frequent alternations at the molecular level are induced by

tissue culture stress in the process of plant regeneration (Table 1). Quantitative DNA changes in rDNA, non-coding repetitive sequences, multicopy gene family and unique sequences and restriction fragments length polymorphism (RFLP) have been reported (Table 1). There are some indications that tissue culture selection for particular regenerative genotypes from complex explant tissues can restore *in vivo* DNA profiles in regenerants from calli showing gross DNA changes (D'Amato 1990). RFLP has been detected in soybean cell culture regenerants, and all polymorphic loci observed appeared to already exist in natural populations (Roth et al. 1989). In maize, Brown (1991 - Table 1) reported deviation in calli RFLP in relation to explant-donor plants and reappearance of the former DNA banding patterns in regenerants. Cullis & Cleary (1986a - Table 1) reported similarity in quantity of repetitive DNA between genotrophs and their callus regenerants, but substantial reduction in amount of these DNAs in calli. Thus, to recreate these type of events and sizes of fragments (RFLPs), it appears that very specific DNA rearrangements must be taking place, I would call these rearrangements cell selection for regeneration. Nelke et al. (1993) using the Jeffreys' 33.6 probe detected, in Southern blots of red clover genomic DNA, an extremely high rate of mutation in a specific region of the genome in regenerative somaclonal variants. This region of the DNA seems to be involved in tissue culture cell selection for regeneration

Table 1 DNA changes during plant tissue culture

species	plant tissue	methodology	observations	references
alfalfa	cell suspension exposed to herbicide with glutamine-synthetase (GS) inhibition activity; cells lines and their regenerants with improved resistance to the herbicide	Southern hybridization with soybean GS cDNA as probe	11-fold amplification of the GS gene in somatic clones in comparison to the controls, the amplified DNA stable for more than 200 generations in the absence of selective pressure	Donn et al., (1984)
alfalfa	callus regenerants (immature ovaries) and 3 successive tissue culture generations (leaves)	DNA slot blotting; probes: pea 4S rDNA and 2 alfalfa repetitive	DNA amplifications and deletions (quantities of the same sequence can change bi-directionally in culture)	Kidwell & Osborn, (1993)
carrot	callus	electrophoresis of restriction enzyme digested DNA	reduction in repetitive DNA restriction fragments	Arnholdt-Schmitt (1993)
carrot	plantlets and their calli	video-densitometry of repetitive DNA restriction fragments after agarose gel electrophoresis	gross elimination of repetitive DNA in calli and DNA re-amplification in plantlets	Arnholdt-Schmitt (1995)
flax	9 genotrophs, their calli and regenerants (leaves)	DNA slot blotting; probes: rDNA, 2 satellite DNAs, 5 random repetitive DNAs (all from flax)	reduction in repetitive DNA of calli in relation to genotrophs and regenerants	Cullis & Cleary (1986a,b)

Table 1 (cont)

lilia	bulb callus, 2 years old subcultured aseptic regenerants and the same regenerants but grown extra year in garden	denaturation profiles (DNA melting curves) blot hybridization with satellite DNA from lilia chalcone synthase gene from snapdragon and 18S rDNA from parsley	bulb calli and aseptic regenerants showed: loss of nuclear DNA, loss of satellite DNA, reduced copy number of synthase gene, increased amounts of rDNA	Deuming & Clermont (1989)
maize	calli from immature embryos and regenerants	RFLP analysis with ATP/ADP translocator gene, sucrose synthase gene and waxy genes as probes	RFLP banding pattern deviations in calli from 2 maize lines and restoration of control DNA profiles in regenerants	Brown et al., (1991)
pea	in vivo and in vitro regenerated vegetative apices and whole shoots	DNA slot blotting densitometry; probes: 18S + 25S and 5S flax rDNA; highly repetitive DNA (HIR), medium repetitive DNA (MR) and unique DNA (U) from pea	reduction in 18S + 25S and 5S rDNA and U DNA in meristems of regenerants; amplifications of 18S + 25S rDNA, HIR and Mit DNA in shoot tissue of regenerants	Cecchini et al., (1992)
potato	2nd vegetative generation of callus protoplast derived	Southern hybridization with probe homologous to potato 25S rDNA	reduced amount of rDNA in 2 out of 12 plants	Landsmann & Uhrig (1985)
potato	leaf-disc regenerants, apical meristem derived plants and micro-propagated shoots	RFLP probed with potato rDNA fragment, 2 unknown low copy cDNAs and patatin gene (4 restriction enzymes) densitometry	qualitative and quantitative variations of rDNA in leaf-disc derived regenerants; RFLP variants for patatin gene and low copy genes present only in leaf-disc derived regenerants	Potter & Jones (1991)

Table 1 (cont)

rice	regenerants derived from immature embryo callus (28 and 67 days on callus medium)	RFLP analysis (Hind III digests); probes: ATP/ADP translocator gene, sucrose synthase gene, waxy gene, dehydrogenase gene, actin gene and rDNA genes	DNA polymorphism very low for short culture period regenerants (6% for actin gene)	Muller et al., (1990)
rice	cell suspension cultures and seedlings from different cultivars	dot hybridization of nuclear DNA with 9 highly repetitive DNA (including rDNA) and 1 interspersed rice DNA sequence	all selected DNA sequences were amplified in culture	Zheng et al., (1987)
soybean	suspension cultures from 2 cultivars and their F1 sexual hybrid intact plants	RFLP with soybean genomic DNA fragments as probes	RFLP allelic differences at various loci after culture RFLP alleles changes were the same as RFLP alleles found in vivo	Chowdhury & Vasil (1993)
sugarcane	embryogenic and non-embryogenic callus and suspension cultures and their regenerants	RFLP with maize 18S rDNA and wheat NOR locus probes	qualitative and quantitative DNA variations for 18S rDNA, no variations with NOR locus probes	
tobacco	pith tissue cultures after 0, 48, 96 and 144 hrs	DNA melting profiles CsCl gradient for DNA buoyant density determination	quantitative variations of satellite DNA during in vitro culture	Durante et al., (1983)
tobacco	pollen culture doubled-haploid regenerants	CsCl equilibrium	3 fold increase in satellite DNA in doubled-haploids in relation to the original line	De Paepe et al., (1982/1983)

Table 1 (cont)

tomato	regenerants from leaf-disc callus	RFLP with chlorophyll a/b binding protein gene (Cab) cDNA as probe	polymorphism for the Cab locus detected in 2 out of 17 regenerants tested	Nambisan et al., (1992)
wheat	cell suspension culture	In situ hybridization with wheat rDNA fragment and rye tandem repeat DNA as probes; Southern with cereal retro-element (dispersed DNA repeat)	most of rDNA copies lost in culture; changes in copy number of repetitive DNA; stable banding patterns of retroelement DNA	Leitch et al., (1993)
wheat	immature embryo callus regenerants and their sexual progeny	analysis of rDNA Inter-genic spacer in NOR loci Southern hybridization of <i>TaqI</i> and <i>EcoRI</i> - <i>Bam</i> III digests with rDNA spacer repeat as probe	changes in NOR loci in 1 out of 3 cultivars in relation to explant donor plants; heritable reduction in rDNA spacer repeats number	Brelman et al., (1987b)
wheat x rye (triticale)	callus (immature hybrid embryo) regenerants and their selfed progenies	In situ hybridization probes: 2 rye repetitive DNA sequences	telomeric amplification of repetitive DNA in 3 hybrid regenerants	Laplan et al., (1988)
wheat x rye (triticale)	regenerants from immature embryo callus	Southern hybridization with wheat rDNA spacer fragment; "C-banding" of chromosomes	rDNA stable but heritable reduction in the rDNA spacer sequence in NOR locus	Brettell et al., (1986)

(see Chapter 4). It has been suggested that temporary DNA amplifications leading to the specific changes in developmental patterns can result from differential DNA replication (Nagl 1979). Furthermore Nagl (1972; 1979) showed that DNA amplifications occurred in the parenchyma of *Cymbidium* protocorms cultivated *in vitro*. Such cultures showed 2 distinct cell populations. One resulted from regular endopolyploidization of both hetero- and euchromatin, and the other from endopolyploidization of heterochromatin. The chromatin amplification process took place during the first week after subculture and could be inhibited by the inhibitor of DNA synthesis, hydroxyurea. Microscopic observations of the long-term culture (2 weeks or more) subjected to ³H-thymidine pulse-chase experiment in the presence of hydroxyurea indicated that amplified DNA of nuclei is not stable, but released into cytoplasm and degraded. Presumably all origins of DNA replication can act as sites of amplification of nearby sequences. If the amplified units integrate into the chromosome, a new genome is created containing a new multigene family(s) (Donn et al. 1984). The authors imply that unstable DNA amplifications observed in plant genomes in response to environmental stress can cause epigenetic changes in gene expression. Some of the DNA amplifications in cultured calli and regenerants (Table 1) could be a consequence of differential DNA replication. Observed DNA amplifications as well as DNA deamplifications, especially in callus, can arise by mechanisms such as mitotic

recombination, late heterochromatin replication or sister chromatid exchanges (see section of this chapter - gross chromosomal rearrangements). Reports on DNA changes in tissue culture and the interpretation of the results have to be viewed critically. As communicated by Deumling and Clermont (1989), gross chromatin diminution (Table 1) in aneuploid lily bulb protoplast-derived callus and regenerants can be caused by the exceptionally big genome of this species (approximately 2.5×10^7 kb) and represents an interesting exception. The obvious genetic disturbances like the higher frequencies of chromosome abnormalities in callus culture from polyploids or the cytological instability associated with protoplast regeneration, might have strong impact on the results. The DNA reductions resemble changes in the ciliate genome during formation of the macronucleus (Prescott 1985). It has been shown that the expression of genes in *Drosophila* can be influenced by changes in the DNA surrounding the genes (Dutton & Chovnick 1988). Bennet and Gustafson (1982) demonstrated that repeated DNA sequences may affect the development of seeds in triticale, a hybrid of wheat and rye. The ends of rye chromosomes contain telomeric heterochromatin blocks which are absent in comparable regions of wheat. Triticale seeds are often abnormal and shriveled. The authors identified some lines producing less shriveled seeds. The rye chromosomes of those lines showed loss of telomeric heterochromatin blocks and the extent of the loss was correlated with decrease of shriveling. This region of

heterochromatin had no effect on seed shriveling when present exclusively in rye.

Methylation

Methylation may play an active role in tissue-specific regulation of gene expression (Cedar 1988) excluding house-keeping genes which seem to be constitutively unmethylated (Bird 1986). Bestor (1990) hypothesized that DNA methylation in large genomes of eucaryotes, including plants, evolved for protection from the possible deleterious impact of genome expansion. According to the author, compartmentalization of the genome into an unmethylated fraction for interaction with regulatory factors and a larger methylated fraction that is maintained in a repressed state permits appropriate gene regulation by decreasing the amount of DNA to be scanned by sequence specific regulatory proteins. Demonstration that eucaryotic transcription factors and certain proteins do not bind to methylated DNA suggested a mechanism by which methylation may inhibit gene expression (Dyner 1989). Generally, hypermethylation is associated with gene inactivation and hypomethylation is associated with gene activity. Plant regeneration from tissue culture via callus involves two successive developmental processes, de-differentiation and re-differentiation, probably accompanied by changes in DNA methylation status, analogous to several animal systems (Brown 1989). Methylation of cytosine alone (5mC) as well as cytosine contained within the trinucleotide

CpNpG accounts for differences in methylation sites between plants and animals. In animals, only cytosine in CpG couplets are methylated (Hepburn et al. 1987). Many studies of animals have shown that the transcriptional control regions of genes that are expressed in a given cell type are undermethylated compared to the same non-expressed sequences in other cell types.

Most studies of methylation/demethylation during tissue culture regeneration are based on Southern analysis where genomic DNA is digested with isoschizomeric enzymes. These are pairs of enzymes that recognize the same DNA sequence, but differ in sensitivity to DNA methylation of the restriction nuclease recognition site. For example, the isoschizomers, *Msp* I and *Hpa* II cleave at CCGG sites, but *Hpa* II will not cut DNA where the cytosine residues of the sequence are modified to 5'CmCCGG-3' or 5'mCCGG-3' by methylation. While *Msp* I will cut if the internal C is methylated, methylation of the external C, 5'-mCCGG-3' makes *Msp* I inactive (Busslinger et al. 1983). Similar methylation sensitivity exists in the isoschizomers, *EcoR* II and *BstN* I. *EcoR* II will not cut if the cytosine in the trinucleotide sequence CTG or CAG is methylated while *BstN* I will (Gruenbaum 1981). Extended hypermethylation of DNA sequences, sometimes accompanied by hypomethylation of other DNAs, of plant genomes in cell culture has been found (Brown 1989; Kaeppler & Phillips 1993; Cecchini et al. 1992; Arnhold-Smith 1993). Hypermethylation in tissue culture might

be a reflection of the process of disconnection of potential lethal genes during stress conditions (Muller et al. 1990). Brown (1989) performed Southern analysis on *Msp* I and *Hpa* II digested genomic DNA from maize tissue culture regenerants using structural and house-keeping genes as hybridization probes. Alternation in the methylation patterns were common and were detected even in inbred line regenerants from the same callus. Once methylation patterns were established, they were stable up to 3 generations of selfed progeny. In a similar study, but with different maize lines and DNA probes (sucrose synthase and alcohol dehydrogenase cDNA clones and 18 single-copy *Pst*I genomic clones) Kaeppler and Phillips (1993) detected alternations in DNA methylation. They were stably inherited and frequently homozygous in original regenerants which implies a non-random mutational mechanisms. Arnhold-Schmitt (1993) demonstrated an increase in DNA methylation in carrot root callus by analysis of DNA profiles generated by digestion with 2 pairs of isoschizomeric enzymes, *Eco*R II and *Bsp*N I, and, *Hpa* II and *Msp* I. The increase in DNA methylation was not correlated with the observed decrease in the number of repetitive DNA fragments during callusing. Cecchini (1992) compared the length of DNA fragments after isoschisomers digestion of genomic DNAs from control- and callus-regenerated pea plants. One line showed an increase in methylation for internal and external cytosines of the CCG sequence. Tissue culture-induced reduction in methylation in soybean was communicated

by Quemada (1987). This author established that soybean 5S RNA genes contain 2 CCGG sites, which when unmethylated can be digested by both *Msp* I and *Hpa* II but methylation of internal cytosine allowed only *Msp* I digestion. A 5S RNA clone was used as a hybridization probe in Southern analysis of the *Msp* I and *Hpa* II digested genomic DNA of root tissue cell suspension cultures and their donor plants of 2 soybean cultivars, and their F₁ sexual hybrid. Results suggest the absence of methylation in cultured cells but the presence of methylation in the intact plants. After a prolonged culture period, 5S RNA genes of the cells slowly regained resistance to a *Hpa* II cut, suggesting a remethylation process. Morrish and Vasil (1989) did not find any quantitative and qualitative methylation changes in embryogenic and non-embryogenic calli and explant donor plants of napiergrass.

The 5 azacytidine, a chemical analogue of m5C, was recently discovered as a potent demethylating agent. Employing the 5-azacytidine in culture medium, and Southern hybridization of isoschhisomer restricted DNA, Bezdek et al. (1991) showed demethylation of highly repeated DNA sequences in tissue culture of tobacco. The presence of azacytidine in medium for regeneration of maize protoclonal cultures had a negative effect on regeneration (Brown 1989). Nelke (unpublished) applied azacytidine in medium to induce regeneration of non-regenerative red clover callus cultures. No regeneration was observed. Azacytidine did not have any negative effect,

however, on embryogenesis and regeneration of regenerative somaclonal variant (MacLean & Nowak 1989). Foreign genes integrated into the plant genome can be reactivated in tissue culture after addition of azacytidine to the medium as has been showed by John and Amasino (1989) in an *Agrobacterium*-transformed tobacco plant. Blot hybridization of tobacco mRNA to an antisense oncogene transcript probe indicated activation of a silent hypermethylated oncogene.

Interpretation of the methylation state of genomes in tissue culture on the basis of the Southern analysis using isoschisomers for digestion of genomic DNA can be obscured by common *in vitro* DNA rearrangements. Employing DNA slot blotting, Cecchini (1992) detected a reduction even in the quantity of unique sequences in the pea genome after tissue culture regeneration (Table 1). In Southern hybridization experiments of this type only the use of developmentally specific genes as probes could demonstrate the presence or the absence of methylation-dependent gene expression. The biochemical mechanism of methylation is understood in contrast to demethylation biochemistry. The chemical removal of the methyl moiety from the cytosine base is considered to be thermodynamically impossible (Frank et al. 1987). It is possible that the demethylation state is imprinted in the plant genome and only the methylation processes can occur. Castiglione et al. (1995) investigated DNA methylation patterns of soybean metaphase chromosomes using specific monoclonal antibodies to 5mC. They detected differences in

methylation between corresponding regions of homologous chromosomes. This result implies that in plants some genes might be expressed only on one homologous chromosome and be silent on the other. Therefore, the expression of totipotency of plant cells might depend on the selection of cells with more active unmethylated genes and, as such, capable of differentiation into a mature plant.

V. Changes in cytoplasmatic genomes

Although the majority of genetic information is coded by nuclear DNA, essential functions linked to cellular energy are encoded by the chloroplast (cpDNA) and mitochondrial genomes (mtDNA). During *in vitro* culture, extrachromosomal genomes have shown variable degrees of instability.

Chloroplasts

Mutations in tomato cpDNA were detected by RFLP analysis (Evans 1984). Stability of the chloroplast genome was reported by Brears et al. (1989) in callus-derived sugar beet regenerants by comparing *Sma* I-digested chloroplast DNA profiles generated after agarose gel electrophoresis. When rice plants regenerated from pollen culture were analyzed by Southern hybridization with chloroplast-derived probes, large deletions in cpDNA were detected (Harada et al. 1991).

Mitochondria

Some variations in mtDNA have been found in plants regenerated in tissue culture. A large amounts of non-coding DNA give flexibility to retain mutations without disrupting functions. The multipartite structure of plant mt genomes with a master circle containing several sites of intermolecular recombination suggest one manner in which rearrangements may occur (Lonsdale et al. 1988). Another possibility for rearrangements is sequence duplication of repeats (Lonsdale et al. 1988). Rearrangements in mt DNA between parent, calli and cultured plants of tobacco (Dale et al. 1981), hexaploid wheat (Rode et al. 1988; Hartmann et al. 1994), wild barley (Shimron-Abarbanel & Breiman 1991), sugar beet (Dicalova et al. 1993), sorghum (Kane et al. 1992) were demonstrated by Southern blotting with different mitochondrial probes. All the alternations were detected in non-coding sequences of mitochondrial genomes; however, coding sequences of mitochondrial DNA's remained stable throughout cultures. The variability in mitochondrial DNA detected in tissue culture regenerants may arise from reciprocal recombination events in the specific unstable regions of the genome (Hartmann et al. 1994). As has been suggested by Shimron-Abarbanel and Breiman (1991), the selection mechanism restricts the regeneration of plants to cells having an unaltered mitochondrial coding capacity. Initiation of de-differentiation processes is associated with increased respiration expressed in increased numbers of

mitochondria by the cells' demand for mitochondrial genes products. Mitochondrial genes are among the most conserved genes known.

VI. Conclusions

In order to control the occurrence of variation in the plant genome due to tissue culture, an understanding of the biological processes, both phenotypic and genotypic, is required (Larkin et al. 1989). An understanding of the genetic changes during the process of regeneration would be a valuable contribution to the science of plant breeding. The literature shows that due to the development of DNA based technology, genomic research has advanced gradually in the past 15 years. It has been recognized, for example, that mitosis, a basic process of *in vitro* propagation, is not conservative. It shows many variations and suggests great fluidity. On the other hand, reported heritable changes in flax (Cullis 1986 a,b) and other plants (Bassi 1990) are attributed to alternations in environmental conditions. Those observations resurrect the mechanism of evolution proposed by Lamarck almost two centuries ago. The process of plant adaptation takes place through mutational mechanisms. The same mutations occur naturally, apart from possible differences in degree and/or frequency, and can be detected in somaclonal variants (soybean-Roth et al. 1989; maize-Brown 1991; flax-Cullis 1986a,b; review by d'Amato 1985). Accumulated botanical data indicates that changes in cell

metabolism might be an original source of mutations in plants *in vivo* and *in vitro* (d'Amato 1990). For example, Cullis (1977) observed that flax plants grown under different nutrient concentrations generated genetic changes which in one generation became heritable and did not reverse when conditions were reversed.

Shapiro (1991) proposed a concept of the genome as a complex interactive information system; hence, it is able to process outside information. In plant cell and tissue culture, this information can be received via environmental stress to generate somaclonal variation. Thus, the current concept of "constant genome" should be replaced by "fluid genome", a true dynamic system in which function can be adjusted as the conditions require.

The hypothesis

De-differentiation and re-differentiation processes which occur in tissue culture during callus formation from somatic tissues and plant regeneration from callus, respectively, are associated with qualitative and quantitative changes in genomic DNA of red clover. The hypothesis that some of these DNA changes generate somaclonal variants with valuable character traits was tested in this thesis.

Objectives of the study

The regeneration trait in red clover is very rare. The regenerative mutant (F49R) was selected from callus culture of a non-regenerative genotype (F49M) (MacLean & Nowak 1989). The first objective of this thesis was to identify molecular markers for regeneration to produce genetically diverse red clover lines. The second objective was to evaluate somaclonal variation as a tool for red clover improvement by:

1. studying the frequency and stability of tissue culture-derived genetic changes using DNA fingerprinting;
2. RFLP analysis of *in vitro* selected genotypes tolerant to proline-analogues;
3. analyzing quantitative and qualitative changes of repetitive DNA sequences during *in vitro* plant regeneration;
4. investigating a molecular basis of improved cold tolerance in red clover regenerative somaclonal variants by assaying the expression of cold-induced genes.

CHAPTER 2: MATERIALS AND METHODS

I. Plant material

Seeds of red clover cultivars Florex, Arlington, Lakeland, Atlas, Altaswede, Prosper I and germplasm CRS-1 were obtained from Dr. Bert Christie, Agriculture Canada Research Station, Charlottetown, Prince Edward Island, Canada. Genotypes F49Rh1-15 are single hypocotyl-derived regenerative clones, genotypes F49Rp are leaf petiole-derived regenerative clones, genotype F49M is epicotyl-derived non-regenerative mother plant-explant donor for F49Rh and F49Rp (Figs. 2.1 and 2.2) (MacLean & Nowak 1989). F₁ plants were generated from field-produced seeds of crosses between F49Rh clones and non-regenerative genotypes of cultivar Florex. G2a and G2b were produced by backcrossing (by hand) F₁ non-regenerative genotype G with clone F49Rh2. This plant material was maintained as tissue culture plantlets and mature plants grown in the greenhouse in 20cm pots on peat, perlite, soil (1:1:1) mix. All listed above plants, except genotypes F49Rp, were selected and provided by Drs. Jerzy Nowak and Nancy MacLean, Nova Scotia Agricultural College, Truro, Nova Scotia, Canada (MacLean & Nowak, 1989). Genotypes F49Rp were selected by the author.

II. Tissue culture

Detection of the regenerative trait, plantlet multiplication and maintenance were as described by MacLean &

Figure 2.1 Outline of an *in vitro* screening protocol for regeneration of red clover genotypes and selection of single hypocotyl derived clones (F49Rh). Seeds were surface sterilized in 2% w/v sodium hypochloride, then germinated and grown to 7-9 day-old seedlings on growth regulator-free agar medium (without sucrose) in GA7 culture vessels (Magenta Corp., Chicago) Epicotyl from the particular seedling was placed on growth regulator-free agar medium in culture tubes (150 mm x 25 mm) and grown into plantlets. Hypocotyl segments from the same seedling was used to evaluate regeneration capacity of hypocotyl callus. The segments were put on callus initiation medium (auxin rich - up to 4 mg/l) in sterile 24 cell well plates (Corning) with 2 ml medium per well (A). The 4-week-old callus was transferred to the plate containing somatic embryogenesis induction medium (auxin poor) (B) and, somatic embryos, if formed, were transferred (after 4 weeks) to the plate with shoot development induction medium (C). Finally, generated shoots were put in culture tubes (on growth regulator-free medium) to grow into regenerative plantlets (1, 2, 3, ...n). F49M - epicotyl-derived non-regenerative plant, an explant source for hypocotyl-derived regenerative clones F49Rh (cv. Florex) the only one which regenerated from 642 genotypes screened. Clones are plantlets regenerated from the same explant callus For detailed culture conditions and media composition see Plant Cell Rep. (1989) 8: 395-398 and Appendix.

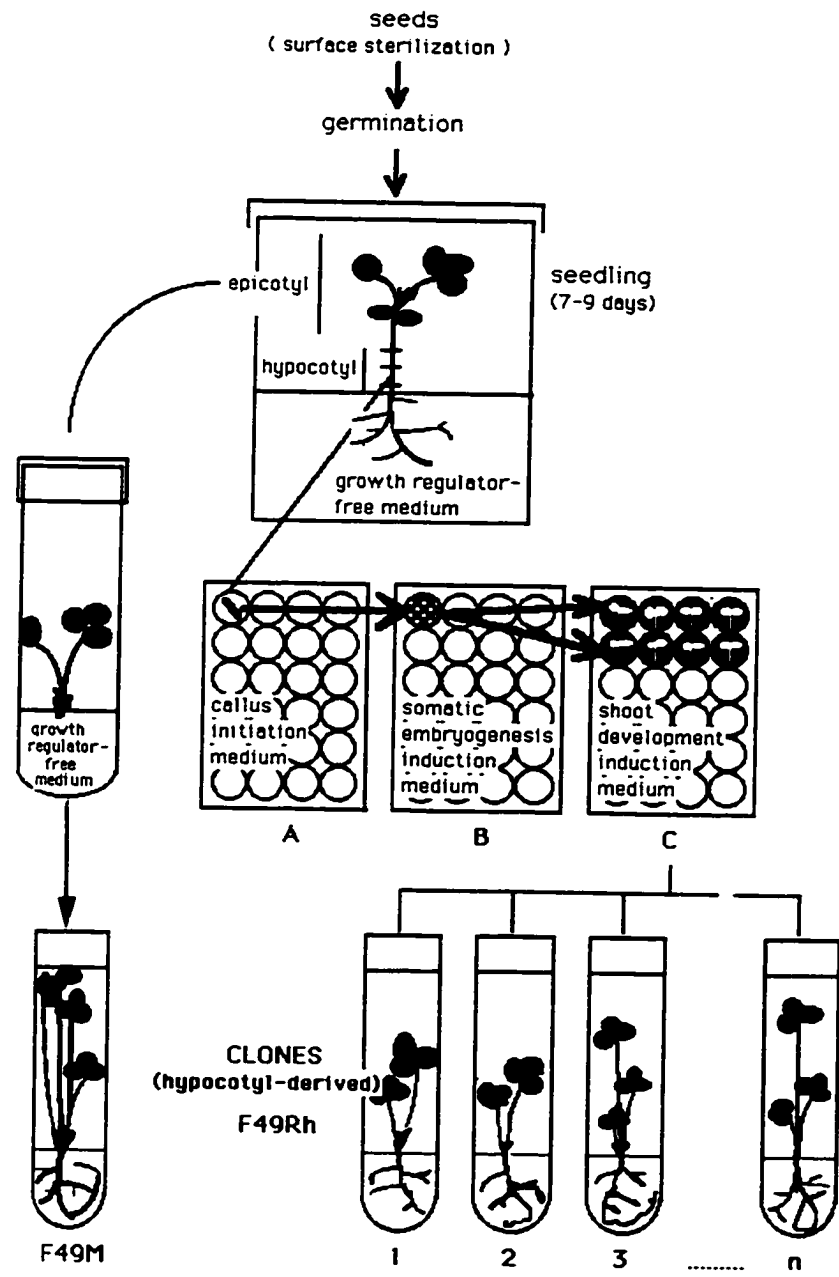


Figure 2.1

Figure 2.2 Screening procedure for selection of leaf petiole- derived regenerative clones (F49Rp). Petiole sections were excised from non-regenerative epicotyl-derived F49M plantlet growing in culture tubes (150 mm x 25 mm) on growth regulator-free agar medium and placed on callus initiation medium (auxin-rich) in sterile 24 cell well plate (Corning) with 2 ml medium per well (A). After 4 weeks callus was transferred to the wells of plate containing somatic embryogenesis induction medium (auxin-depleted) (B). Somatic embryos, if formed during 4 weeks period, were placed in cell well plate with shoot development induction medium (C). After 4 weeks of growth shoots were anchored in growth regulator-free medium in culture tubes and grown to leaf petiole-derived clones F49Rp (1, 2, 3, ...n). For details on the regeneration protocol see Collins and Phillips (1982) and Appendix.

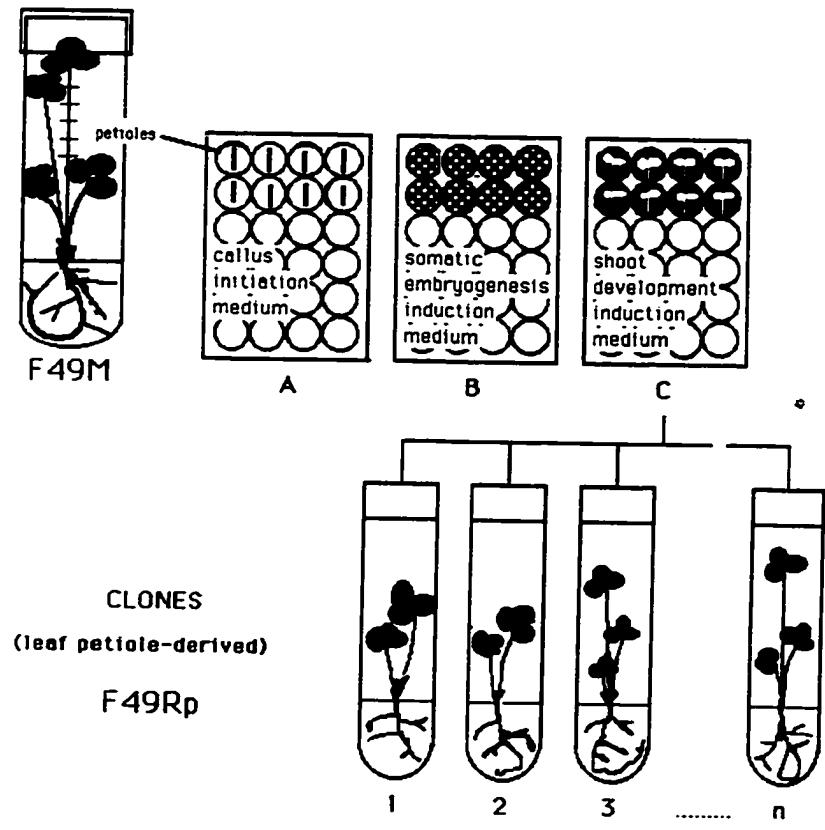


Figure 2.2

Nowak (1989). Leaf petiole explants of F49Rh clones were used to produce the second generation of regenerants which were the explants donors for regeneration of the third generation plantlets. A three-step protocol with four-week-long passages on L2 (callus-inducing), SEL (somatic embryo-inducing) and SPL (shoot formation-inducing) media was employed (Collins & Phillips, 1982; see also Figs 2.1 and 2.2). For media composition see Appendix.

III. Genomic DNA extraction

DNA was extracted from leaf samples of either 1-2 month-old plants grown in the greenhouse or six-week-old tissue culture plantlets according to the procedure of Doyle & Doyle (1990). DNA from callus was isolated by the method of Quemada et al. (1987).

IV. Restriction endonuclease digestion

Genomic DNA was digested in 2 stages by the indicated restriction enzyme using a total of 4 units of enzyme per μg DNA. In the first stage, 3 units/ μg DNA were added and the reaction incubated for 2 h. In the second stage, a further 1 unit/ μg DNA of the restriction enzyme was added and the reaction incubated for at least 2 h at the temperature recommended by the manufacturer (Pharmacia). Digested DNA was purified by extraction with one volume of phenol/chloroform, precipitated by ethanol, air-dried and then redissolved in TE buffer.

V. Agarose gel electrophoresis

Digested genomic DNA samples (20 µg) were fractionated by gel electrophoresis on either 1% agarose gels at 1.5 volts/cm for 20-26 hrs or on 0.7% agarose gels at the same voltage for 44-60 hrs. A 1x Tris-acetate-EDTA buffer was used for electrophoresis. Gels were stained with 0.5 µg/ml ethidium bromide solution and then photographed.

VI. Southern blotting

DNA was transferred from agarose gels to nylon membranes (Hybond-N, Amersham) by vacuum blotting using a Vacu-Blot apparatus (Pharmacia). DNA was depurinated with 0.25 M HCl for 20 min, denatured with 1.5 M NaCl, 0.5 M NaOH for 20 min, neutralized with 1.5 M NaCl, 1.0 M Tris-HCl, pH=7.6 for another 20 min and then transferred with 20 X SSC (3.0 M NaCl, 0.3 M sodium citrate, pH=7-7.2) for 60 min. Membranes were then washed in 2 X SSC, air-dried for 15 min and baked at 80°C for 2 hr.

DNA dot-blotting

The nylon membrane with a grid of squares with 6-mm sides was wetted with 6 X SSC and laid on the lid of a plastic box. The samples were heat-denatured in a boiling water bath for 10 min before being applied in a 2 µl volume to the membrane. Following application of DNA samples, the membrane was placed for 10 min on a stack of three sheets of

Whatman 3MM paper that were saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH). The membrane was then transferred to a stack of 3MM paper soaked in neutralizing solution (1 M NaCl, 0.5 M Tris-HCl, pH 7.0) and incubated for a further 5 minutes, following by air-drying and baking at 80°C for 2 hr.

VII. Radiolabeling of nucleic acid probes

Genomic DNA was labeled by nick translation using alpha [³²P]- dCTP (3000 Ci mmol⁻¹) (Mundy et al. 1991). Jeffreys' probes, 33.6 and 33.15, (Cellmark Diagnostics) were labeled according to specifications of the supplier using alpha [³²P]- dGTP (3000 Ci mmol⁻¹). The cloned DNA inserts, and the repetitive DNA fragments isolated from a red clover genomic library used for hybridization to the DNA dot blots, were labelled by random oligonucleotide priming (Feinberg & Vogelstein, 1983, 1984) with alpha [³²P]- dCTP (3000 Ci mmol⁻¹). Unincorporated nucleotides were removed from all probes by Sephadex G-50 column chromatography.

VIII. Hybridization analysis of immobilized nucleic acids

Nylon membranes were incubated for at least 1 hour in hybridization buffer containing 0.5 M sodium phosphate, pH 7.4, 7% SDS, 1% BSA, 1 mM EDTA, pH 8 (Westneat et al. 1988) at 58°C or 60°C, then the denatured (by heating in boiling water bath for 5 minutes) radiolabelled probe was added to

hybridization reaction. Hybridization was carried out in the same solution and temperature for 20 to 24 hours using a Hybaid rotating hybridization oven. Membranes were washed at varying stringencies. Genomic DNA blots hybridized to Jeffreys' probes 33.6 and 33.15 were washed with 2 X SSC plus 0.1% SDS for 10 minutes at room temperature, followed by 10 minutes at 58°C with either 0.5 X SSC, 1 X SSC or 2 X SSC for high, medium or low stringency washes, respectively. Genomic DNA blots hybridized to other probes were routinely washed with 1 X SSC plus 0.1% SDS for 15 minutes at room temperature and then twice with 0.5 X SSC, 0.1% SDS at 60°C for 20 minutes. Autoradiographs were developed after 2-10 days exposure of membranes to Kodak X AR-5 film with an intensifying screen at -70°C. To estimate quantitative differences in the repetitive DNAs of different plant genomes and to determine their genomic copy number (DNA dot blot hybridization analysis), equal squares of the membrane containing repetitive and genomic DNA sequence were cut and the radioactivity measured by liquid scintillation spectrometry. The amount of radioactivity for each dilution was plotted versus the amount of DNA applied, and copy number estimated by comparing the repetitive DNA sequence and genomic DNA applied at equivalent hybridization signal. For re-hybridization of DNA blots on nylon membranes with a different probe, the membrane was boiled in a 0.1% SDS solution and then exposed overnight to x-ray film to confirm efficiency of probe stripping.

IX. Library screening

A genomic DNA library of F49M genotype was commercially prepared (Clonotech). The DNA was partially digested with *Mbo* I and then cloned in the lambda vector EMBL 3. The library contains an average insert size of 10 kbp, with very high titer (2×10^{10} pfu/ml). The phage library was diluted to yield a maximum 30,000 pfu for each 150-milimeter plate and grow on the bacterial host strain *Escherichia coli* K802. Plaques were transferred to nylon membranes according to the Sambrook et al. (1989). DNA was denatured for 1.5 minutes in solution containing 1.5 M NaCl and 0.5 M NaOH, then neutralized for 5 minutes in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0), rinsed in 3 X SSC and baked for 2 hours at 80°C. After hybridization with the DNA probes the membranes were washed for 15 minutes in 2 X SSC, 0.5% SDS at room temperature, followed by 2 one-hour-long washes in 1 X SSC, 0.1% SDS at 60°C. Plaques that hybridized strongly to the DNA probes, as seen on the autoradiographs, were selected and used for preparation of lambda DNA by small or large scale plate lysate methods (Sambrook et al. 1989).

X. Cloning methodology

Lambda DNAs from selected EMBL-3 clones were digested with *Sfi* I or *Xho* I restriction enzymes to release inserts carrying DNA sequences of interest. These digests were subjected to agarose gel-electrophoresis and, following ethidium bromide staining, DNA inserts were excised from gel

and recovered after passage through glass powder medium (Gene-Clean-BIO 101 Inc.). To make the DNA inserts blunt-ended for ligation, they were treated with mung-bean nuclease (1 unit/ 10 μ g DNA, Pharmacia) at 37°C for 1 hour and then were subjected to phenol extraction (Sambrook et al. 1989). These lambda DNA inserts were blunt-end ligated into the *Sma* I site of pUC18, dephosphorylated vector, employing conditions recommended by the supplier (Pharmacia) except that the ligation buffer contained 10mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 1 mM rATP, 5 mM DTT, 5% PEG. Ligation products were used to transform MAX Efficiency DH5alpha Competent Cells (Gibco/BRL), following the supplier's protocol. The cells were plated on 2 X YT medium containing ampicillin (50 μ g/ml), X-Gal (50 μ g/ml), and IPTG (0.5 mM). Recombinant plaques, indicated by lack of color, were selected and prepared for DNA sequencing by standard protocols (Sambrook et al. 1989).

XI. DNA sequencing

Double-stranded recombinant DNA plasmids were sequenced by the chain termination method of Sanger et al. (1977) using alpha [³⁵S]-dATP with T7 DNA polymerase and M13 universal primer (Pharmacia T⁷ Sequencing Kit). The reaction products were fractionated by electrophoresis on 6% acrylamide gels.

XII. RNA isolation

Total RNA was extracted according to the method of De Vries et al. (1988). Two to four grams of the plant material were ground in liquid nitrogen and added to 4-8 ml of preheated to 90°C extraction buffer. Extraction buffer contained 1:1 mixture of phenol and 100 mM LiCl, 10 mM EDTA, 1% (w/v) SDS. The sample was rotated for 15 minutes on a shaker at 200 rpm, then 2-4 ml of chloroform was added and the sample was shaken for another 15 minutes, followed by centrifugation at 20,000 x g for 30 minutes at room temperature. The supernatant was withdrawn and LiCl added to a concentration of 2 M and the sample was kept at 4°C for 20-24 hours. The RNA was recovered as a pellet after centrifugation at 12,000 x g for 30 minutes at 4°C. The pellet was washed with 2 M LiCl, then with 80% (v/v) ethanol, and afterwards, air dried and dissolved in TE buffer (pH 7.5). RNA in the sample was quantified by measuring its absorbance (at 260 nm) in spectrophotometer.

XIII. RNA gel electrophoresis

Total RNA (20 µg) was denatured in formaldehyde and separated on 1% agarose/formaldehyde gel for 5.5 hours at 5 V/cm as described by Sambrook et al. (1989). All RNA samples subjected to electrophoresis contained 1 µl of ethidium bromide (1 µg/ul).

XIV. Northern blotting

The denatured agarose gel containing fractionated RNAs was photographed to visualize the quality and quantity of RNA preparations, and then washed 4 times for 15 minutes in diethyl pyrocarbonate (DEPC)- treated water. RNAs were transferred for 1.5 hours onto nylon membrane (Amersham) by vacuum blotting (Vacu-Blot, Pharmacia) using 20 X SSC. Membrane was then washed briefly in 2 X SSC, air dried and baked for 2 hours at 80°C.

XV. Northern hybridization

Conditions for Northern blot hybridization were identical to those of Southern blots. After hybridization, the membrane was routinely washed with 1 X SSC plus 0.1% SDS for 15 minutes at room temperature and then twice with 0.5 X SSC, 0.1% SDS at 60°C for 20 minutes. Autoradiographs were developed after 1-14 days exposure of membranes to Kodak X AR-5 film with an intensifying screen at -70°C.

XVI. Protein extraction

Total proteins were extracted by direct homogenization of plant tissue in dissociation buffer (Nechushtai & Nelson, 1985). The dissociation buffer contained 0.1 M Tris-HCl (pH 6.8), 2 mM EDTA, 2% (w/v) SDS, 2% (v/v) beta mercaptoethanol, 10% (v/v) glycerol and 1 mM PMSF. After 1 hour incubation at room temperature, samples were centrifuged at 30,000 x g for

10 minutes, the supernatant was removed and heated at 70°C for 20 minutes, followed by 10 minutes centrifugation at 30,000 x g. 1 mM PMSF was added to supernatant to be stored at -20°C.

XVII. SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were subjected to electrophoresis on 12.5% acrylamide mini- gels (Bio-Rad) using the Laemmli buffer system (Laemmli, 1970). A kit of molecular weight standards (Bio-Rad) was used as molecular weight standards. After electrophoresis (at 30 mA for 1 hour) gels were either stained in 0.1% Coomassie blue in 40% methanol and 10% acetic acid solution and destained in 40% methanol, 10% acetic acid or their proteins were immediately transblotted on nitrocellulose membrane.

XVIII. Proteins immunoblotting (Western blotting)

Proteins were electroblotted on nitrocellulose membrane at 40 mA for 20 hours at 4°C using Bio-Rad transblot apparatus and buffer recommended by Towbin et al. (1979). Non-specific antibody binding sites on the membrane were blocked by 2 hours of incubation in phosphate buffered saline (PBS : 81 mM Na₂HPO₄, 19 mM NaH₂PO₄ and 150 mM NaCl), pH 7.5 containing 0.1% of Tween-20 (a non-ionic detergent). The membrane was incubated with the polyclonal, primary antibodies (1:1000 dilution) in PBS-Tween-20 buffer for 2 hours and then washed 3 times for 15 minutes with the same

buffer. Anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim) diluted in PBS-Tween-20 was used as the second antibody and membrane was incubated in this solution for 2 hours and washed again 3 times for 15 minutes with PBS-Tween-20. The binding of antibodies to proteins was visualized by treating the membrane with X-phosphate/NBT substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride) supplied by the manufacturer (Boehringer Mannheim).

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CHAPTER 3: DNA FINGERPRINTING OF RED CLOVER WITH JEFFREY'S PROBES

I. Introduction

In a selected red clover mutant, *in vitro* regeneration is thought to be controlled by two complementary genes (MacLean & Nowak, 1992). Incorporation of this trait into the red clover germplasm improved its low temperature tolerance (Nowak et al. 1992). Unfortunately, the regenerative trait in this species is very rare as less than 1% of genotypes regenerate from petiole callus in tissue culture (MacLean & Nowak, 1989; Phillips and Collins, 1979). Moreover, detection of the regenerative trait is laborious and time consuming, involving culture initiation of aseptic tissue and a three-step media protocol, totalling at least thirteen weeks. Identification of molecular markers for regeneration would allow for rapid development of regenerative red clover lines with diverse genetic backgrounds. Such lines could be utilized in the production of cultivars with upgraded persistence and other genetically improved qualities, and for hybrid seed production.

Recently, a class of numerous, dispersed and highly polymorphic repetitive DNA sequences, termed minisatellites or variable number of tandem repeat (VNTR) loci, (Jeffreys et al. 1985a, b; Nakamura et al. 1987) have been shown to be ubiquitous in the eukaryotic genome (reviewed in Wright, 1993). As such, minisatellites have received a great deal of

interest because of their potential as genetic markers for pedigree analysis in selection and breeding programmes (Wright, 1993).

In this chapter, I describe the use of the commercially available multi-locus DNA fingerprint probes, 33.15 and 33.6 (Jeffreys et al. 1985a, b), to search for regenerative trait markers, to detect somaclonal variation, to determine genetic stability in tissue culture and to produce genotypic fingerprints in red clover.

II. Characterization of the DNA fingerprints

In genomic DNA digested with *Dra* I, a 15 kbp fragment (Fig. 3.1A) was detected in F49Rh (lane 2) that was absent in F49M and regenerative F₁ (lanes 1 and 3). In *Rsa* I digested genomic DNA (Fig. 3.1B), an 11 kbp band was present in the DNA fingerprint of F49Rh (lane 2) but absent in the DNA fingerprints of F49M and regenerative F₁ (lanes 1 and 3), while a 2.2 kbp band was present in the fingerprints of F49M and regenerative F₁ but absent from that of F49Rh. In *Hind* III digested DNA (Fig. 3.1C), a 5.4 kbp fragment was detected in the F49Rh DNA fingerprint (lane 2) but not in the fingerprints of F49M and regenerative F₁ (lanes 1 and 3). DNA fingerprints generated by probe 33.6 with *Hind* III and *Dra* I- digested genomic DNA from three consecutive generations of regenerants, F49Rh, F49R1g, and F49R2g were identical (Fig. 3.2A and B). DNA fingerprints of four F₁ plants produced by probe 33.6 in *Hind* III and *Dra* I digests revealed that each

Figure 3.1 DNA fingerprints generated by probe 33.6 in *Dra* I (A), *Rsa* I (B) and *Hind* III (C) -digested genomic DNA of red clover. F49M (lane 1), F49R (lane 2) and a regenerative F₁ plant obtained from a cross between F49R and a non-regenerative cv. Florex genotype (lane 3). Arrows indicate DNA changes detected in somaclonal variant (F49R). Molecular weight markers are 1 kb ladder (BRL).

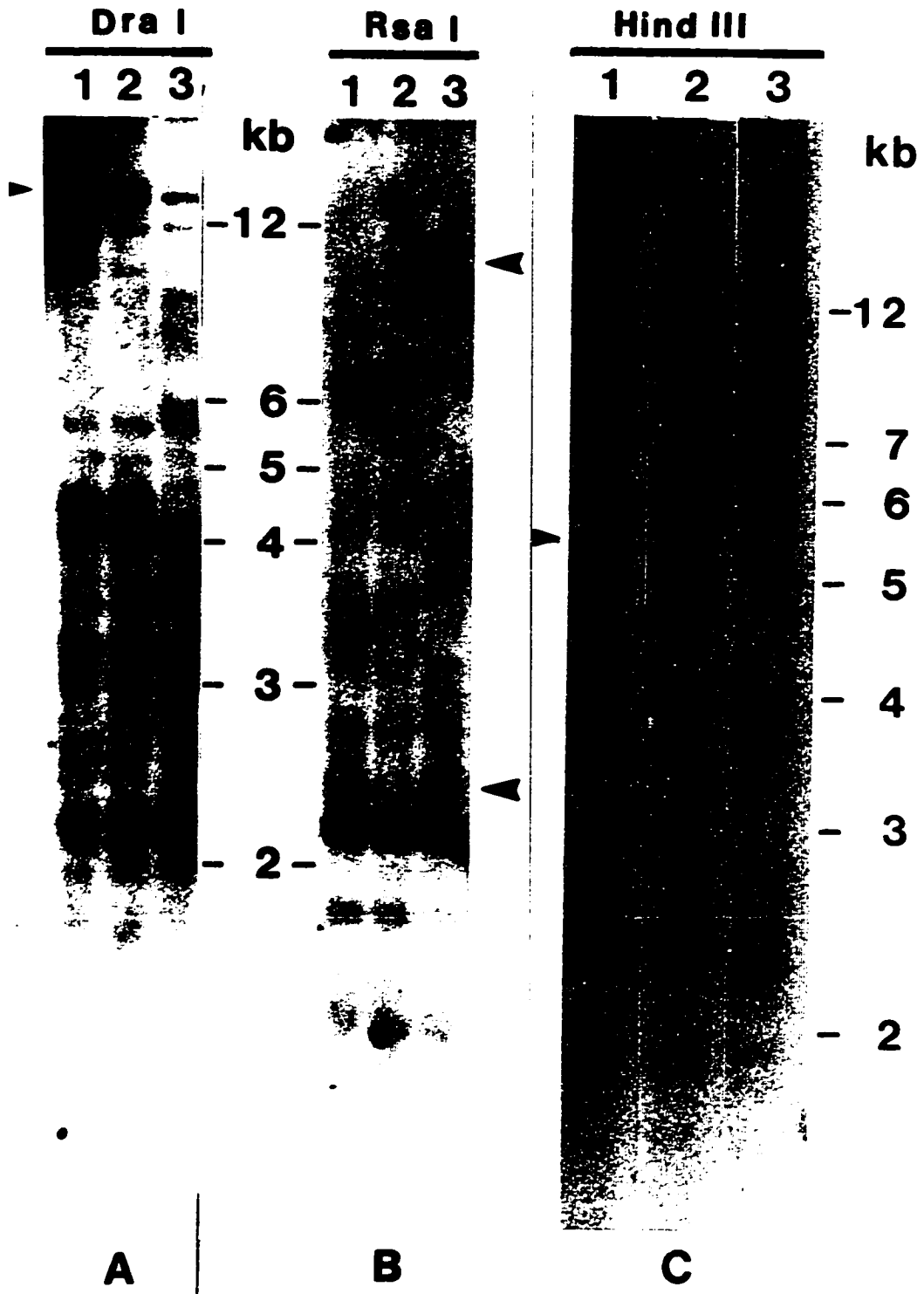


Figure 3.1

Figure 3.2 DNA fingerprints generated by probe 33.6 in *Hind* III (A) and *Dra* I (B) -digested genomic DNA of red clover. F49M (M) and F49R somaclonal variant after 3 consecutive regeneration cycles via callus culture (R, R1g and R2g; g stands for tissue culture generation). Lanes 1 and 2 represent two different regenerants. Molecular weight markers are 1 kb ladder (BRL).

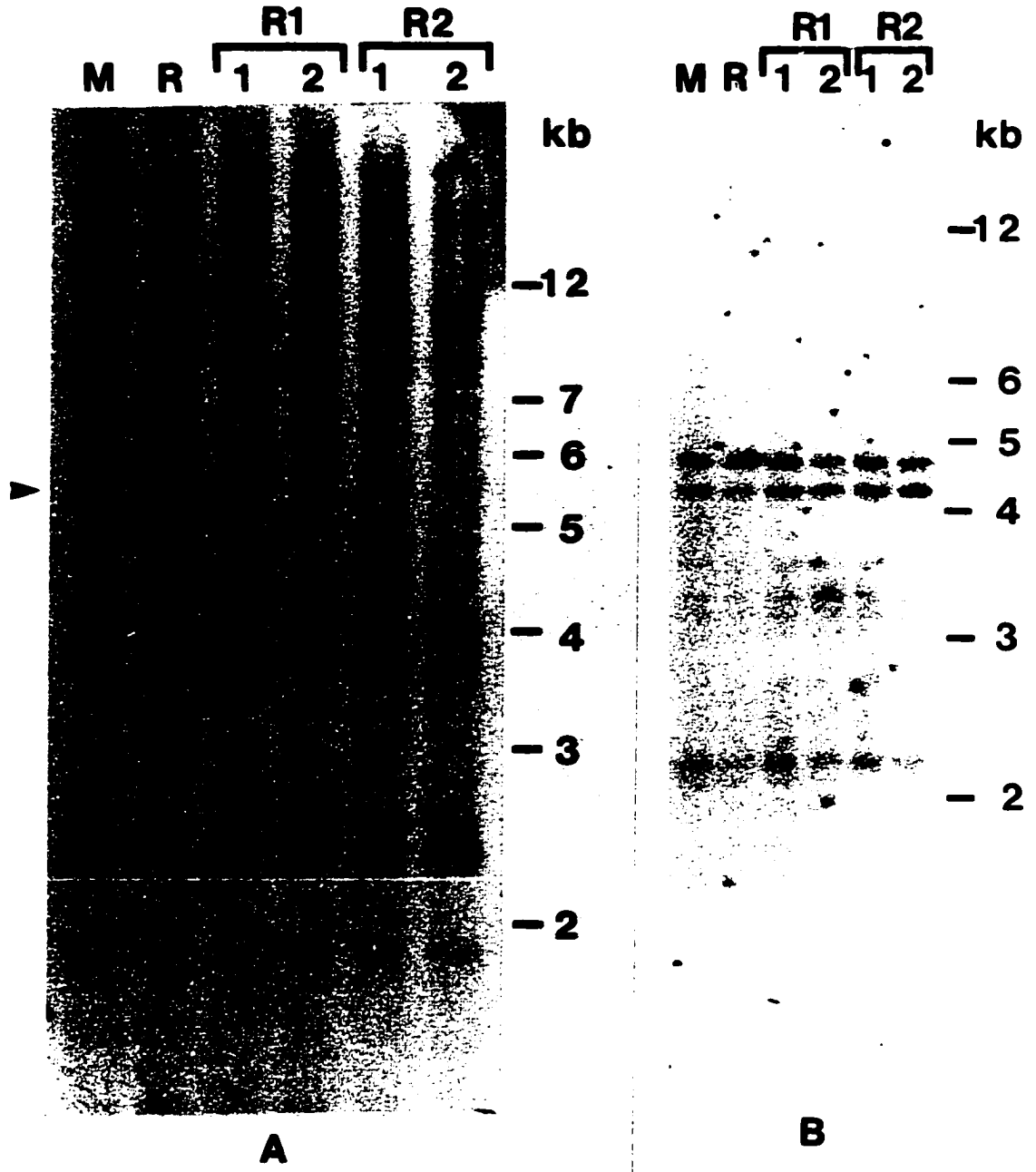


Figure 3.2

Figure 3.3 DNA fingerprints generated by probe 33.6 in *Hind* III (A) and *Dra* I (B) -digested genomic DNA of red clover. Hand-crossed parents, F49R (□) and a non-regenerative cultivar Florex genotype (○), and their four F₁ progenies (◇). Seeds from the cross were grown into F₁ plants and four of them were randomly selected for the experiment. Molecular weight markers shown at the right of the panels are a 1 kb ladder (BRL).

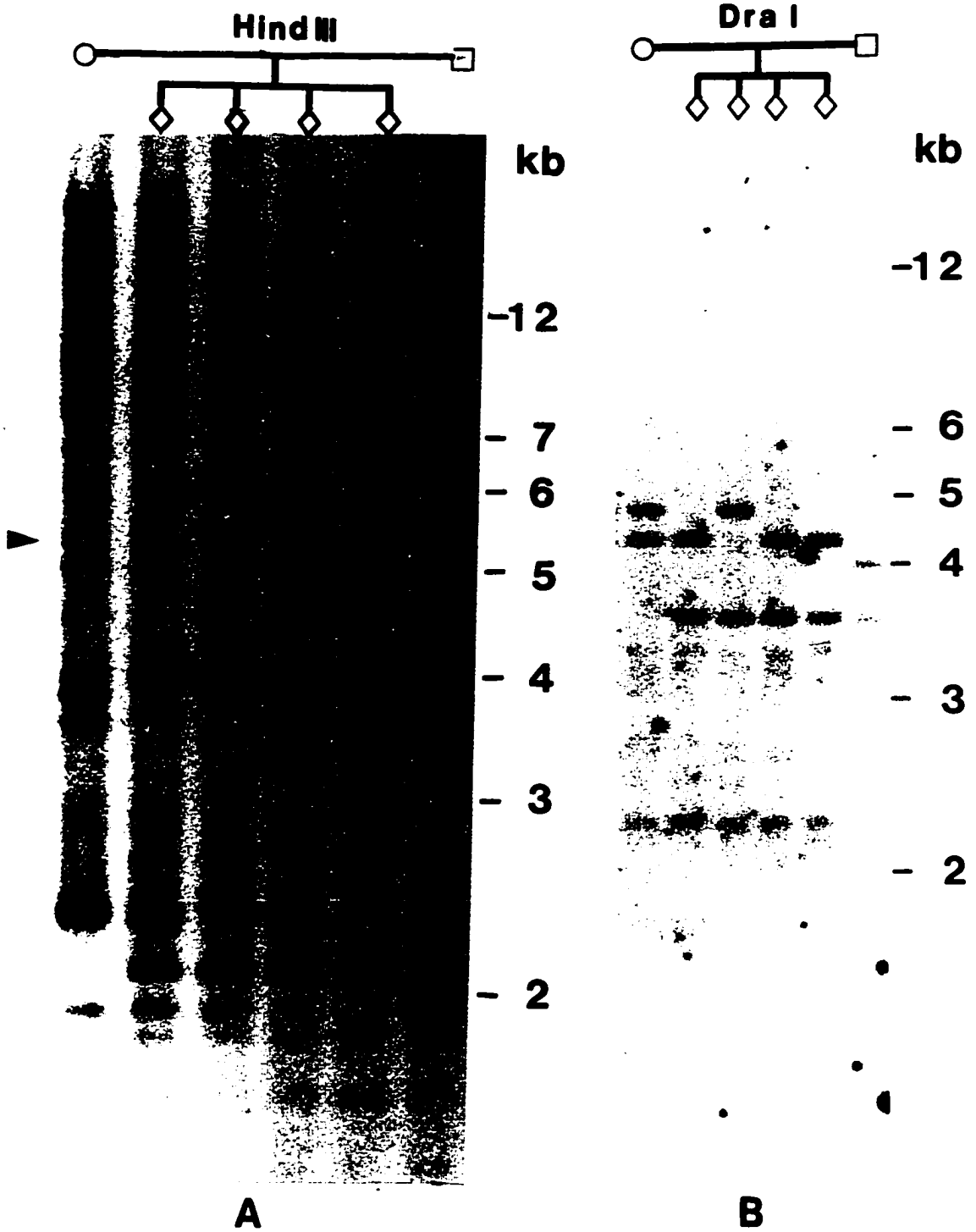


Figure 3.3

Figure 3.4 Multilocus DNA fingerprints of seven cultivars of red clover generated by probe 33.6 in *Hind* III-digested genomic DNA from individual plants. Molecular weight markers shown on the right of the panel are a 1 kb ladder (BRL).

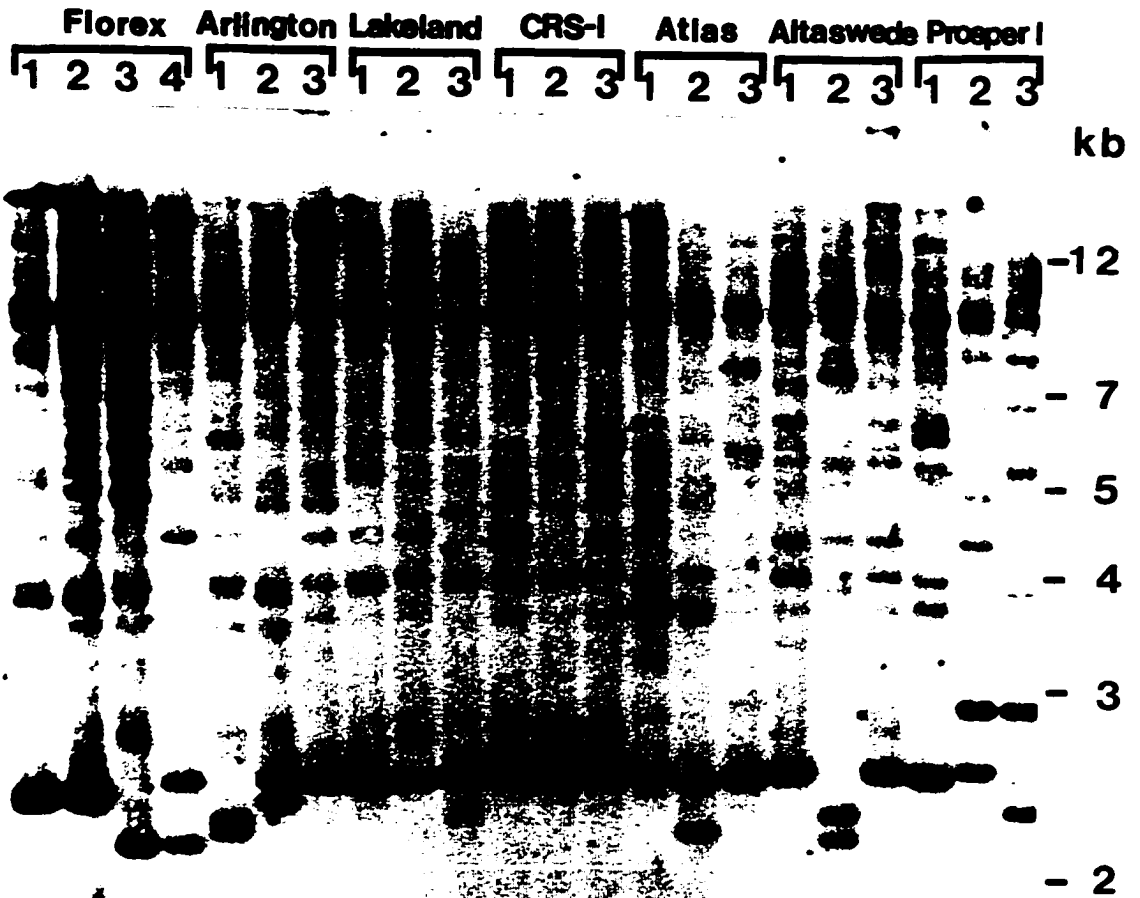


Figure 3.4

Figure 3.5 Multilocus DNA fingerprints of 7 cultivars of red clover generated by probe 33.15 in *Hind* III-digested genomic DNA from individual plants. Molecular weight markers shown on the right of the panel are a 1 kb ladder (BRL).

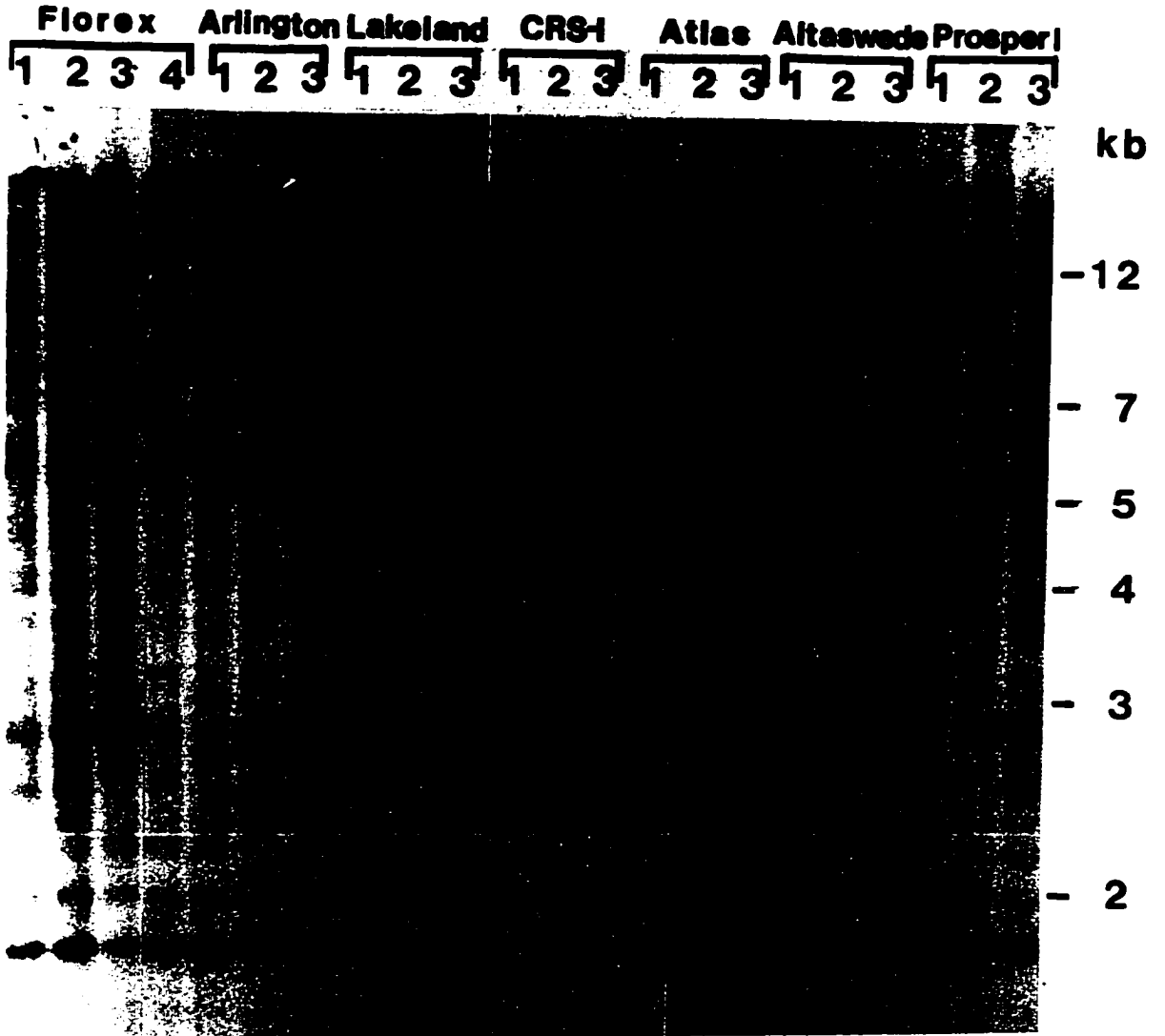


Figure 3.5

polymorphic band was inherited from either one or the other parent (Fig. 3.3A and B). Probes 33.6 and 33.15 generated different fingerprints for the same individual DNAs digested with *Hind* III (Figs. 3.4 and 3.5). Four monomorphic fragments, common to all analysed cultivars and genotypes, were detected with probe 33.15 but none with 33.6. Probe 33.15, however, produced multilocus fingerprints with high background (Fig. 3.5). Higher stringency of washing did not improve the quality of the DNA fingerprints produced by probe 33.15 (data not shown). DNA fingerprints produced by probe 33.6 (Fig. 3.4) gave less background and fewer but better defined bands than those generated by 33.15 (Fig. 3.5). On average 4-11 bands could be scored for each genotype in the 33.6 fingerprints, with 2-3 fragments showing strong hybridization signals. The number of bands detected by this probe was greater when genomic DNA was digested with *Hind* III than with *Rsa* I or *Dra* I. At higher stringency washes, fingerprints produced by the 33.6 probe again showed less background and fewer bands than DNA fingerprints produced by 33.15. In the DNA fingerprints produced by probe 33.6, more bands were seen with 2 X SSC (Fig. 3.1A) than with 0.5 X SSC washing solution (Fig. 3.2B). Both probes, 33.15 (Fig. 3.4) and 33.6 (Fig. 3.5) identified individual genotypes. Genetic variability (V), determined for the fingerprints obtained with probe 33.6, was greater between cultivars than within cultivars. V values for individual cultivars were: 0.417 (Altaswede), 0.485 (each, Atlas and Lakeland), 0.500

(Florex), 0.511 (CRS-1), 0.524 (Prosper 1), 0.548 (Arlington) and for all genotypes of the seven cultivars together, $V = 0.899$.

III. Discussion

DNA fingerprints generated by the Jeffreys' minisatellite hybridization probes, 33.6 and 33.15, indicate the presence of minisatellite-like sequences in the red clover genome (Figs. 3.1-3.5). To date, only a few reports have described the successful application of human minisatellite probes to DNA fingerprinting of plants (Dallas, 1988; Rogstad et al., 1988; Tzuri et al. 1991; Vainstein et al. 1991; Parent & Page 1992; Besse et al. 1993).

In this study, probe 33.6 detected a RFLP in the regenerative somaclonal variant F49Rh, obtained under tissue culture conditions from the non-regenerative red clover genotype F49M (Fig. 3.1). This RFLP was not linked, however, to the regenerative trait as the regenerative F_1 plants assayed ($n=5$, one shown; Fig. 3.1, lanes 3) did not inherit any of the bands distinguishing F49Rh from F49M. *

* At this stage of the research, it was assumed that all F49Rh clones were genetically identical and it was not noted which of the calli clones was a parent for regenerative F_1 . Further experimental work has shown that the *Dra* I fragment detected in the F49Rh DNA fingerprint but absent in F49M (Fig. 3.1A) varies in the molecular weight and organization (1 or 2 bands) between the F49Rh clones (Chapter 4, Fig.

It is entirely feasible that a more extensive survey of the red clover genome using other multilocus fingerprint probes (see Wright 1993) may prove useful in detecting DNA fragments linked to the genes controlling regeneration, such as, for example, DNA rearrangements linked to mortality loss in human cell tumors (Fey et al. 1988).

The fingerprints obtained under the applied conditions demonstrated genetic stability of consecutive generations of the regenerants in tissue culture. These findings support earlier observations that indicated karyotypic and phenotypic stability of the F49Rh somaclonal variant during subsequent cycles of regeneration from leaf petiole explants via callus culture (MacLean & Nowak personal communication, Truro, 1994). The presence of all hypervariable fragments of F₁ plants in one or other of parents indicates that DNA fingerprinting has the potential for pedigree analysis of red clover; multilocus probes can be employed in RFLP linkage to agronomically important traits (Hillel et al. 1990) and single locus probes for offspring identification in controlled crosses.

* 4.1). The conclusion drawn in Chapter 3 (Fig. 3.1A, B, and C) that there is no linkage between regenerative trait and the detected mutation became questionable, especially in the light of the preliminary experimental data which indicated that the linkage exists but is clone specific (Chapter 4, Fig. 4.3).

The poorly defined DNA fingerprints with 33.15 probe may result from the presence of highly reiterated, dispersed DNA motif in the red clover genome exhibiting partial sequence similarity to the minisatellite. High background was also reported when the 33.15 probe was tested on rice (Dallas 1988), rose (Tzuri et al. 1991), rubber tree (Besse et al. 1993) and other organisms (Bentzen et al. 1991).

In summary, the multilocus DNA fingerprint probes of Jeffreys, especially 33.6, in combination with various restriction enzyme and different stringency washes, generate informative DNA fingerprints in red clover. This should prove useful for: (1) the detection of morphologically invisible somaclonal variation; (2) discrimination between epigenetic and genetic changes in micropropagated material; (3) monitoring genetic stability of the regenerative material in tissue culture, particularly critical in hybrid seed production; (4) identification of somatic hybrids after protoplast fusion; (5) in red clover breeding where DNA fingerprints can estimate genetic distance between breeding lines; (6) in determination of linkages between specific RFLPs and useful traits, and subsequent selection of parental genotypes for the production of synthetic cultivars.

CHAPTER 4: HYPERVARIABLE DNA SEQUENCES IN A SELECTED GENOTYPE OF RED CLOVER AND ITS SOMACLONAL VARIANTS

I. Introduction

Differentiated tissues taken from a different positions on a plant have specific functions and usually can not survive on their own. As soon as a cell is determined to serve a specific role in a plant, some genes responsible for totipotency are silenced by specific developmental programs. These processes can not be reversed without genetic changes leading either to silencing of tissue-specific genes or activation and/or enhancement of expression of other genes to restore the cell to an undifferentiated state. These developmental genetic changes include or are accompanied by DNA amplifications, DNA diminutions, DNA transpositions and DNA methylation (see reviews by Karp 1991 and 1995). Although, not all genomic alternations are functional, many of them are independent mutations imposed by environmental stresses. Distinction between tissue culture-induced DNA changes leading to differentiation and those involved in de-differentiation is unclear because of limited understanding of the plant regeneration process at the molecular level. Researchers could gain additional insight into designing tissue culture conditions for improved regeneration frequencies if these processes were understood. This knowledge could have important practical implications, e.g:

in artificial seed production for clonal propagation. Plant tissue culture systems based on adventitious regeneration via callogenesis and/or indirect somatic embryogenesis are the best suited for studying molecular principles of plant regeneration. Those culture systems assure easy distinction and access to tissue from different developmental stages of the plant. Some plant species can regenerate with high frequency; e. g., tobacco, potato, petunia. Other plants are recalcitrant to regeneration, e. g. red clover. Red clover is one of the most intractable species and regenerates from callus with frequency less than 1% (MacLean & Nowak 1989) .

DNA fingerprints generated by Jeffreys' probe 33.6 with *Dra* I digested genomic DNA of the F49Rh clone revealed a one high molecular weight DNA fragment which was absent in the DNA fingerprint of F49M (see chapter 3). Characterization of this mutation was the focus of this study. A high rate of DNA rearrangements has been observed in this region when red clover regenerative somaclonal variants were DNA fingerprinted with Jeffreys' 33.6 probe (Fig. 4.1a,b). Possible clone- specific linkage of the DNA rearrangements to the regeneration trait was examined. Only one common RFLP band linked to proline analogue tolerance has been identified in this highly mutable DNA region.

II. Isolation and characterization of the highly polymorphic DNA region

Dra I and *Hind* III- digested genomic DNA of non-regenerative F49M and clones of F49R regenerative somaclonal variants were DNA fingerprinted with Jeffreys' probe 33.6 (Fig 4.1a,b). Both digests generated variable DNA fingerprinting patterns. The molecular weight of highly variable fragments were, 14-25 and 23-30 kbp, respectively, for *Dra* I and *Hind* III- digested DNA. Detected changes in the size of high molecular weight DNA with 2 different restriction enzymes testify against point mutation(s). Eleven F49Rh and 4 F49Rp clones showed different banding patterns in this highly mutable region as compared to the non-regenerative F49M, (Fig. 4.1a, b).

Interestingly, DNA fingerprints of F49M and its petiole-derived callus (pooled calli from 24 different petioles) are identical (Fig. 4.2) indicating that callusing alone does not generate the RFLP. It still has to be shown if specific DNA rearrangements in this "hot spot" are required for the regeneration process to occur. However, the frequency of regeneration from F49M derived explants is higher than that might be expected from the occurrence of spontaneous mutations in eukaryotes, which is roughly estimated for 10^{-5} - 10^{-6} events per locus per generation. Six out of 192 F49M petioles subjected to the tissue culture regeneration protocol (McLean & Nowak 1989) produced regenerative clones-F49Rp (Fig. 2.2). Two high molecular weight restriction fragments were detected

Figure 4.1 DNA fingerprints generated by probe 33.6 in *Dra* I (a) and *Hind* III(b) digested genomic DNA of F49M (lane 6), regenerative clones F49Rp (lanes 1-4) and F49Rh (lanes 5, 7-17). Numbers indicate individual plants. 7 describes clone 5 which passed through an additional regeneration cycle via callus culture. Molecular size marker are high molecular weight markers (Gibco-BRL). See Figs. 2.1 and 2.2 for explanation of F49M, F49Rh and F49Rp.

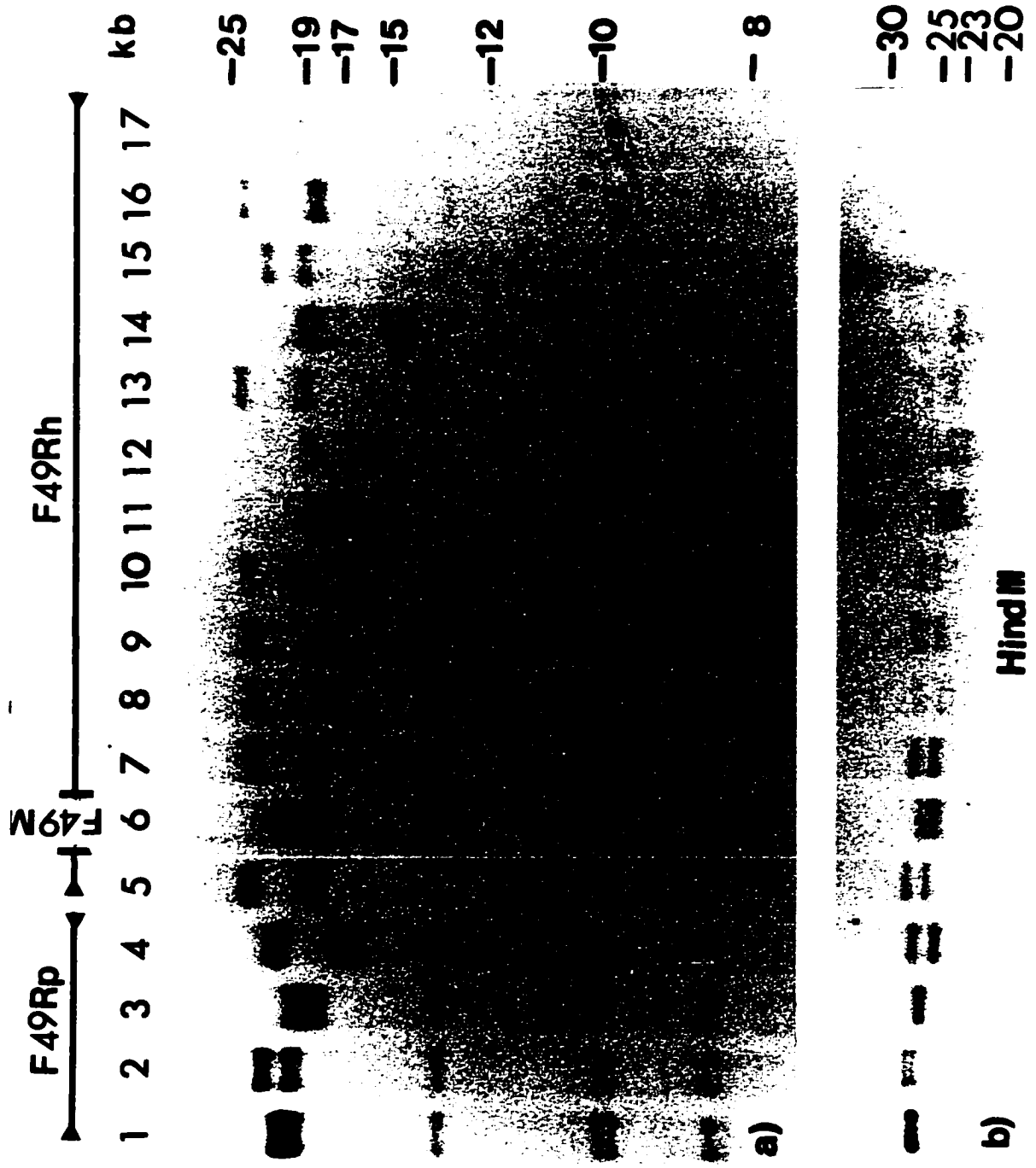


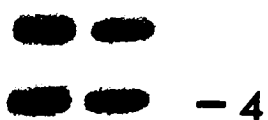
Figure 4.1

in all analysed F49Rh clones (Fig. 4.1,lanes: 7-17) and in only two from four F49Rp clones (Fig. 4.1,lanes: 2 and 4). Two F49Rp clones (lanes: 1 and 3) had only 1 band in this "hot spot" (Fig. 4.1a, b). Clone F49Rh1 (lane 5) and its next generation tissue culture regenerant (lane 7) showed the same *Dra* I high molecular weight banding patterns. This result confirms previous observation (see chapter 3) of genetic stability of DNA rearrangements in regenerative somaclonal variants *in vitro* once regeneration has occurred. Identical banding patterns in this mutational region were detected for F49Rh clones 1, 3 and 10 (18 and 24 kbp bands for lanes: 5, 9 and 16) and 3 other F49Rh clones 4, 7 and 11 (19 and 23 kbp for lanes: 10, 13 and 17).

DNA rearrangements detected in somaclonal variants (Fig. 4.3) seem to be linked to the regeneration trait, as based on evidence from limited inheritance patterns. DNA fingerprints generated by Jeffreys' 33.6 probe with the *Dra* I digested genomic DNA from parents, a specific regenerative clone (lane 3) and a non-regenerative genotype (lane 4), and their regenerative progeny (lanes 1 and 2) demonstrated that both offsprings inherited the 19 kbp band from the regenerative parent. Lane 5 shows an unrelated regenerative F₁ that exhibits no band linkage to extrinsic regenerative clones. DNA fingerprinting of a larger number of regenerative and non-regenerative progeny and their parents would be required to provide stronger evidence for clone RFLP linkage to the regeneration trait in the red clover somaclonal variants.

Figure 4.2 DNA fingerprints from plantlets (P) and callus (C) of F49M genotype after *Dra* I- digestion of genomic DNA and Southern hybridization with Jeffreys' 33.6 probe. Molecular size marker are high molecular weight markers (Gibco-BRL).

F49M
P C kb
-23
-19
-12



DraI

Figure 4.2

III. Identification of a common RFLP band linked to proline analogue tolerance

Red clover regenerative genotypes with enhanced *in vitro* plantlet freezing tolerance were selected (Nowak et al. 1992) and screened for somaclonal variants resistant to proline analogues (Nowak et al. 1994). The objective of this study was to find a genetic marker for proline-overproduction.

Amino-acid analogues are frequently used in tissue culture as selecting agents for overproducers of specific aminoacids. The analogues act as competitive inhibitors of particular amino-acid utilization in protein synthesis and other metabolic pathways. Cells or callus capable of growing on analogue-supplemented media must overproduce specific amino-acid in order to survive. This selection technique requires material which can regenerate plants from callus and/or cell suspension cultures. Accumulation of proline makes plants more resistant to environmental stresses, especially those leading to cellular dehydration; e.g., salt, cold and drought (Ober & Sharp 1994; Van Swaij et al. 1987). Cold- stress tolerant barley and potato mutants have been selected when screened for trans-4-hydroxy-L-proline (Hyp) resistance (Van Swaij et al. 1987 and Kueh & Bright 1981). Another proline analogue, L-azetidine-2-carboxylic acid (Azc) has been used to generate barley mutants with 10-30 fold higher levels of proline accumulation (Keuh & Bright 1982).

Glutamate is the primary precursor for proline synthesis in stressed cells (Fig. 4.4). Expression of this pathway is

Figure 4.3 DNA fingerprints of red clover genotypes with Jeffreys'probe 33.6. *Dra* I digested DNA consisting of handcrossed parents, lane 3- F49Rh9 and lane 4-F₁NR (non-regenerative genotype from cross of unknown F49Rh and non-regenerative cv. Florex plant), their 2 regenerative offspring, lanes 1 and 2, and F₁R (regenerative genotype from cross of unknown F49Rh and non-regenerative cv. Florex plant)-lane 5. Arrow indicates a clone specific band linked to the regeneration trait. Molecular size marker are high molecular weight markers (Gibco-BRL).

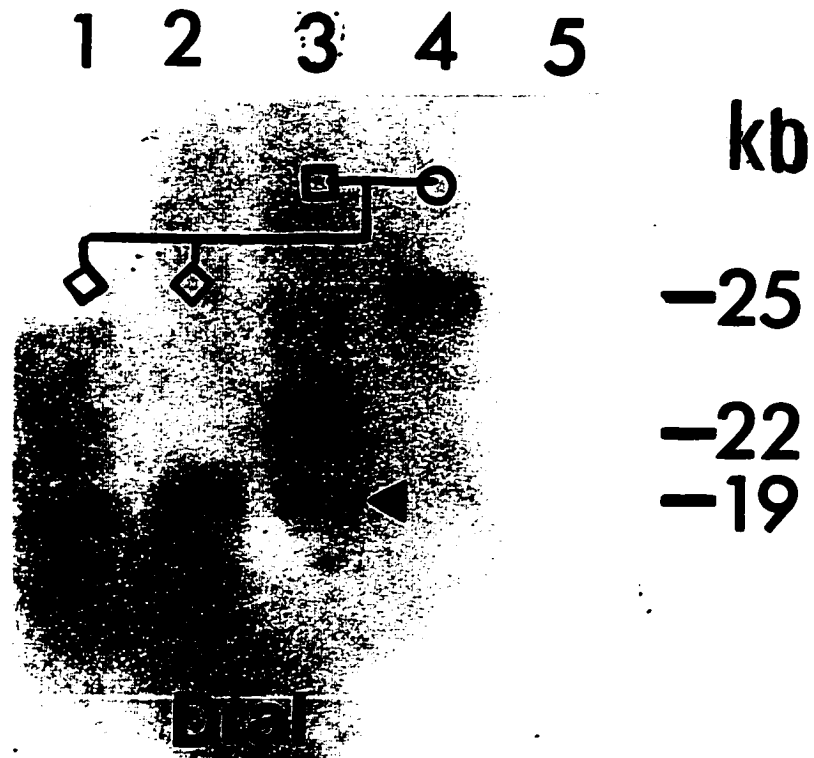


Figure 4.3

regulated at pre- and post-translational levels and genetic data implicate single genes in proline overproduction (Sumaryati et al. 1992). Phosphorylation of glutamate in the first reaction step in the biosynthetic pathway of proline is enhanced by γ -glutamyl kinase. This enzyme is subjected to feedback inhibition by proline. One strategy for tissue culture selection for proline overproducers relies on the loss of this feedback inhibition. Bacteria with increased growth rates on NaCl-enriched medium contained several-hundred-fold more proline than the wild type (Csonka 1989). Potato mutants with improved tolerance to NaCl and freezing stress were regenerated on hydroxyproline-enriched medium and were shown to contain high endogenous levels of proline (van Swaaij et al. 1986). It is highly probable that proline overproducers selected in tissue culture are generated by mutational events leading to the loss of the feedback inhibition of γ -glutamyl kinase or other enzyme(s) in the proline biosynthetic pathway.

Proline analogues, Hyp and Azc, have been used as an *in vitro* selection pressure for selection of cold tolerant variants of red clover (Nowak et al. 1994). The rationale of the project was that the combination of regenerative ability and high accumulation of proline during fall hardening will produce red clover germplasm with improved winter survival. The screening procedure is outlined in Figure 4.5. Selected variants and seedling genotypes tolerant to proline analogues, some of them proline overproducers, exhibited

Figure 4.4 Primary pathway for proline biosynthesis in plants. Reaction 1 and 2 are catalysed by pyrroline carboxylic acid synthetase. Reaction 3 is spontaneous, and reaction 4 is catalysed by pyrroline 5-carboxylate reductase. Arrow - the activity-catalysing glutamate phosphorylation reaction (δ -glutamyl kinase) is feed-back inhibited by proline (Bartels & Nelson 1994).

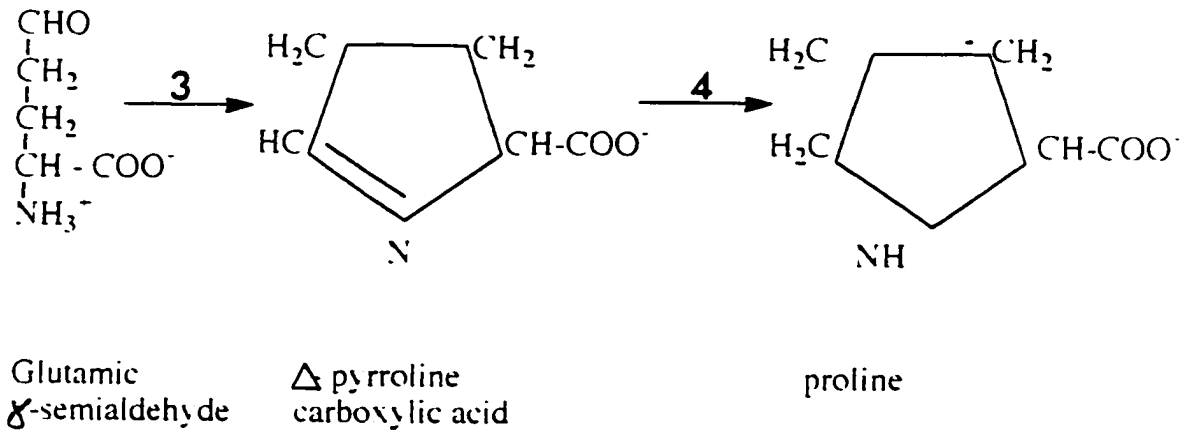
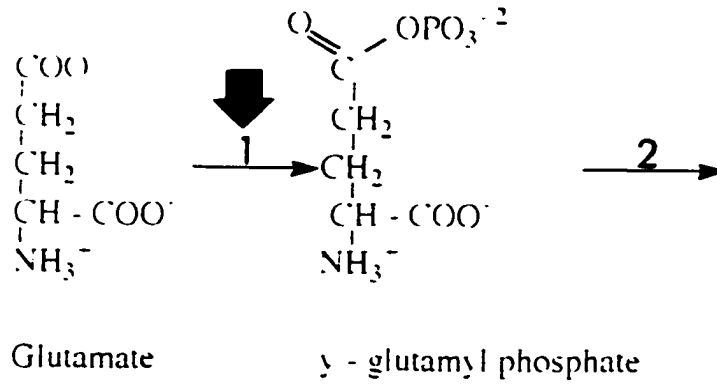


Figure 4.4

upgraded cold tolerance in both *in vitro* and *in vivo* freezing tests (Nowak et al. 1994)). Four genotypes of red clover, composed of 2 genotypes (G1a and G2a) selected on proline analogues (one on Azc and the other on Hyp), 1 seedling from cultivar Florex resistant to Azc, and 1 non-selected regenerative variant F49Rh, were subjected to DNA fingerprinting with Jeffrey's probe 33.6 after genomic DNA was digested with Dra I (Fig. 4.6). Two selections and the Azc tolerant seedling, but not, the regenerative control, shared a 20 kbp band as seen on this DNA fingerprint. F₁ populations were generated by the intercrosses of the analogue-tolerant genotypes for resistance to Azc and screened for Azc tolerance using *in vitro* callus test (Fig. 4.5). Seedlings were selected from F₁ synthetic lines which were tolerant to the proline analogue, Azc, and capable of regeneration from somatic cells under tissue culture conditions.

IV. Attempts to isolate and sequence regeneration related DNA fragments from a red clover genomic library

The presence of the regenerative trait in red clover is associated with enhanced cold stress tolerance (Nowak et al. 1992). This trait is very rare in red clover and it would be difficult to introduce it to the existing cultivars as outcrossing red clover is heterozygous for many genes and, during crosses, loses dominant alleles for other important

Figure 4.5 Screening procedure of red clover germplasm for tolerance to proline analogue, azetidine-2-carboxylic acid (Azc). Seeds were surface sterilized and germinated on growth regulator-free medium in GA7 culture vessels (Magenta Corp. Chicago). Germinated seedlings (5-7 days old) were dissected and the hypocotyl placed in 24-cell well plates (Corning) containing callus induction medium supplemented with 0.1 mM Azc. The epicotyl was placed on growth regulator-free maintenance medium in culture tubes (150 x 25 mm) to grow into plantlets. After 4 weeks on analogue media, the hypocotyl callus was evaluated for growth. Petiole segments from those plantlets, whose hypocotyl callus exhibited tolerance to Azc, were placed on callus-inducing medium supplemented with 0.15 and 0.30 mM Azc. Petiole callus was evaluated for growth after 4 weeks and divided into tolerant and non-tolerant populations. Twelve non-tolerant plantlets were also incorporated into the petiole evaluation as controls.

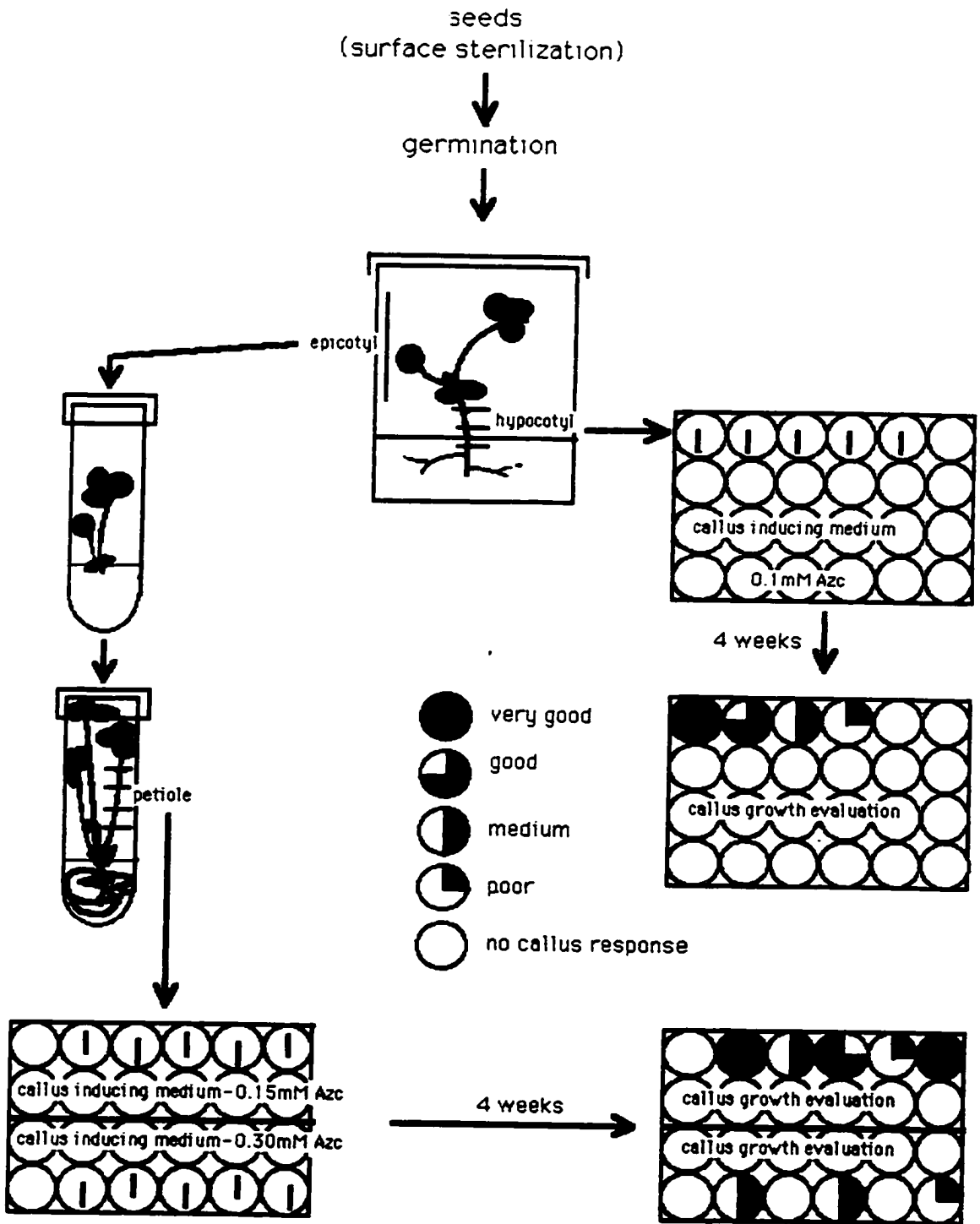


Figure 4.5

characters. Isolation of gene(s) related to regeneration in this species and their utilization in genetic transformation alone or together with genes coding for other valuable traits would be of a great significance.

It is likely, that DNA rearrangements in the mutational "hot spot", detected with the Jeffreys' 33.6 probe (see Fig. 4.1), have led to the selection of regenerative clones. F49Rp1 clone has been chosen as the DNA source for isolation and sequencing of the 22 kb RFLP band detected in the highly mutagenic region by DNA fingerprinting with Jeffreys' probe 33.6 (Fig. 4.1a - lane 1). DNA fingerprinting of the F49Rp1 clone showed only one band (22 kbp) compared to two bands (18 and 20 kbp) seen in the same region on the DNA fingerprint from F49M non-regenerative control (Fig. 4.1a - lanes 1 and 6). It has been calculated that 10 mg of *Dra* I digested F49Rp1 DNA should yield approximately 200 ng of one genomic copy of the 22 kbp fragment. *Trifolium pratense* cells contain 1 pg of DNA per diploid genome and 1 pg equals 9.65×10^5 kbp (Bennett & Leitch 1995). The 21-23 kbp region was excised from a preparative, 0.7%, agarose gel stained with ethidium bromide. The DNA was electroeluted and digested with *Pal* I to generate blunt ended, smaller fragments. After phenol extraction, the digested DNA was cloned into *Sma*I pUC18 and introduced into host cells (DH5alpha-Gibco-BRL) for recombinant selection. Attempts to clone *Pal* I fragments were unsuccessful as no recombinants were obtained on X-Gal selection media. It is possible that the minute quantities

Figure 4.6 DNA fingerprints of red clover genotypes selected on proline analogues. *Dra* I-digested genomic DNA was hybridized with Jeffreys' probe 33.6. Lanes, 1 - non-selected regenerative variant (F49Rh); 2 - selected from F49Rh on Azc; 3 - selected from F49Rh on Hyp; 4 - seedling from cv. Florex resistant to Azc. Molecular size marker are high molecular weight markers and a 1kb ladder (Gibco-BRL).

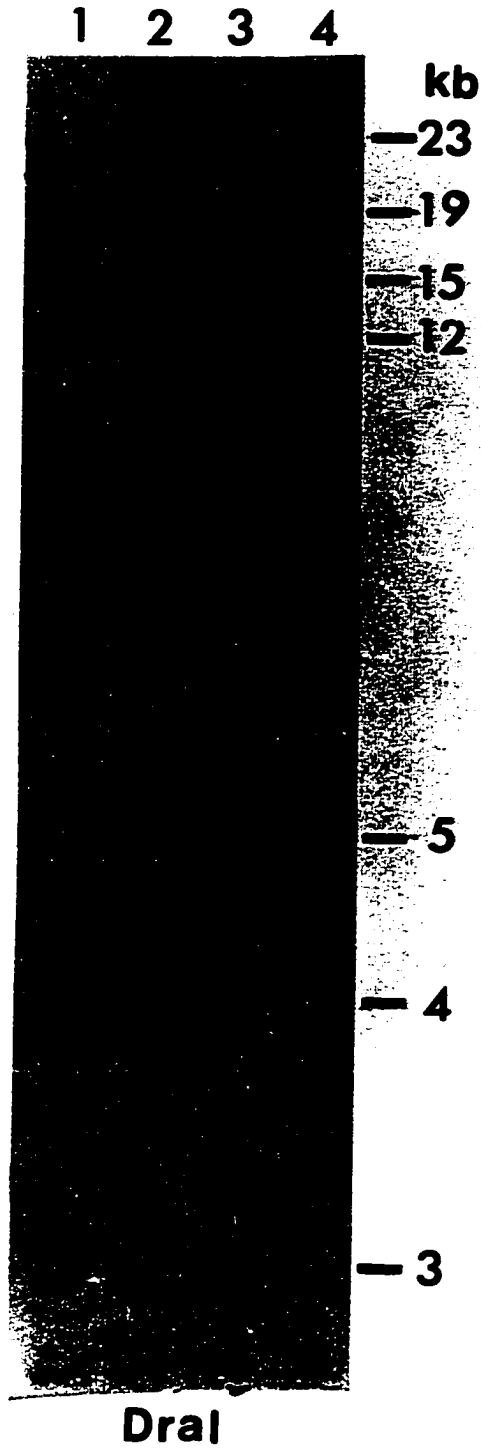


Figure 4.6

of the *Dra* I and later *Pal* I DNA were lost during the purification procedure. Therefore, a different strategy was designed.

This time 15 mg of *Dra* I-digested DNA of F49Rp1 (Fig. 4.1a) was subjected to agarose gel electrophoresis to isolate the 22 kbp DNA fragment. The electroeluted DNA was radiolabelled by nick translation with [³²P] dCTP and used to screen a lambda genomic library constructed from DNA of F49M Florex genotype. The membranes with clones that hybridized to the nick-translated DNA probe were retested with Jeffreys' probe 33.6. Seven clones hybridized to both probes. DNA was isolated from lysates of those lambda clones (Sambrook 1989), digested with *Sfi* I and subjected to electrophoresis, in 2 replicas, on 0.7% agarose gel (Fig. 4.7a). Both gel replicas were subjected to two separated Southern analyses, one using Jeffreys' probe 33.6 and the second using the 22 kbp *Dra*I generated "hot spot" DNA probe (Fig. 4.7 b,c). Four lambda clones were identified that contained 14 kbp inserts hybridized to the both probes. The assumption was made that they carried the same DNA insert. Surprisingly, all DNA fingerprints, including F49M, had the same banding pattern when the 14 kbp DNA insert from the lambda clone (Fig. 4.7 a - lane 1) was used to reprobe (after 33.6 probe) the Southern blot of *Dra* I digested genomic DNA of F49M, F49Rh and F49Rp clones. Hybridization to the mutational "hot spot" bands was not observed (Fig. 4.8a). DNA fingerprints generated with the 14 kbp DNA insert did not show any resemblance to DNA

fingerprints obtained with 33.6 probe (Fig 4.8b). These results indicate that the isolated lambda clone did not contain a highly variable region of DNA.

V. Discussion

A specific genetic change occurred in a highly mutable DNA region which seems to be linked to the expression of the regenerative trait in red clover. Interestingly, in the genotype F49, this DNA region is prone to rearrangements in regenerative variants obtained under tissue culture conditions but is stable in non-regenerative F49M callus. These observations might reflect the activity of a stress-induced transposable element in the sequence of events leading to de-differentiation and regeneration in tissue culture. However, even assuming that the transposon induction took place, it does not prove that this particular induction is required for *in vitro* regeneration. Transposable elements have an ability to transpose, affect gene expression and cause chromosomal rearrangements (see Chapter 1 - transposition). Petrov et al. (1995) have shown in their study of hybrid dysgenesis in *Drosophila virilis*, that mobilization of a single transposon may activate other unrelated transposable elements of the genome. Moreover, McClintock (1984) has showed that transposition in maize takes place in a precise, developmentally regulated manner. DNA transpositions and/or quantitative changes in repetitive DNA observed in plants during *in vitro* (see chapter 5)

Figure 4.7 Results of the isolation procedure of the highly mutable DNA region: **a** - Agarose gel electrophoresis of *Sfi* I- digested red clover Lambda clones selected from EMBL genomic library hybridizing to Jeffreys' 33.6 probe and to electroeluted 22kbp *Dra* I-band of calliclone F49Rp1 (see fig.4.1a); **b** -Southern transfer of the *Sfi* I-digested clones hybridized to Jeffreys' 33.6 probe; **c** - Southern transfer of the *Sfi* I-digested clones hybridized to the 22kbp *Dra* I DNA region of calliclone F49Rp1. Arrows indicate 14kbp lambda insert that hybridized to both probes. Molecular size marker are high molecular weight markers (Gibco-BRL).

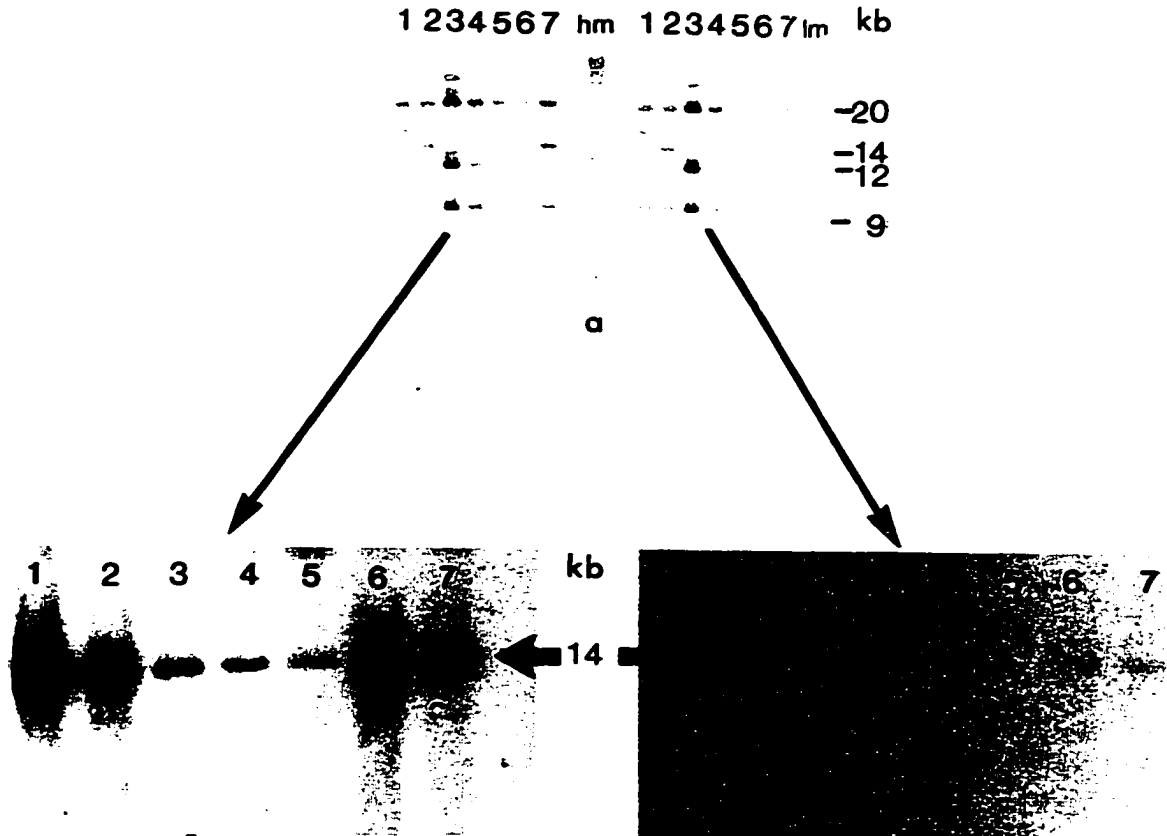


Figure 4.7

Figure 4.8 Southern hybridization of red clover clones F49Rp (lanes 1 and 2.), F49Rh (lane 3) and non-regenerative F49M (lane 4) Genomic DNAs were digested with *Dra* I, Southern blotted and hybridized to a) nick translated 14kb lambda insert., b) Jeffreys' probe 33.6. Molecular size marker are 1 kb ladder (BRL).

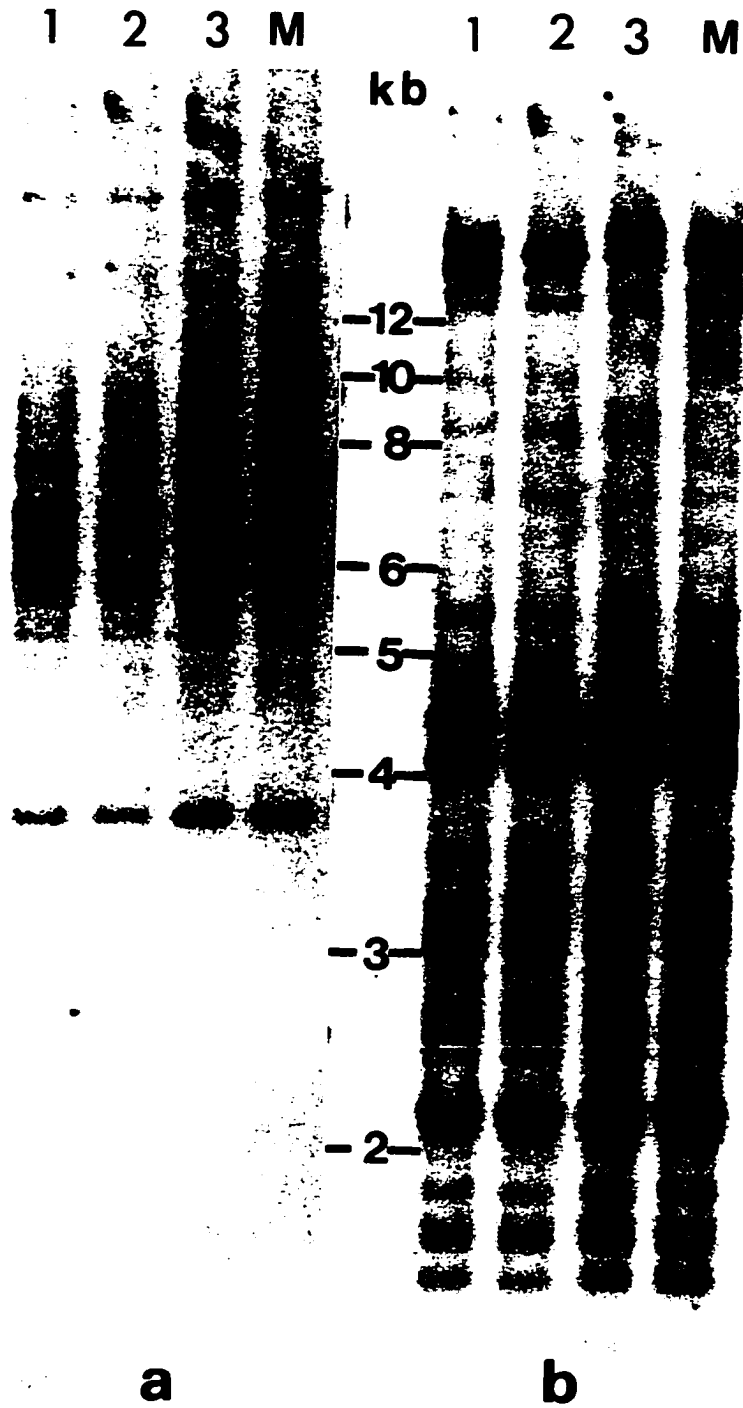


Figure 4.8

regeneration might impose some form of positional effect which enforces the processes of de-differentiation and re-differentiation to occur (Kidwell 1993). In *Drosophila*, the change in the DNA at a specific site of the genome can alter expression of genes in close proximity (Dutton & Chovnick 1985). Regulatory DNA sequences, like enhancers and/or silencers might be moved in relation to gene promoters via DNA rearrangements causing alternations in gene expression. Higher gene expression levels (up to 400-fold) in transgenic plants of sugarcane and carrot were readily generated by altering the number of enhancer elements and their proximity to the promoter region in the beta-glucuronidase reporter gene (Rathus et al. 1993). Rearrangements of chromosomal DNA might put genes under the influence of different promoters to allow the processes of de- and re-differentiation to occur. Plant regeneration from tissue culture might be governed by selective termination of some replicons and/or by rearrangement of their order of replication in the process of genome duplication.

The DNA changes in "the hot spot" of the red clover genome described here seem to be required for expression of totipotency but the question of how the gross genomic DNA rearrangements arise during tissue culture plant regeneration remains unanswered.

CHAPTER 5: TISSUE CULTURE-INDUCED REPETITIVE DNA CHANGES IN A RED CLOVER GENOTYPE AND ITS SOMACLONAL VARIANTS.

I. Introduction

Within the angiosperms, nuclear DNA content can vary from approximately 10^5 kb for *Arabidopsis* to nearly 10^8 kb for *Lilium* species (Smyth 1991). Independently of the genome size, only about 7.5×10^4 kb of DNA is composed of genes required for plant development, growth and reproduction. On average, in higher plants, 0.1-1% of the DNA codes for actively expressed genes (Flavell 1980). More than 75% of all DNA sequences are repetitive fragments, 50 bp or longer (Flavell 1982). Repetitive DNAs are classified according to their genomic organization; they are either interspersed or clustered tandem arrays. Transposons and retrotransposons are the most abundant interspersed DNA sequences of plants (see chapter 1 - molecular changes in nuclear genome). Transposons amplify to no more than several hundred copies per genome but retrotransposons can occur in up to 240,000 copies per genome (Smyth 1991). Clustered sequences include coding and non-coding DNA repeats. Coding clustered repetitive DNAs embrace sequences of ribosomal and histone genes. There are approximately 2000 copies of the rDNA repeat in the pea genome (Ellis et al. 1984) and 2400 in the flax genome (Cullis & Cleary 1986b). In common with many other plant rDNAs, they have a repeat length of 8-12 kbp.

The 5S genes of flax, arranged in tandem arrays of a 350-370 base pair repeating sequence, were shown to represent 4% of the genome (Cullis & Cleary 1986b). The non-coding clusters of tandem repeats are subdivided into three classes according to the size of the repeat and are designated as satellite, minisatellite and microsatellite DNA sequences. Satellite DNA is organized in clusters of tandem arrays of highly repetitive sequences usually positioned near centromeres and telomeres (Charlesworth et al. 1994). The fraction of a plant genome occupied by satellite DNA varies greatly from 15% for flax (Cullis & Cleary 1986a), 10-12% for cotton (Zhao et al. 1995) to 1.5 % for *Arabidopsis thaliana* (Smyth 1991). Minisatellites occur as tandemly-arrayed repeats (9 - 65 bp) with very variable mean array length, and microsatellite sequences are present as arrays of short nucleotide repeats (1-4 bp) (Wright 1993). Minisatellite and microsatellite monomers form clusters of tandem arrays often dispersed throughout the genome and these types of repetitive elements, together with retrotransposons, seem to be predominant in plant genomes. It has been shown by reassociation kinetics study (Murray et al. 1978) that repetitive DNA sequences present in pea genome in about 10, 000 copies are interspersed at intervals of less than 1300 nucleotides throughout 75% of the genome. A total of 83 of 103 repetitive DNA sequences isolated from cotton (200 - 800 bp) were interspersed repeats with genomic copy numbers ranging from 4, 000 to 100, 000 (Zhao et al. 1995).

Interspersed repeated sequences are generated by mechanisms called transposition or retroposition depending on the mode of amplification and movement (Smyth 1991). Tandemly repeated sequences are generated during genome duplication by slippage of DNA synthesis, rolling circle replication and unequal crossing-over (Charlesworth et al. 1994).

Repetitive DNA sequences are particularly prone to changes such as amplification, under-replication or decrease in copy number (Flavell 1986). Quantitative variations of nuclear DNA occur *in vivo* and *in vitro* (reviewed by Bassi 1990). There have been number of reports indicating diminutions and amplifications of repetitive DNA during the regeneration process as a main source of somaclonal variation (reviewed in Table 1). Investigation of quantitative changes of repetitive sequences during the process of *in vitro* de-differentiation was an objective of this study.

II. Isolation of repetitive DNA sequences from a red clover lambda genomic library

A red clover lambda genomic library was screened by filter hybridization using a radiolabelled F49M genomic DNA as a probe. Twenty four plaques, that hybridized strongly to the F49M genomic DNA probe were selected and used for preparation of lambda DNA by the small-scale plate lysate method (Sambrook et al. 1989). Inserts, ranging from 10 to 17 kbp, were excised from 0.7% agarose gel after digestion of

lambda DNA with the *Sfi* I restriction enzyme and electrophoresis. Recovered DNA, after passage through glass powder medium (Gene-Clean-BIO 101), was digested with the restriction endonuclease, *Pal* I. The digests (10 μ l of total 50 μ l each) were separated on 1.8% agarose gels, Southern blotted and hybridized to nick translated F49M genomic DNA (Fig.5.1). Five repetitive DNA fragments, as indicated by the strong radioactive signal in the autoradiography, were chosen for further characterization (Fig. 5.1). The *Pal* I digests (40 μ l) of selected DNA clones were subjected to electrophoresis on agarose gel and bands of interest (1.2 kbp for clones 1, 3, and 4; 2.5 kbp for clone 8; and 4.5 kbp for clone 19) were excised from gel and purified using the Gene-Clean (Gene-Clean-BIO 101). Names were assigned to the purified repetitive DNAs : rc1, rc3 and rc4 for 1.2 kbp fragments of clones 1, 3 and 4; rc8 for 2.5 kbp fragment of clone 8 and rc19 for 4.5 kbp fragment of lambda clone 19.

III. Genomic organization of the repeated DNA sequences

When *Eco*R I digested genomic DNA purified from F49M and 4 regenerative clones was Southern blotted and hybridized separately to probes rc1, rc3 and rc4 (lanes 1,3 and 4 - Fig. 5.1) the same banding patterns were detected on 3 autoradiograms (Fig. 5.2 a-c). Cross-hybridization between these clones has been demonstrated later by a DNA dot blot

Figure 5.1 Southern hybridization of *Pal* I-digested repetitive DNAs isolated from F49M Lambda genomic library. Radiolabelled genomic DNA from F49M was used as hybridization probe. Lanes 1-19; different lambda DNA clones. Arrows indicate repetitive DNA fragments used in this study. Molecular size markers are 1 kb ladder (BRL).

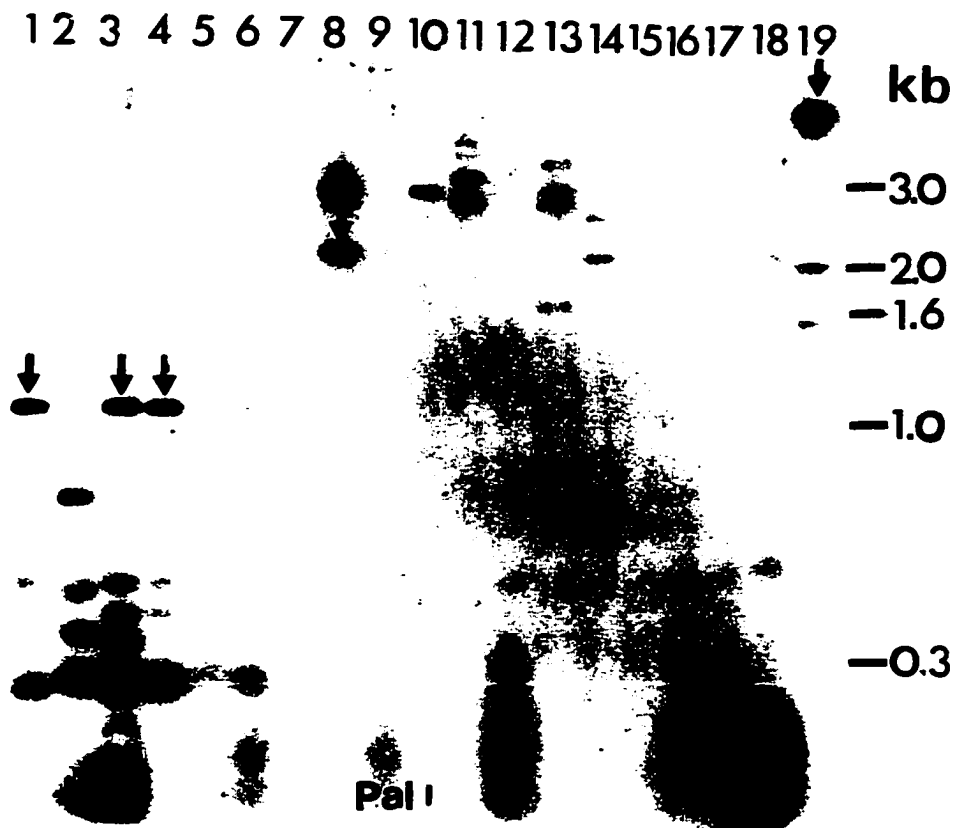


Figure 5.1

experiment (Fig. 5.5). A 3.0 kbp DNA band was preponderant in all genomic digests. The 3.0 kbp fragment corresponds to a distinct band detected by ethidium bromide staining before DNA Southern transfer (data not shown). The autoradiographs also displayed other bands of low intensity and one of them, a 2.2 kbp band, was only present in the genomic DNA of F49M (lane 1) and F49Rh1 (lane 2) (with the intensity of this band much greater in F49Rh1), but absent in the other 3 clones genomic DNA digests (lanes 3-5) indicating tissue culture-derived qualitative genetic variation (Fig. 5.2). Four DNA bands were detected by probe rc8, when hybridized to trans-blotted EcoRI digested genomic DNAs from F49M and 7 regenerative clones (Fig. 5.3). Even though similar amounts of DNA from each sample were used in this experiment, the differences in bands intensities between F49M (lane 1) and the regenerative clones, as well as among them (lanes 2-8), are striking and might result from amplifications and/or de-amplifications of this repetitive DNA during *in vitro* plant regeneration. The weak intensity of the DNA bands in F49Rp7 and F49Rp8 (lanes 7 and 8) might reflect incomplete DNA digestions as indicated by strong hybridization signals in the region corresponding to the agarose gel wells. The Southern blot of *DraI* -digested genomic DNAs from 8 red clover genotypes was hybridized to rc19 probe. The autoradiogram showed DNA smears and a few distinct bands in all digests (Fig. 5.4). Organization of the distinct DNA bands was similar for all red clover genotypes analysed,

Figure 5.2 Southern blot hybridization of *EcoR* I-digested genomic DNA of F49M (lane 1) and 4 regenerative clones (F49Rh1, F49Rh2, F49Rp8 and F49Rp9; lanes 2-5) to 1.2kb repetitive DNA fragments of lambda clones 1- rc1 (a), 3-rc3 (b) and 4- rc4 (c). Molecular size markers are 1 kb ladder (BRL).

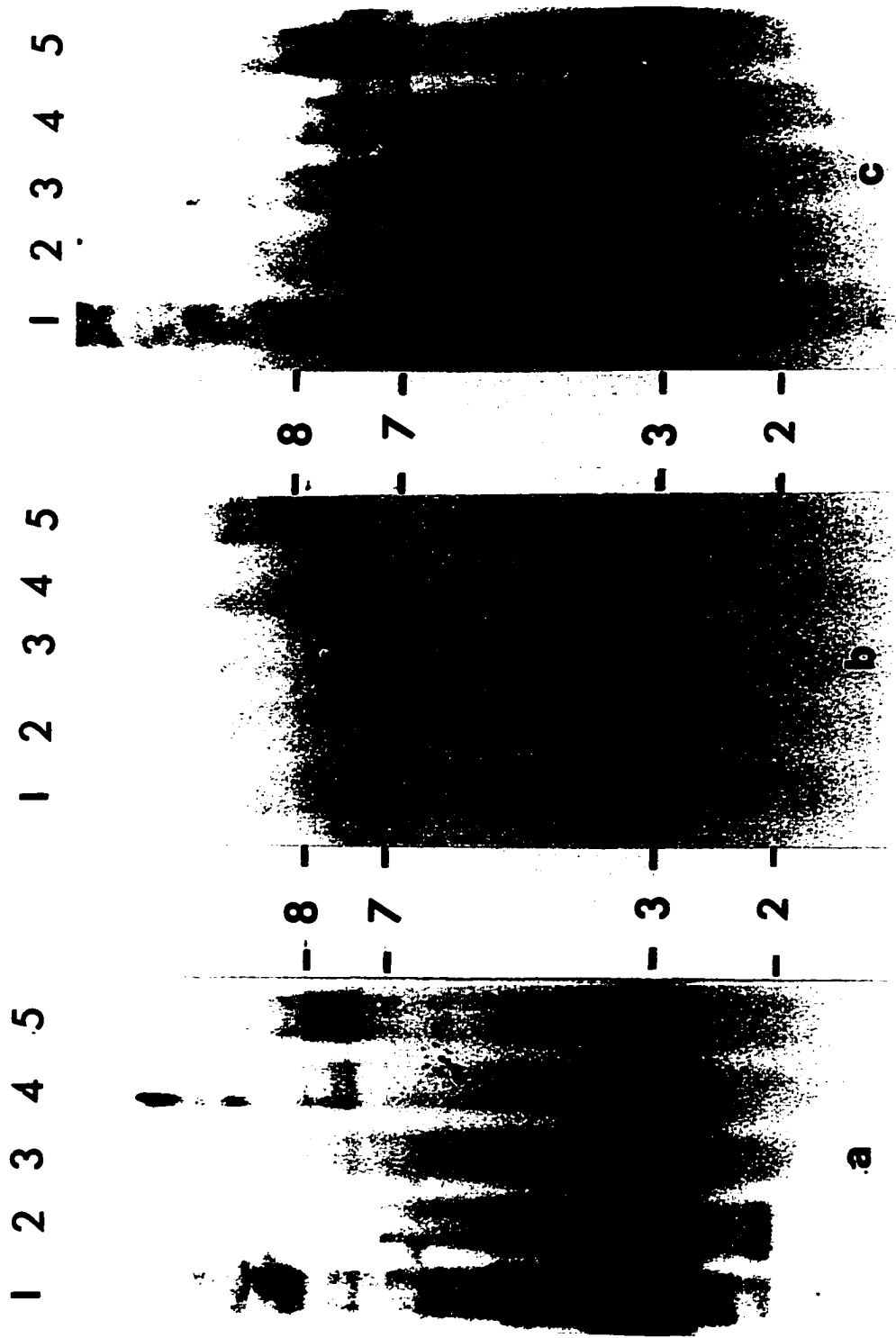


Figure 5.2

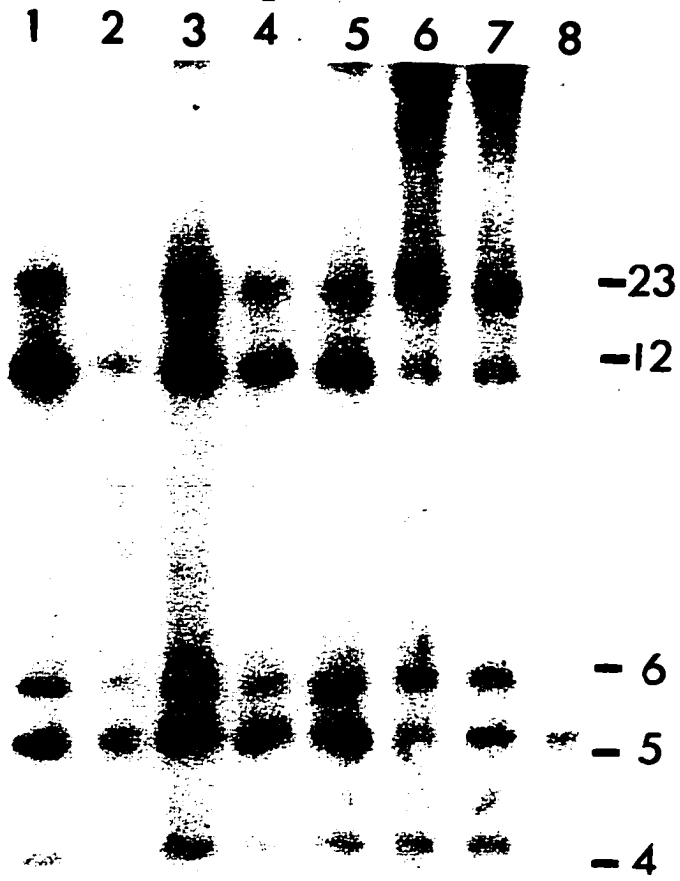
embracing 2 different cultivars (only Florex genotypes shown). The analysed repetitive DNA clones were determined to be abundant in red clover genome but their function is unknown.

IV. Quantitative and qualitative distribution of repetitive DNA in plantlets and calli of regenerative and non-regenerative genotypes.

Quantitative determination of DNA was conducted using leaves taken from six-week-old tissue culture plantlets as well as 3-week old leaf petiole callus of selected genotypes. Genomic DNA was probed with 4 repetitive clones by dot hybridization (Fig. 5.5). Quantification of DNA (ng) was conducted comparing intensity of fluorescence of the samples photographed on a UV box after spotting and mixing with ethidium bromide serial dilutions of "gene-clean" refined DNA samples and standard DNA (λ DNA) onto "Sanwrap" (Sambrook et al. 1989). Genomic DNA used in dot blotting was EcoRI digested and "Gene-cleaned" and 300 ng was applied per spot into nylon membrane. In each hybridization, tubulin cDNA from *Chlamydomonas reinhardtii* (300ng) and 3 dilutions (1; 1/10; 1/100; 2 μ l=1) of each of 4 probes were applied on the membrane as a negative control and as a test of the probe's cross-reactivity. Tubulin cDNA was a gift from Dr. C. D. Silflow - Department of Genetics and Cell Biology, University of Minnesota.

Dramatic reduction of repetitive DNA was recorded during

Figure 5.3 Southern hybridization of *EcoR* I-digested genomic DNA from F49M (lane 1) and 7 regenerative clones (F49Rh1, F49Rh2, F49Rh3, F49Rh10, F49Rh18, F49Rp8 and F49Rp9; lanes 2-8) to 2.5 kb *Pal*I repetitive DNA fragment of lambda clone 8 (rc8). Molecular size marker are *Hind* III-digested lambda DNA and 1 kb ladder (BRL).



EcoRI

Figure 5.3

Figure 5.4 Southern hybridization of *Dra* I-digested genomic DNA of F49M (lane 1) and 7 regenerative clones (F49Rh1, F49Rh2, F49Rh3, F49Rh10, F49Rh11, F49Rh13 and F49Rh18; lanes 2-8) to 4.5 kb repetitive DNA fragment of lambda clone 19 (rc19). Molecular size markers are 1 kb ladder (BRL).

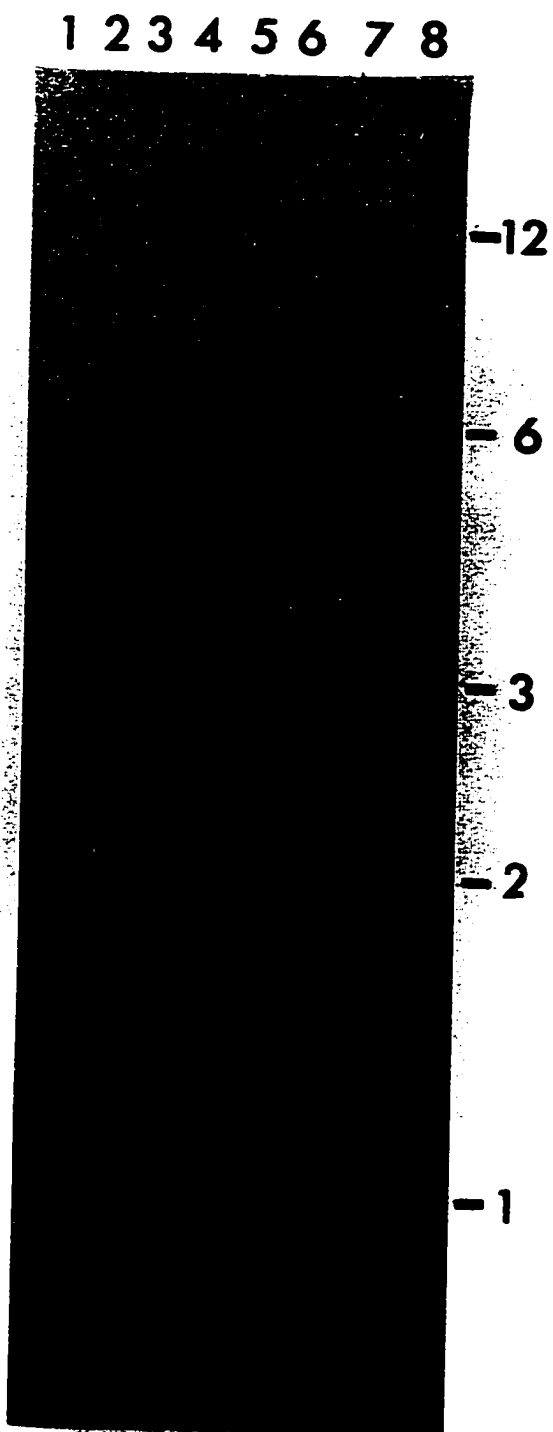


Figure 5.4

the transition from differentiated (explant) to the de-differentiated (callus) state (Fig. 5.5). Quantitative differences in all tested repetitive DNA were detected between leaves and calli, ranging from 1.1-1.5 fold for clone rc19 to 3.6-6.1 for clone rc8. Calli of both F49M and 3 regenerative clones showed lower content of all 4 tested repetitive DNAs compared to their plantlets. F49M callus showed higher quantitative loss of repetitive DNAs than F49Rs calli. DNA diminutions in calli detected by homologues clones rc1 and rc4 (see later in this subchapter for explanation) were similar (2.6-3.9 fold for rc1 and 2.1-3.6 fold for rc4). Content of tested repetitive DNAs in leaves of 3 F49R calliclones was similar (maximum 25% difference for rc4) and did not differ much from F49M leaves (maximum 38% for rc8, when mean value for 3 F49R clones was used for comparison). As F49M is a non-regenerative genotype, this observation implies that re-differentiation process in tissue culture is accompanied by amplification of repetitive DNA sequences reduced during de-differentiation events (callusing). High DNA sequence identity was found between rc1, rc3 and rc4 sequences. They bound to the same restriction fragments of genomic DNAs (Fig. 5.1 a-c) and rc1 and rc4 cross-hybridized to each other (Fig. 5.5). DNA sequences of rc8 and rc19 did not cross-reacted with each other and with rc1 and rc4 clones (Fig. 5.5).

Approximate copy number of repetitive DNA sequences in leaves and calli genomes was estimated by quantitative dot

Figure 5.5 Dot hybridization of genomic DNA from plantlets (P) of F49M (M) and 3 regenerative F49R clones (R1 = F49Rh1, R2 = F49Rh2 and R3 = F49Rh3) and their 3-weeks-old petiole-derived calli (C). Genomic DNA (300 ng - in replicas for each DNA sample) and 3 different dilutions (1; 1/10 and 1/100) of repetitive DNA fragments isolated from F49M red clover Lambda genomic library (rc1, rc4, rc8 and rc19) were spotted onto each nylon membrane. The same 4 repetitive DNA fragments (rc1, rc4, rc8 and rc19) which were applied on each membrane were used individually as radiolabelled probes in 4 separated hybridizations. After autoradiography, dots were cut out from nylon membrane to determine radioactivity hybridized. Genomic DNA dots are in replicas and quantitative data is the average of 2 determinations. Numerical data are the ratios of counts for plantlets and their calli (P/C) calculated for four different genotypes (M, R1, R2 and R3) and 4 different hybridization probes (rc1, rc4, rc8 and rc19).

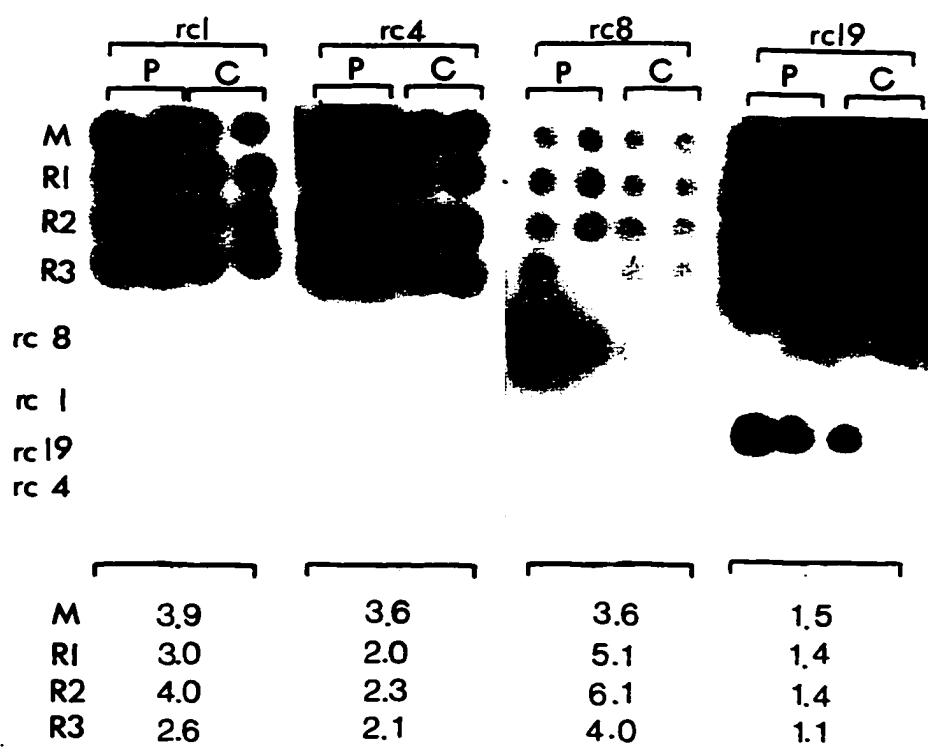


Figure 5.5

spot hybridization to known amounts of genomic DNA and different dilutions of the repetitive DNA-containing clones using the radiolabelled repetitive DNA-containing clones as probes. Repetitive DNA of rc1, rc4, rc8 and rc19 constituted respectively 2%, 1.9%, 0.3% and 1% of genome of plant cells in the differentiated state. Quantitative over-representation (2-fold) of the DNA repeats of clones rc1 and rc4 was observed in leaves versus calli when Southern hybridizations of *Dra* I digests of genomic DNA extracted from plantlets of the regenerative clones F49Rh and their calli were performed using the clones rc1 and rc4 as hybridization probes (Fig. 5.6 a,b). This result is in agreement with results of the dot blotting experiments (Fig. 5.5). The similar DNA bands intensities for *Dra* I -digested genomic DNA from the plantlet and its callus observed on the ethidium bromide-stained agarose gel after the electrophoresis indicate that the same quantities of DNA were loaded on the gel (data not shown). Qualitative DNA variations during cells transition to undifferentiated state were not detected (Fig. 5.6 a,b).

VI. Discussion

The results demonstrate that substantial quantitative changes in reiterated DNA sequences occur in tissue culture of red clover explants. Reduction in the copy number of repetitive DNA in calli was observed for all 3 unrelated clones rc1, rc8 and rc19 which were shown to contain DNA

Figure 5.6 Southern hybridization of of *Dra* I-digested genomic DNA from plantlet (P) of regenerative clones (R1 = F49Rh1 and R2 = F49Rh2) and their calli (C) hybridized to 1.2kb *Pal* I DNA fragments of lambda clones 1 (rc1) - a) and 4 (rc4) - b). Molecular size markers are 1 kb ladder (BRL).

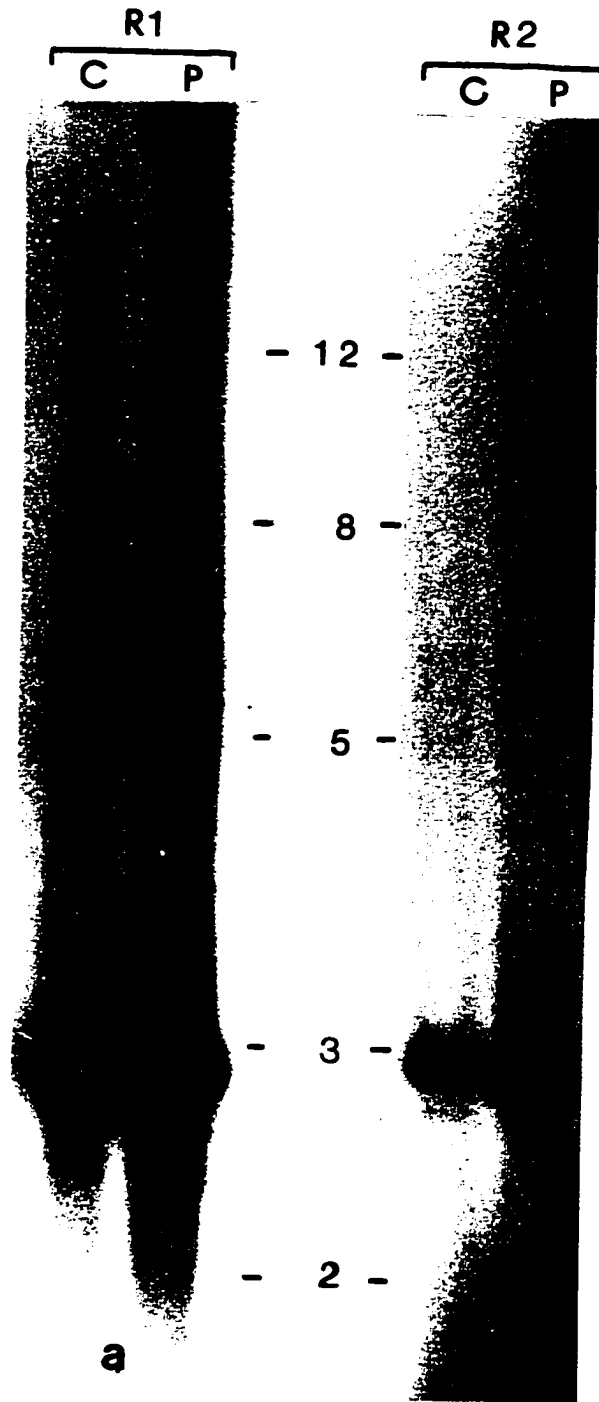


Figure 5.6

repeats. Analogous findings were reported for carrot (Steward et al. 1964; Arnholdt-Schmitt 1993, 1995), pea (Cecchini et al. 1992), lilia (Deumling & Clermont 1989), flax (Cullis & Cleary 1986a,b), maize (Nagl 1988). Deumling and Clermont (1989) concluded from their study that diminution of highly repetitive DNA sequences in cell culture of lilia (*Scilla siberica*) is a prerequisite for regeneration. In this study, copy number of some repetitive DNA sequences of explants is restored in regenerants following quantitative reduction of these sequences during callusing. Brown et al. (1991) observed the same pattern of repetitive DNA diminution in maize callus and a restoration of these DNAs after de-differentiation and organogenesis began. Brown (1991) speculates that the selection for one particular regenerative genotype in the genetic mosaic of cells in callus culture might explain detected fluctuations in quantity of DNA repeats. Arnholdt-Schmitt (1995) used video-densitometric analysis for scanning quantitative changes in the *Bst**N* I restriction fragments of the carrot genome, (shown to contain repetitive DNA - Arnholdt-Schmitt 1993a), in plantlets and their calli with high cell-division activity and older calli at the stationary growth. He demonstrated gross elimination of all analysed repetitive sequences at rapid callus cell growth and their re-amplification, some to higher degree, during transition to stationary growth. The cell-division rates were not influenced by extent of the loss of repetitive DNA.

According to the author, elimination of repetitive DNA during the de-differentiation process in tissue culture might cause structural activation of replicon origins. In the red clover study, 3-week-old proliferating callus was analysed, but a detailed time course of DNA variations was not investigated.

The above-described DNA variations take place independently of each other in different families of repetitive DNA sequences resulting in alternation in their relative proportion in the genome. Quantitative changes of repetitive DNA are particularly evident in plants and are most often associated with the developmental processes such as differentiation and de-differentiation (Natali et al. 1986; Nagl 1990; Altamura 1987; Cecchini et al. 1992) or ageing (Nagl et al. 1983; Nowak 1994). Can the expression or non-expression of specific sets of genes be controlled by conformational effects imposed by changes in non-coding DNA ? It should be noted that DNA modifications cited here occur in somatic cells of plants which unlike animals, have the capacity to be totipotent and regenerate new individuals from somatic cells. Non-random developmental DNA modifications events in plant tissue culture do not have an equivalent in the animal kingdom which is unable to reproduce by somatic cells.

**CHAPTER 6: COLD-TOLERANT SOMACLONAL VARIANTS
OF RED CLOVER (*Trifolium pratense* L.)
EXHIBIT ENHANCED EXPRESSION OF A COLD-
INDUCED GENE FOR A GLYCINE-RICH PROTEIN.**

I. Introduction

During the course of evolution, some plants acquired resistance to freezing stress (see review by Thomashow 1990). These plants exposed to low temperatures, undergo physiological adaptation, called cold acclimation, which increases their cold tolerance (see review by Guy 1990). Some plant species are more cold tolerant than others. Cold tolerance can be improved by classical genetic crosses and selection and by *in vitro* techniques. Nowak et al. (1994) showed that upgraded cold stress tolerance in red clover is associated with the ability of plants to regenerate. Moreover, they produced somaclonal variants overproducing proline (using analogues of this amino acid as a selective pressure in cell culture of red clover) with improved cold resistance (Nowak et al. 1994). These examples from one laboratory illustrate how many factors can contribute to improved cold tolerance and how complex the acclimation process (physiological, biochemical and molecular aspects - see review by Thomashow 1990) is itself. Genetic evidence suggests that cold tolerance, induced by cold acclimation, is a quantitative trait controlled by number of additive genes (Guy 1990). A constantly growing number of genes that

respond to cold at the transcriptional level have recently been isolated using differential hybridization screening (e.g., Carpenter et al. 1994; Laberge et al. 1993). The function(s) of the encoded products of some of these genes has been predicted from sequence homology with known functional proteins. The characterized genes encode 2 groups of proteins (based on localization in a plant cell); structural, with amino-terminal signal peptides (Showalter, 1993) and cytoplasmatic, without signal peptide sequences. Hydroxyproline-rich proteins (HPRP) and glycine-rich proteins (GRP) are the 2 major classes of cold-induced structural proteins and are associated with the plant cell wall and membranes (Sachetto-Martins et al. 1995; Marcus et al. 1991; Ferullo et al. 1994). The latter group include hydrophilic late-embryogenesis proteins (Bartels & Nelson 1994) and proteins with an amino-terminal RNA-binding domain (Carpenter et al. 1994; Kenan et al. 1991).

Recently, 8 alfalfa genes coding for polypeptides induced by cold acclimation of this species were identified and cDNA sequences of 3 of them, MsCOR A, B and C, were determined (Laberge et al. 1993; Monroy et al. 1993; Castonguay et al. 1994, Ferullo et al. 1997). These 3 genes are members of gene families and exhibit enhanced mRNA levels in cold-acclimated alfalfa. Alfalfa MsCOR A protein is most likely a cell wall GRP with characteristic for this class domains containing quasi-repetitive glycine (Laberge et al. 1993). The MsCOR B protein is a nuclear GRP of an unknown

function with glycine evenly distributed throughout the deduced amino acid sequence (Monroy et al., 1993). A MsCOR C protein is structural HPRP and associated with cell membranes (Castonguay et al. 1994).

The objectives of this study were to : (1) detect genes homologous to MsCOR A, B, and C in red clover; (2) evaluate the steady-state level of messenger RNA for MsCOR A, B, and C in cold-acclimated and non-acclimated regenerative (F49R) and non-regenerative genotypes; (3) determine proteins levels of MsCOR A by immunodetection in cold-acclimated and non-acclimated regenerative (F49R) and non-regenerative genotypes and, (4) investigate the genomic organization of the MsCOR A, B, and C genes in red clover

I. Screening of red clover lambda genomic library by heterologous hybridization to alfalfa cDNAs of cold-regulated genes

Complementary DNAs of alfalfa MsCOR A, B and C, were utilized in this study as hybridization probes. Hybridization probes were prepared by *Eco RI* digestion of recombinant plasmid containing the MsCOR A, B and C cDNAs. After 1% agarose gel electrophoresis of the digests, the inserts were excised from the gel and purified using Gean-Clean (Gene-Clean, BIO 101) and these DNAs were radiolabeled and used as probes to screen a red clover lambda genomic library (120 000 pfu) by filter hybridization. Forty plaques hybridized to the alfalfa cDNA probe MsCOR A, 3 to MsCOR B and 25 to MsCOR C. Based on the average lambda library insert size for red clover of 10 kbp and assuming random distribution of the genes throughout the genome and taking into account the haploid C value 4.8×10^8 bp, I estimate that there are 14 copies of MsCOR A, 1 copy of MsCOR B and 9 copies of MsCOR C-like sequences in the red clover genome. These results indicate that red clover genes, homologous to the A and C alfalfa genes are most likely (see subchapter IV) members of gene families, while the gene homologous to alfalfa MsCOR B appears to be a single-copy gene.

II. Expression of cold-induced MsCOR A, B, and C genes in red clover

For this study, hypocotyl-derived F49R somaclonal variant clones were used. The crowns of the greenhouse grown 1-2 months-old potted plants were dissected for analysis. The plants were left to re-grow for 1 month and then were cold-acclimated for 6 weeks at 5°C and samples of the crowns were excised again. RNA was isolated from the crowns (De Vries et al. 1988) and Northern analysis were performed essentially as described by Sambrook et al. (1989). Northern analysis of total RNA has shown that acclimation exclusively induced the expression of MsCOR A (Fig. 6.1a). Under the applied experimental conditions, there was no difference in the steady-state level of MsCOR B mRNA between acclimated and non-acclimated F49R (Fig. 6.1b) and no messenger RNA was detected for the MsCORC gene in either non-acclimated or acclimated plants (Fig. 6.1c). The MsCOR A and B cDNA probes hybridized to single RNA transcripts of approximately 0.9 and 0.7 kbp, respectively, indicating approximately the same sized transcripts for these genes in alfalfa and red clover.

III. Differential accumulation of MsCOR A transcripts and protein in cold-acclimated, non-regenerative and regenerative red clover somaclonal variants

Plant maintenance, cold-acclimation and Northern blot-hybridization studies described here were identical to those

Figure 6.1 Northern analysis of 3 MsCOR genes homologs transcripts in cold-acclimated (a) and non-acclimated (n) F49Rh (R); a) - MsCOR A, b) - MsCOR B, c) - MsCOR C. 30 μ g of each RNA sample was subjected to gel electrophoresis. Molecular weight markers shown are 0.24 to 9.5 kb RNA ladder (BRL).

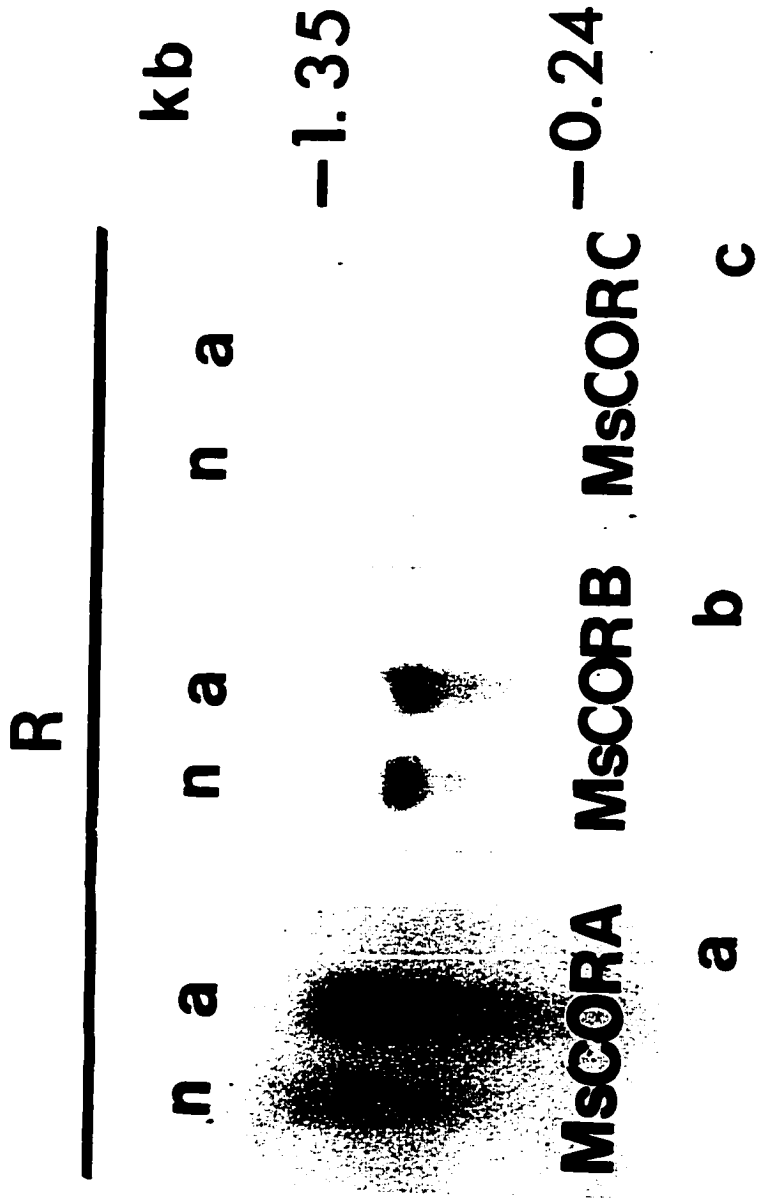


Figure 6.1

applied above. Crowns from 12 genotypes were analysed, namely F49M, F49Rh1, F49Rh2, F49Rh4, 7 F₁ plants (3 regenerative and 4 non-regenerative F₁ genotypes from seeds), which were generated by crosses between F49Rs and non-regenerative genotypes of cultivar Florex, and 1 regenerative backcross produced by hand-pollination of a F₁ non-regenerative genotype with F49Rh2. Northern autoradiographs and ribosomal RNA 28S and 18S bands visualised on agarose gel by ethidium bromide staining were subjected to densitometry scanning analysis using Scan Jet II cx/t (Hawlett-Piccard) and "Biosoft" computer software (U.K.). Northern blot densitometry readings were then corrected for the quantitative differences in RNA loading on the gel.

Total proteins were extracted from cold-acclimated and non-acclimated F49M and F49R subjected to electrophoresis on a 12.5% acrylamide gel using the Laemmli buffer system (Laemmli, 1970). Two gels were run simultaneously, one for Commassie blue staining, and the other for protein electroblotting onto nitrocellulose membrane using a transblot apparatus (Bio-Rad). For immunoassay, polyclonal antibodies against MsCOR A fusion polypeptide (Ferullo et al. 1997) was used (the antibodies were kindly provided by L. Vezina from Agriculture and Agri-Food Canada, Research Station, Sainte-Foy, Quebec). Anti-rabbit IgG conjugated with alkaline phosphatase was used as the second antibody. The binding of antibodies to proteins was visualized by

treating the membrane with substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitro-blue tetrazolium chloride (NBT) as recommended by manufacturer (Boehringer Mannheim).

The steady-state level of MscOR A mRNA was determined in non-acclimated and cold-acclimated non-regenerative F49M and its regenerative clone F49Rh by Northern hybridization (Fig. 6.2). This experiment showed 3-fold higher accumulation of the gene transcripts in cold-acclimated F49R genotype over cold-acclimated F49M genotype. Moreover, Western blot analysis, of total proteins from the same plants, using polyclonal antibodies against MscOR A fusion polypeptide (Fig. 6.3) detected approximately 28 kDa polypeptide (lane 4) in the protein extract from cold-acclimated F49R. The molecular weight of the protein is similar (32.2 kDa) to that detected by Western blotting in alfalfa (Ferullo et al. 1997). Based on the derived amino acid sequence of the alfalfa MscOR A cDNA, the MscOR A protein was predicted to have a molecular weight of 21.8 kDa (Ferullo et al. 1997). Post-translational modification of the protein might account for the discrepancies in the protein sizes for both species with the size 0.9 kbp of their precursor mRNA, and cDNA sequence in alfalfa (Fig. 6.1a). The immunoblot with anti-MscOR A also showed 2 bands of 40 and 55 kDa, with low-intensity staining in all analysed samples. Fusion MscOR A protein (containing the amino acid sequences deduced from its cDNA) used for raising anti-MscOR A was contaminated by some

Figure 6.2 Northern analysis of MsCOR A mRNA levels in cold-acclimated (a) and non-acclimated (n) non-regenerative genotype (M) and its regenerative somaclonal variant (R). Fourty μg of each RNA sample was subjected to gel electrophoresis. Molecular weight markers shown on the right of the panel are 0.24 to 9.5 kb RNA ladder (BRL).

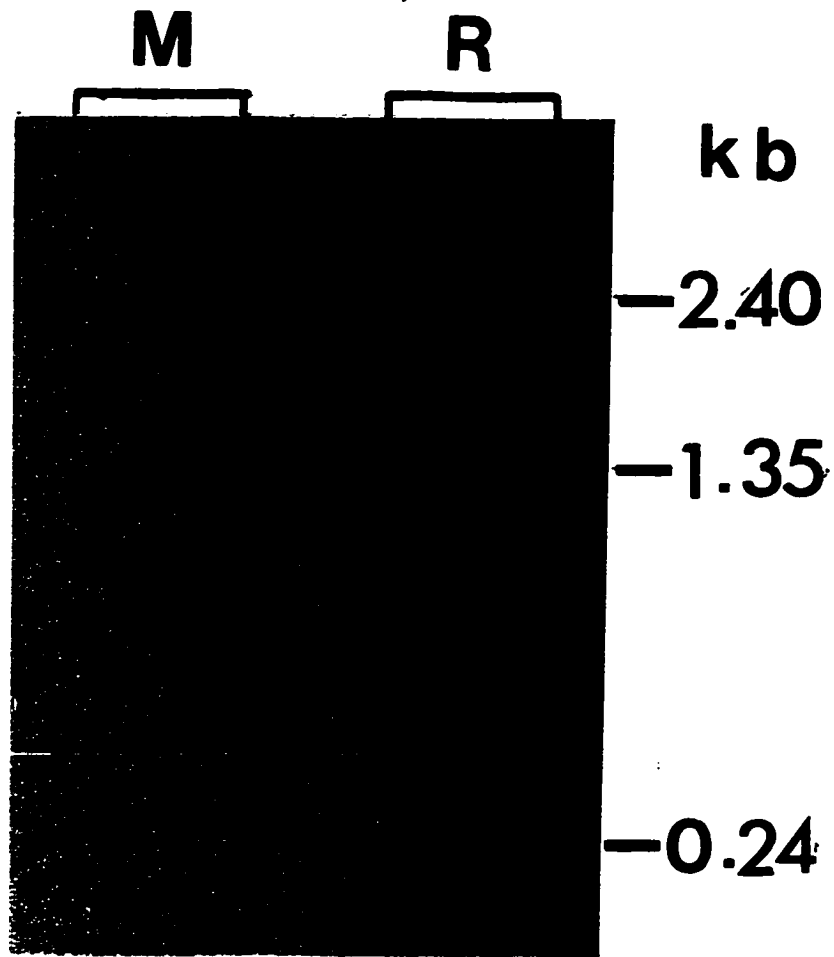


Figure 6.2

Figure 6.3 Western of protein extracts from non cold-acclimated (lanes 1 and 2), cold-acclimated (lanes 3 and 4) and de-acclimated (lanes 5 and 6) F49M (M) and its somaclonal variant F49R (R). Polyclonal anti- MsCOR A was used for immunodetection. Molecular weight markers shown on the right of the panel are SDS-PAGE low range molecular weight standards (Bio-Rad).

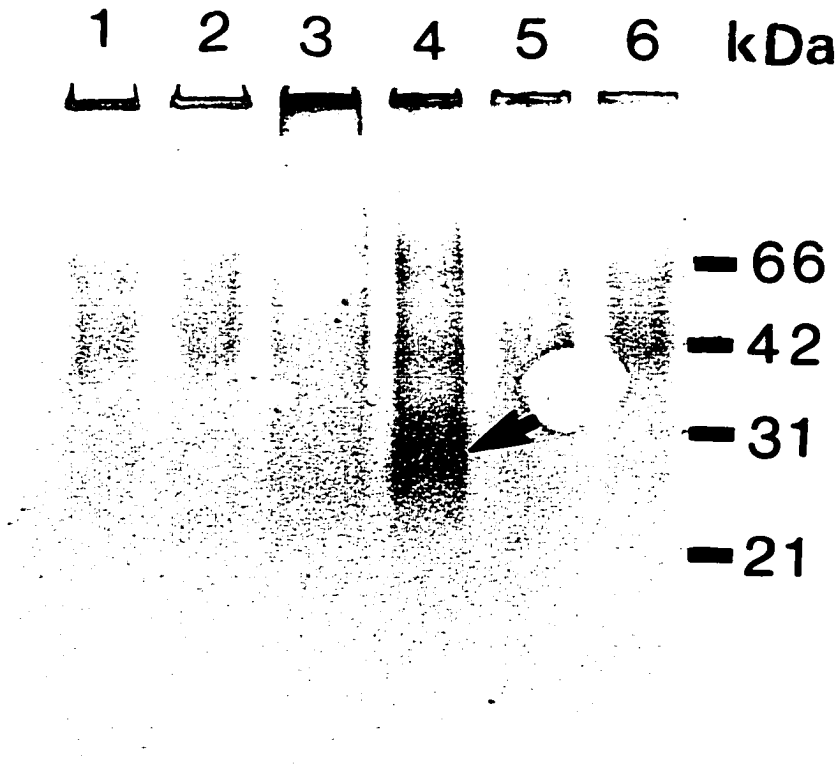


Figure 6.3

other minor protein species (Ferullo et al.1997). Presence of antibodies against proteins other than the MsCOR A in the antisera might explain observed non-specific immunoreactions.

MsCOR A gene transcripts were detectable in all cold-acclimated (a) and unacclimated (n) plants (Fig. 6.4). All plants gave enhanced expression of the gene after cold treatment (from 1.3 fold for F49M-lanes 11n and 11a to 4.9 fold for F₁C regenerative genotype-lanes 3n and 3a). The higher steady-state levels of MsCOR A mRNA in the cold-acclimated plants seems to be associated with regeneration but the transcripts levels varied from 1 genotype to another. Accumulation of the MsCOR A gene transcripts for cold-treated non-regenerative plants were 1.3 to 1.7 fold higher compared to non-regenerative controls (a/n ratios). For regenerative plants, the enhanced levels of MsCOR A mRNA ranged from 1.8 to 4.9 (a/n ratios; average 3- fold enhancement). These detected variations in the steady-state levels of MsCOR A mRNA among cold-hardened regenerative genotypes might reflect differences in the genetic background of the tissue samples (e.g., different stages development). The regenerative F₁ progeny (lanes 8n and 8a), from a cross between regenerative clone F49Rh2 (lanes 10n and 10a) and a non-regenerative genotype (G2a-lanes 7n and 7a), showed 3.9-fold higher abundance in MsCOR A gene transcripts after cold acclimation (Fig. 6.4). The non-regenerative F₁ progeny (lanes 9n and 9a) from the same cross showed only 1.3-fold increase in mRNA of MsCOR A after cold treatment.

Figure 6.4 Northern analysis of MsCOR A homolog transcripts accumulation in cold-acclimated (a) and non-acclimated (n) regenerative (R) and non-regenerative (NR) red clover genotypes. Lanes 1n and 1a up to 11n and 11a represent 11 different genotypes (which were cold-non-acclimated (n) and cold-acclimated (a)) which include 3 regenerative (lanes 1, 3 and 5) and 3 non-regenerative (lanes 2, 4, 7), F₁ genotypes from field seeds, 2 regenerative F49Rh calliclones (F49Rh4 - lane 6, and F49Rh2 - lane 10), non-regenerative F49M (lane 11) and regenerative (lane 8) and non-regenerative backcross (lane 9) generated by pollination of non-regenerative F₁ from field seed (lane 7) with regenerative calliclon F49Rh2 (lane 10). For each genotype ratio (a/n) of the Northern blot densitometry readings for cold-acclimated (a) to non-cold-acclimated (n) states was calculated (densitometry readings used for ratios calculation were corrected for the quantitative differences in RNA loading on the gel - see subchapter II). Forty µg of each RNA sample was subjected to electrophoresis.

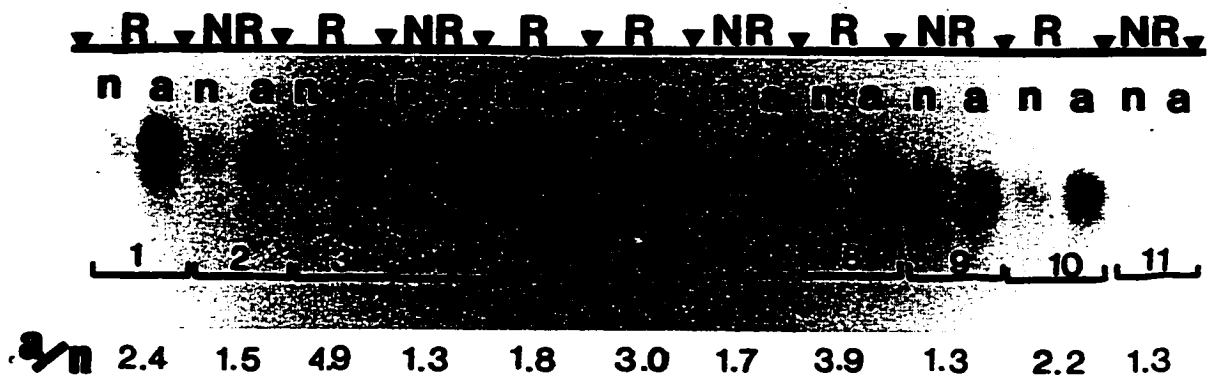
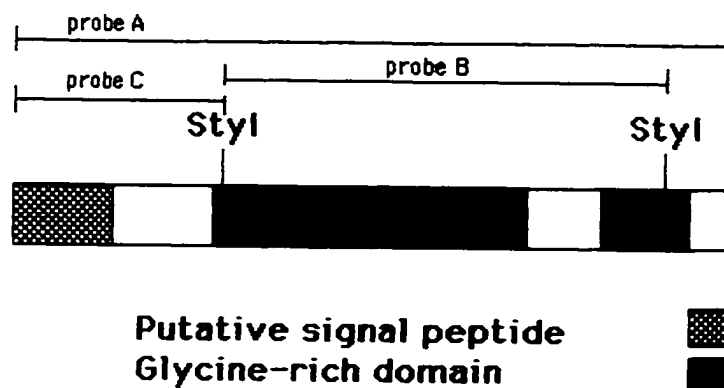


Figure 6.4

IV. Organization of the MsCOR A, B, and C genes in the non-regenerative F49M genotype and its regenerative F49R calliclones.

Genomic organization of MsCOR genes, especially MsCOR A, in non-regenerative F49M and regenerative F49Rs clones was examined by Southern-blot hybridization to explain observed differences in steady-state level of MsCOR A mRNA under cold treatment in regenerative somaclonal variants compared to non-regenerative F49M (see subchapter III). Southern blot hybridization analysis performed on *EcoR* I- digested red clover genomic DNAs revealed differences in banding patterns between non-regenerative F49M (lane M) and their 2 regenerative calliclones (lanes R1 and R2) using as hybridization probes the cDNAs for MsCOR A (probe A - Fig.6.5) (Fig. 6.6a), and MsCORC (Fig. 6.7b). No difference was for MsCOR B (Fig. 6.7a). DNA fingerprint-like profiles were generated by the alfalfa MsCOR A and MsCOR C cDNA hybridization probes. The MsCOR B cDNA detected 2 identical bands for all 3 genotypes. Southern blots of *Pal* I and *Hind* III genomic DNA digests of the same 3 red clover genotypes showed 1 and 2 bands, respectively, when hybridized to MsCOR B cDNA (data not shown). These results suggest a re-organization of the MsCOR A and C genes of red clover during somaclonal propagation which preceded regeneration. Southern blot hybridization of *Eco RI* -digested genomic DNA from seal using the alfalfa cDNA as a hybridization probe generated a multi-band pattern (data not shown). The nucleotide sequence

Figure 6.5 Amino acid sequence domains (a) and nucleotide sequence (b) of the alfalfa cold-induced cDNA, MsCOR A, showing the glycine-rich and glycine-poor coding regions of the cDNA used as a hybridization probes in Southern blot analysis. In (a), cDNA fragments used as probes in hybridization experiments are identified. Probe A - the entire cDNA of MsCOR A; Probe B - cDNA fragment rich in the triplet GGT (5'-3' sequence between nucleotides 198 and 591 bp); Probe C - cDNA fragment poor in the triplet GGT (5'-3' sequence between nucleotides 1 and 197 bp). In (b), thick underlines indicate the start and stop codons; thin underlines indicate *Sty I* restriction sites to generate different hybridization probes.



a)

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1  acaaaatatacacagttaaacagatattgaacaatggattcgaaaaa
47  ggcaatcctcatgctaagcctcttggctatggctcttatttcctcag
94  tgatgtccgctagggacttaactgaaacttcaccgatgctaaaaa
140  ggaggttggtgaaaagactaatgaagtaaatgatgccaatatggt
186  ggtggctacaaccatggtgggtgggttacaatggtggaggtataacc
232  acggtggtggctacaaccacggtggaggtggttaccacaatggtgg
278  tggcggttacaaccacggtggaggtggttacaatggtggtgggggt
324  cacggtggtcacggtggaggtggttacaatggtggtggaggtcacg
370  gtggtcatggtggaggtggttacaatggtggtggaggtcacggtgg
416  tcatggcgggtgctgaatctgttgccgtgcaaaactgaggaaaagact
462  aatgaagtaaatgatgccaatattgaggtggtagtaattacaaccg
508  atggtagaggtggctacaaccacggtggaggtggttacaaccacgg
554  tggaggtggtcacggtggacacggtggacatggtggccatggtggt
600  cacggtggacacggtgccgtgcaaaactgaggacaacactcaaaact
646  gatcataatgatatacatcatgcaccatgcacttttctcagtaatat
693  atgcatgaataaaaaataatgttgtatggtgatataaaaaaaaaaggc
740  atacatagatgtgcagcagttcagttatgggtattatctatcttg
787  ccactatatagtatagatagatattcatcagtggaatcatgtata
834  aatttgcacagactatgtatttgt

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b)

Figure 6.5

Figure 6.6 Organization of the MsCOR A gene homolog in red clover. *Eco R I* -digested genomic DNA from non-regenerative F49M (M) and its regenerative clones F49Rh1 (R1) and F49Rh2 (R2) was subjected to Southern blotting analysis and hybridized to either the entire cDNA of MsCOR A (Probe A - Fig. 6.5) (a), cDNA fragment rich in the triplet GGT (Probe B - Fig. 6.5) (b), or cDNA fragment poor in the triplet GGT (Probe C - Fig. 6.5) (c). Markers shown on the right of the panel are 1 kb ladder (BRL).

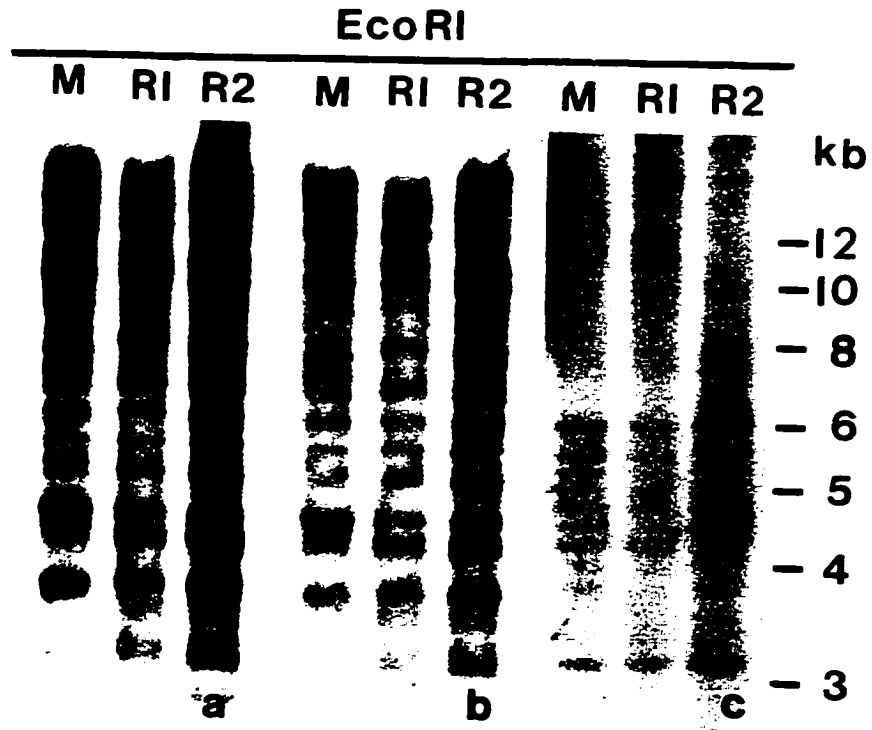


Figure 6.6

Figure 6.7 Organization of the MscOR B (a) and MscOR C (b) genes homologs in red clover. *Eco* R I-digested genomic DNA from non-regenerative F49M (M) and its regenerative clones F49Rh1 (R1) and F49Rh2 (R2) was subjected to Southern blot and hybridized to MscOR B probe (a) or MscOR C probe (b). Markers shown between the panels are 1 kb ladder (BRL).

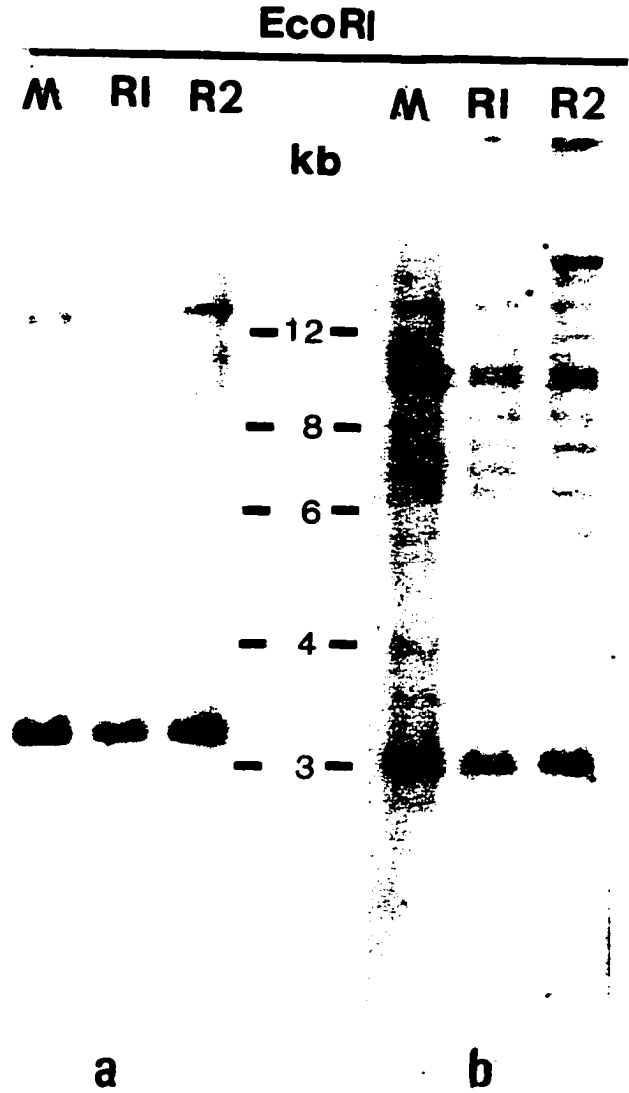


Figure 6.7

of MscOR A cDNA contains tracts of the trinucleotide GGT coding for glycine in the protein-coding region (Fig. 6.5) which might detect dispersed microsatellite repeats in the seal and red clover genomes, thereby generating the "DNA fingerprint"-like profile in these Southern blots (Fig. 6.6 a). Hybridization of the alfalfa MscOR A cDNA to both, gene homolog and dispersed repetitive DNA sequences of red clover might explain changes in the restriction fragment patterns in somaclonal variants (Fig. 6.6 a - lanes: R1 and R2). The MscOR A cDNA was digested with the restriction endonuclease *Sty* I to generate the cDNA fragments rich in the triplet GGT (5'-3' sequence between nucleotides 198 and 591 - probe B - Fig. 6.5) and poor in the triplet GGT (5'-3' sequence between nucleotides 1 and 197 bp - probe C - Fig. 6.5). The fragments were resolved on 2% agarose gel, and after staining excised and "gene cleaned" (BIO 101, La Jolla, CA). Southern hybridization analysis was performed with the same genomic DNA and experimental condition as described for Figure 6.6a, but with cDNA fragments rich in the triplet GGT (Fig. 6.5 - probe B) and poor in the triplet GGT (Fig. 6.5 - probe C) DNA fragments, instead of whole 0.9 kbp cDNA insert (Fig. 6.5 - probe A), as hybridization probes (Fig. 6.6 b and c). The probes A (entire cDNA) and B (GGT-rich) produced identical DNA profile (Fig. 6.6 a and b). The probe a (GGT-poor) hybridized to the same DNA fragments in F49M and 2 F49R genotypes, but the banding pattern was completely different to those obtained with other 2 DNA probes (Fig. 6.6 c).

V. Cloning and sequencing the MsCOR A homolog from a F49M red clover lambda genomic library

Two recombinant DNA bacteriophages from a red clover lambda genomic library that strongly hybridized to the MsCOR A cDNA probe in the filter hybridization experiment were selected for further analysis. To release the inserts, lambda DNA was digested with *Xho* I and then Southern blotted with the cDNA sequence. The cDNA sequence, poor in the GGT triplet (Fig. 6.7 - probe a), was used as a hybridization probe. Two lambda DNA fragments from 2 different recombinant DNA clones, one of 5.8 kbp and another of 2.8 kbp, hybridized strongly and exclusively to the probe (data not shown) and they contain the gene DNA sequences (the genomic DNA sequence of the MsCOR A gene of alfalfa contains 1 small intron placed between the coding sequences for the signal peptide and the glycine-rich domain - S. Laberge, personal communication, St.-Foy, 1995). The large-scale lysate plate method (Sambrook et al. 1989) was used to generate 15-20 µg of DNA from these 2 lambda DNA inserts. These were blunt-end ligated into pUC18 *Sma* I. Ligation products were used to transform competent *E. coli* strain DH5alpha (Gibco/BRL) and cells were plated on 2 x YT media containing X-gal. Colorless recombinant plaques were identified and the plasmids isolated. Inserts were released by digestion with *Sal* I and *Ban* II and the digests subjected to agarose gel electrophoresis. Most of the inserts were 1.5kb in size (Fig. 6.8). Five plasmids with the inserts were sequenced by

Figure 6.8 Agarose gel electrophoresis of *Sal I*- and *Ban II*- digested pUC18 to release cloned DNA inserts carrying supposingly red clover MscOR A homologous sequences. a - p - different recombinant clones, s - 1 kbp marker, v - pUC18. Molecular size markers shown to the left of the panel are 1 kb ladder (BRL).

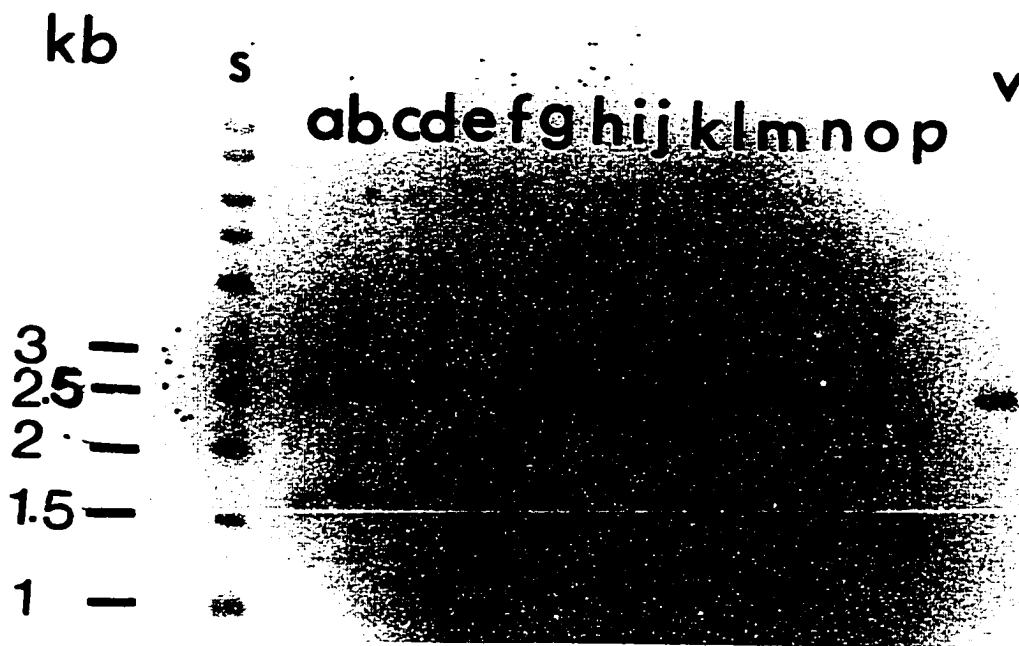


Figure 6.8

chain termination method (Sanger et al. 1977). The recombinant plasmids when sequenced appeared to be fragments of the pUC18 vector originating distally from the cloning site.

VI. Discussion

Red clover genes, homologous to the alfalfa genes coding for the cell wall proteins GRP (MsCOR A) and HPRP (MsCOR C), are organized, like in alfalfa, into multigene families. However, the MsCOR B gene, unlike its alfalfa homologue (Monroy et al. 1993), seems to be present as single copy gene in the red clover genome. Southern-blot hybridization with MsCOR A cDNA as a probe detected changes in DNA banding patterns between non-regenerative F49 M and its regenerative somaclonal variants. These DNA rearrangements resulted from changes in non-coding repetitive DNA in tissue culture (see chapter 5) and were not due to genomic reorganization of the red clover gene. The highly polymorphic DNA banding patterns demonstrated by Southern blot hybridization with MsCOR C cDNA, rich in the triplets CTT and/or CCA (Castonguay et al. 1994), as a probe might reflect a strong hybridization of CCT and/or CCA sequences of the probe to non-coding repetitive DNA very prone to changes in tissue culture.

MsCOR A gene transcripts accumulate in red clover in reaction to cold acclimation. This finding is in agreement with the results of parallel study conducted by Laberge (personal communication, St.-Foy, 1995) who noted a 15 times

higher level of the MsCOR A transcripts in the cold-acclimated red clover plants as compared to the non-acclimated, both, in the potted material used in this study and in field-hardened plants. This increase in the steady-state level of MsCORA mRNA might indicate direct link between the gene expression and cold tolerance. Unfortunately, we do not know if changes in MsCOR A expression are at transcriptional level or alternations are caused by stress-induced variations in mRNA turnover or both. This link has to be demonstrated, and utilization of the antisense RNA technology to determine the link, has been suggested (Castonguay et al. 1993). Previous studies have reported that some genes of multigene families can be differentially expressed even though their genomic organization is unchanged. Somaclonal variants were identified with altered expression of multigene family genes coding for gliadins in wheat (Evans 1989), for hordeins in barley (Breiman et al. 1987), for esterases in potato (Allicchio et al. 1987). *In vitro*-generated mutants often express proteins absent in donor plants. Larkin(1987) identified wheat somaclonal variants producing an additional set of beta-amylase enzymes and speculated that repressed genes were activated in tissue culture. Krasnuk (1978) found specific esterase isozymes present only in cold tolerant cultivars of alfalfa. Some copies of the repressed MsCOR A gene homologs in F49M might have been activated in F49R tissue culture regenerants as a result of e.g. genomic reorganization outside the gene.

Differential gene expression of cold-acclimated F49M and its regenerative somaclonal variant might be caused by mutation in the promoter region of the MsCOR A homolog gene or in cis-acting elements, which can regulate gene induction under stress (Shinozaki et al. 1996). It has been shown by Castiglione et al. (1995) by means of monoclonal antibodies to 5-mC that corresponding sites of homologous chromosomes of soybean differ in methylation. This observation implies that regenerants could be selected in tissue culture with more active, demethylated copies of genes. It is very tempting to draw a linkage between the presence of the regenerative trait and enhanced expression of MsCOR A homolog gene in cold-treated red clover. However, plant tissue culture is mutagenic and very often plants are recovered with several monogenic mutations (Evans & Sharp 1983). We have been attempting to sequence the MsCOR A homolog from a F49M red clover lambda genomic library but recombinant plasmids when sequenced appeared to be fragments of the pUC18 vector originating distally from the cloning site. These sequences were likely inserted at the cloning site by a rearrangement event resulting from an induced recombination rate in the presence of repetitive DNA arrays (Harris 1995, Whal et al. 1991). If red clover MsCOR A gene is sequenced, we would elucidate the molecular basis of the enhanced expression of this gene in the regenerative, cold tolerant variant, F49R. Furthermore, it would be feasible to use various constructs

of the gene to improve cold tolerance of red clover genotypes by transgenics.

SUMMARY

De-differentiation and re-differentiation processes that occur in tissue culture during callus formation from somatic tissues and plant regeneration from callus, respectively, are associated with quantitative and qualitative changes in genomic DNA of red clover. The hypothesis that some of these DNA changes generate somaclonal variants with valuable character traits was tested in this thesis.

The work of MacLean and Nowak (1989) showed that genetic variation induced in a plant cell and tissue culture can be successfully used to select totipotent mutants (F49R - possessing genetic competence for plantlet regeneration) in a species like red clover recalcitrant to regeneration. Plant regeneration is required for most genetic manipulations of crop plants like haploid lines production, protoplast fusion or genetic transformations. Identified competence for *in vitro* regeneration can be introduced into non-regenerative agronomically valuable genotypes of red clover by sexual crosses. The mapping of genes for regeneration trait would help to identify and characterize these genes at molecular level. In this study DNA rearrangements in "the hot spot" of F49R genome were detected by DNA fingerprinting with Jeffreys' probe 33.6. These rearrangements seem to be linked to regeneration trait, as based on the evidence from limited inheritance patterns. If the results are confirmed by DNA fingerprinting of larger number of regenerative and non-

regenerative progeny and their parents, the linkage could serve as molecular marker for regeneration allowing rapid development of regenerative red clover lines with diverse genetic backgrounds. Such lines could be used for improving persistence and for hybrid seed production in red clover. Somaclonal variation can be successfully used for mutant selection. Interestingly, in the same genomic "hot spot" where DNA rearrangements associated with regeneration trait were detected the RFLP marker linked to proline analogue tolerance was identified.

Frequencies of somaclonal variation in regenerated plants are very difficult to assess because most of the tissue culture derived DNA changes occur in non-coding repetitive sequences that may not have any effect on phenotype. The changes in quantity of repetitive DNA seem to be associated with differentiation and de-differentiation of plant cell in tissue culture. Substantial quantitative changes in repetitive DNA during red clover regeneration were observed and these changes have been found to take place independently of each other in different families of repetitive DNA sequences resulting in alternation in their relative proportion in genome. These findings imply that that expression or non-expression of specific sets of genes might be controlled by conformational effects imposed by changes in non-coding DNA. These findings are of great importance for efficient application of genetic transformation techniques as changes in repetitive DNA

during plant regeneration from transformed cells or protoplasts might cause observed low genetic transformation success.

It has been shown in this study that expression of multigene family can vary in regenerative somaclonal variants. Cold-acclimated F49R and its regenerative progeny showed induced expression of the MsCOR A gene (associated with improved cold-tolerance) compared to cold-acclimated F49M and non-regenerative F49R progeny. Genomic organization of the gene in F49R compared to non-regenerative F49M (explant source for regenerative mutants F49R) was unchanged. This data implies that some copies of the gene were repressed in F49M and activated in F49R tissue culture regenerants.

Experimental data derived from this thesis is in support of somaclonal variation as a part of breeding programs for red clover improvement. DNA changes associated with de-differentiation and re-differentiation processes in tissue culture produce regenerants with new agronomically important traits that can be developed in the elite clones (regenerative variants with enhanced expression of MsCOR A). Somaclones can be a valuable source of germoplasm to find genetic markers or to construct linkage maps for specific genes of interest for identified traits or to isolate these genes. In this study DNA rearrangements linked to regeneration trait and RFLP marker for tolerance to proline analogue (azetidine-2-carboxylic acid) were identified. The results, in particular those from the study on changes in

repetitive DNA during tissue culture regeneration, indicate that better understanding of regeneration process at a molecular level is needed for more controlled introduction of desirable genetic diversity into red clover.

APPENDIX

Tissue culture techniques

1. Culture media

1.1 Callus and regeneration initiation medium B5C (Beach & Smith 1979)

1.2 Somatic embryogenesis induction medium B5E (Beach & Smith 1979)

1.3 Callus initiation medium (L2) (Collins & Phillips 1982)

1.4 Somatic embryogenesis induction medium (SEL) (Collins & Phillips 1982)

1.5 Shoot development induction medium (SPL) (Collins & Phillips 1982)

F49Rh clones were generated on the following media sequence:

B5C - 4 weeks

B5E - 2x 4 weeks

SPL - 4 weeks

F49Rp clones were generated on the following media sequence:

L2 - 4 weeks

SEL - 4weeks

SPL - 4 weeks

Table 2. Recipes for 1 litre of media (mg)

component	B5C	B5E	L2	SEL	SPL
NH ₄ NO ₃			1000	1000	1000
KNO ₃	2500	2500	2100	2100	2100
CaCl ₂ ·2H ₂ O	150	150	600	600	600
MgSO ₄ ·7H ₂ O	250	250	435	435	435
KH ₂ PO ₄	-	-	325	325	325
(NH ₄) ₂ SO ₄	134	134	-	-	-
NaH ₂ PO ₄ ·H ₂ O	150	150	85	85	85
KI	0.75	0.75	1	1	1
H ₃ BO ₃	3	3	5	5	5
MnSO ₄ ·H ₂ O	10	10	15	15	15
ZnSO ₄ ·7H ₂ O	2	2	5	5	5
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.4	0.4	0.4
CuSO ₄ ·5H ₂ O	0.025	0.025	0.1	0.1	0.1
CoCl ₂ ·6H ₂ O	0.025	0.025	0.1	0.1	0.1
NaFeEDTA	43	43	25	25	25
inositol	100	100	250	250	250
nicotinic acid	1	1	-	-	-
pyridoxine·HCl	1	1	0.5	0.5	0.5
thiamine·HCl	10	10	2	2	2
adenine	-	-	-	2	-
benzylaminopurine	-	-	0.1	-	-
2,4-D	2	-	-	0.01	-
picloram	-	-	0.06	-	0.002
naphthaleneacetic acid	2	2	-	-	-
kinetin	2	2	-	-	-
sucrose	20000	20000	25000	25000	25000
pH	5.5	5.5	5.8	5.8	5.8

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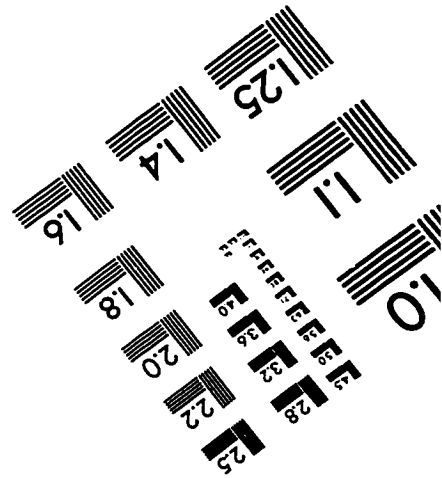
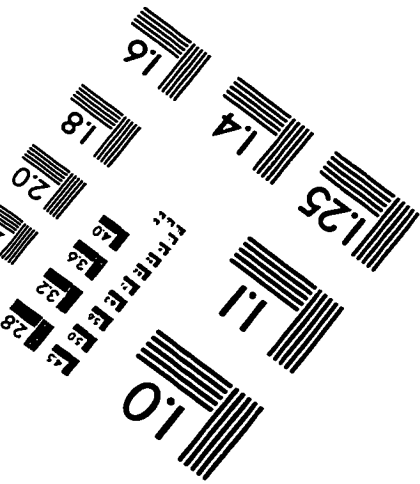
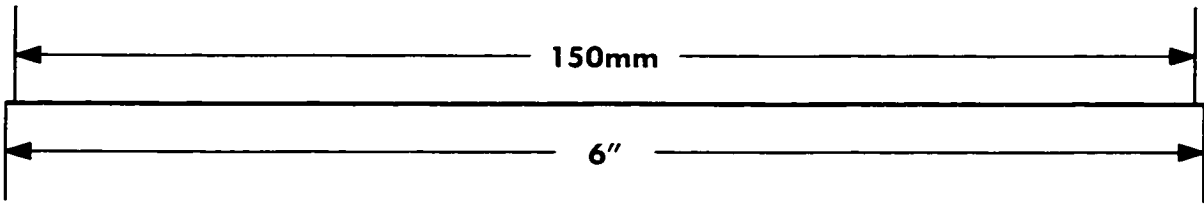
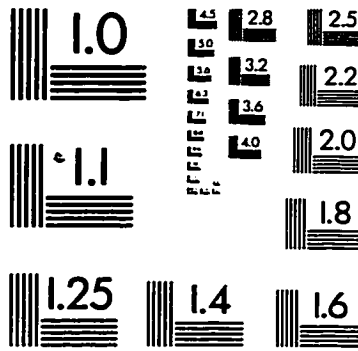
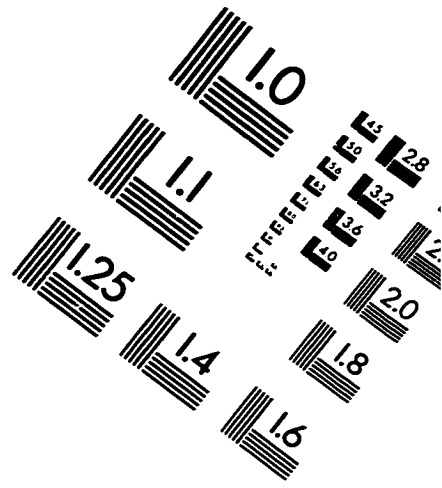
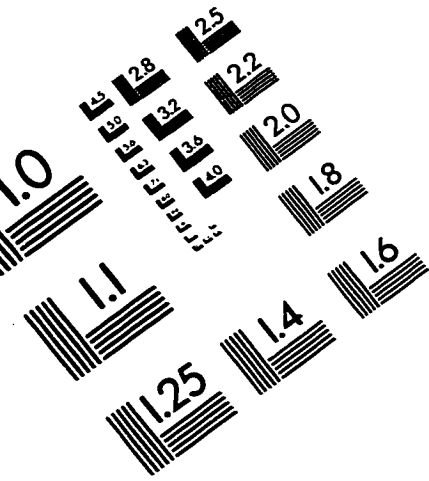
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IMAGE EVALUATION TEST TARGET (QA-3)



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