MOLeCULAR AND GEneTIC VARIATION IN CELL CULTURES AND REGENERATED PLANTS OF PETUNIA HYBRIDA.

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A thesis presented for the degree Doctor of Philosophy

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This thesis is dedicated to my wife, Stella, and to my parents. Without their encouragement and support it would never have been completed.
Leaves from Petunia hybrida have been used to initiate callus cultures amongst which phenotypic variation, including habituation and pigment synthesis, has been observed. Plants regenerated from callus cultures show phenotypic as well as karyotypic variation. Out of twelve callus regenerants three plants had the normal diploid chromosome number, two were aneuploid and seven had the tetraploid chromosome number. The phenotypic variation affected characters including leaf shape, flower size and pigmentation, internode length, gross morphology and flowering date. Much of the variation was found to be heritable and its extent correlated with the duration of callus culture. Phenotypic variation in plants regenerated from short term (2 month) cultures appears to result primarily from karyotypic differences whilst other genetic differences seem to contribute more to the widespread variation seen amongst regenerants from longer term (>lyr) callus cultures. Phenotypic differences were seen, however, in the two diploid progeny obtained from a short term culture suggesting that specific genetic differences may arise early in callus induction.

The organisation and methylation of a range of repeated DNA sequences has been studied in normal Petunia hybrida plants, in callus cultures and in regenerated plants. Whilst there was no evidence for major changes in the copy number or organisation of most of these sequences, one regenerant was found to have an unusual ribosomal RNA gene (rDNA) repeat
length and the mechanisms by which it may have arisen are discussed. The majority of repeated DNA sequences were seen to show extensive cytosine methylation in normal Petunia plants, however rDNA was found to have some unmethylated CCGG sites. The extent of rDNA cytosine methylation was found to be extremely variable between callus cultures. Despite this variability, rDNA methylation patterns appeared remarkably stable both during the subculture cycle and throughout prolonged (~10 months) callus culture. It is proposed that conditions at the time of callogenesis fix the extent of rDNA methylation in that callus. No variation in rDNA methylation was observed in regenerated plants suggesting that cell selection may largely eliminate different rDNA methylation patterns during regeneration or that specific changes in rDNA methylation are induced upon regeneration. The possibility that cell culture-induced alterations to DNA methylation patterns may contribute to somaclonal variation is discussed.
I hereby declare that I alone have composed this thesis and that, except where indicated, the work presented within was my own.
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NOTES AND ABBREVIATIONS

ATP  Adenosine 5'-'triphosphate
bp   Base pair(s)
DNA  Deoxyribonucleic acid
g    Gram(s)
x g  Average relative gravitational force
hr   Hour(s)
kb   Kilobase pairs i.e. 1000 bp
min  Minute(s)
mm   Millimetres
mg   Milligram(s)
pg   Picogram(s)
RNA  Ribonucleic acid
rRNA Ribosomal ribonucleic acid
rDNA Ribosomal DNA, genes encoding ribosomal RNA; see below
s    Second(s)

v/v  Volume per volume (as percent)
w/v  Weight per volume (as percent)
An indication of DNA fragment sizes is made alongside all autoradiographs. This is based on the migration of Hind III digested DNA from bacteriophage λ. This marker DNA was not radio-labelled and hence the resultant bands were not represented on the autoradiograph. Consequently these values should not be regarded as particularly accurate and are provided solely for general guidance.

Genes encoding the four nuclear ribosomal RNA's in plants are found in two distinct types of sequences. The 18S, 25S and 5.8S encoding sequences exist in clustered arrays and are transcribed as a polycistronic precursor whilst the 5S encoding sequences are found at a different chromosomal location. Unless specifically qualified, when rDNA is referred to in this document it refers to the DNA sequences encoding the polycistronic messenger. Sequences encoding the 5S rRNA are always referred to as 5S rDNA.
SECTION 1. INTRODUCTION

1.1. THE PLANT GENOME

GENOME SIZE.

One feature common to higher eukaryote genomes is the quite remarkable amount of DNA that they contain. In contrast to the situation in prokaryotes, which typically contain $4 \times 10^{-3}$ pg of DNA per genome, even the smallest eukaryote genomes, such as that of *Saccharomyces cerevisiae*, contain about $2 \times 10^{-2}$ pg of DNA whilst the largest genomes, such as are found in the lily family, can contain over 100 pg (Lewin, 1985). Additional DNA might be expected in eukaryotes to provide the additional coding capacity necessary to specify their more complex constitution. However, the increase in DNA content seen in most of the higher eukaryotes appears to greatly exceed the amount expected to account for their greater complexity. Even more surprising is the observation that there would appear to be no correlation between the complexity of a eukaryote and the amount of DNA comprising its genome. Amphibians are not considered any more complex than mammals yet the amphibian genome can be 45 times the size of its mammalian counterpart (Lewin, 1985). Plants represent the kingdom showing the greatest variation in haploid genome content. *Arabidopsis thaliana* has but 0.2 pg of DNA per 1C haploid genome whilst *Fritillaria assyriaca* has 140 pg (Bennett & Smith, 1976). Even within a genus there can be an outstanding variation in genome size, the genus *Crepis* having species with a tenfold difference (Flavell, 1982). This peculiarly eukaryote phenomenon where similar types of organism have genomes containing significantly different amounts of DNA has been termed the C-value paradox (Ohno, 1971).
The possession of such large amounts of DNA has at least two consequences. Firstly it confronts the organism with the apparently deleterious requirement for more replicative DNA synthesis at every cell division whilst, secondly, it provides the possible advantage of providing extra coding capacity, though precisely how this would benefit an organism is unclear. It has been suggested that the additional coding capacity might provide a reservoir of nucleic acid in which chance mutations might act forming novel and beneficial new genes and hence fuelling evolution. Additionally, the organisation of repeated DNA sequences is such as to permit genome reorganisations in ways which may generate new gene combinations or new gene environments. The fact remains, however, that Arabidopsis has evolved quite satisfactorily without this facility. Alternatively, it has been suggested that this DNA represents "selfish genes" of no benefit to the organism but concerned only with their own propagation. In truth, at the moment we have little idea of the real function of the apparently redundant DNA that constitutes such a large fraction of many eukaryote genomes. Undisputibly, however, it exists and is one of the most notable features of their nuclear make-up.

GENOME ORGANISATION.

When the organisation of the genome is studied different classes of DNA sequence elements can be identified both on the basis of their abundance and on the pattern of their dispersion throughout the genome. Study of the abundance of different sequences in the plant genome is possible by measurement of thermal renaturation kinetics (Murray et al., 1978). When DNA in solution is heated above its melting temperature (Tm) the strands of the duplex become separated resulting in single stranded molecules. On cooling, the strands reanneal and the duplex is reformed. The speed at which this reannealing can occur is determined by the concentration of specific single strands in solution and hence by
the degree of repetition of the DNA sequences involved. This results from the fact the reaction observes second order kinetics. The speed of renaturation of a sequence is indicated by its $C_{o}t_{m}$ value this being the product of concentration and time at which half of these sequences will have reannealed. Sequences that are highly repeated will anneal more rapidly, and hence have lower $C_{o}t_{m}$ values, than sequences present in lower abundance in the population of DNA molecules. When plant DNA is melted and reannealed the renaturation profile indicates the presence of four principal classes of sequence on the basis of their speed of renaturation. Whilst these broad categories are a convenient way of describing the nature of repetition of a particular sequence it should not be forgotten that they are but fractions of the whole spectrum of copy numbers. Certain sequences, for example members of a large gene family, may fall between two of the classifications

1) Foldback Sequences.
This class of DNA reanneals almost instantly ($C_{o}t_{m} < 10^{-5}$ moles of nucleotide seconds per litre). The speed of reassociation results from the intra-molecular reannealing of palindromic DNA sequences. Due to the intra-molecular nature of the reaction it follows first order kinetics and its rate is hence independent of concentration. DNA sequences in this class range from 300 to 1200 nucleotides in length and may include transposable elements possessing terminal inverted repeats. In wheat about 20% of the genome contains foldback sequences interspersed with less repetitive DNA sequences between 300 and 1000 base pairs long (Bazetoux et al., 1978).

2) Highly Repetitive Sequences.
This class of molecules reanneals very rapidly ($C_{o}t_{m}$ values of between $10^{-2}$ and 2 mol s $l^{-1}$) and hence represents extremely abundant sequences present at levels of about $10^{6}$ copies per genome depending upon the species considered. These sequences tend
to be short (≈ 10^2 bp). Whilst some of these repeats are interspersed throughout regions of low copy number sequences, the majority of these sequences are present in tandem arrays containing very large numbers of repeats which may be separable, in caesium chloride density gradients, as satellite DNAs by virtue of their atypical base composition. In Secale cereale the most abundant satellite consists of two copies of a 140 bp sequence interspersed with an unrelated 230 bp sequence. Such satellites can sometimes be shown to be associated with blocks of heterochromatin such as are found at chromosome telomeres, centromeres or knobs (Barnes et al., 1985). These highly repeated sequences are thought to be transcriptionally inactive but may have a role in chromosome pairing.

3) Moderately Repetitive Sequences. 
This class of sequence reanneals with C_t° values of between 2 and 10^2 mol s l^-1. Some of the sequences are longer than those in the highly repetitive class ranging up to the order of a few kilobases in length. This class of sequences includes the genes for histones, ribosomal- and transfer-RNA's.

4) Unique Sequences. 
This class of sequence is the last to reanneal (C_t° values approx. 1000 mol sec l^-1). It includes protein coding genes but also many unexpressed sequences. The term is somewhat misleading in that these sequences can be present in more than one copy and, indeed, up to tens of copies per haploid genome.

The pattern of dispersion of repeated sequences can be analysed by studying the rates of reassociation of DNA molecules of different lengths as well as by techniques using specific cloned sequences and Southern hybridisation analysis (Southern, 1975). In plants interspersed repeats are on average less than 1 kb long and these are located between single copy regions of the order of 1-2 kb although there are slight differences between the different plant
species that have been studied. Some repeated sequences are present in tandem arrays at just one or a few loci, such as those comprising ribosomal RNA genes, whilst others are dispersed throughout the genome. A class of dispersed highly repeated sequences present singly throughout the mammalian genome is the short interspersed nuclear elements or SINEs. These are sequences of less than 500 nucleotides that are repeated $10^6$ or $10^8$ times per haploid genome. It is thought that they represent sequences dispersed by a process involving reverse transcriptase and then reintegration, termed retroposons. The repeated sequences in rice with homology to both tRNA and 5S rRNA genes (Wu and Wu, 1987) could be considered as plant SINEs and plants also contain sequences comparable with the long interspersed nuclear elements (LINEs), which are another feature of mammalian genomes. LINEs represent dispersed moderately repetitive sequences which are much larger being anything from 1 to 10 kilobase pairs in length. They are present in numbers of about $10^4$ per genome. Cin4, one of the many transposable element families known to exist in maize, is one example of the LINE type of sequences in plants (Schwarz-Sommer, et al., 1987). This and other transposable elements are further examples of dispersed repeats present primarily as single copies scattered throughout the genome.

1.2) FLUIDITY OF THE GENOME.

RAPID GENOME CHANGE.

In recent years it has become evident from the study of a number of systems that the eukaryote genome can no longer be considered a fixed entity that is inherited unchanged from generation to generation with only very long term evolutionary processes having any effect on its composition. All eukaryote genomes would appear to have the capacity for quite pronounced yet rapid changes that can mean the genomes of offspring are radically altered from that of the parent. In plants in particular, frequent genome changes
would appear to be the norm rather than the exception. This may be a consequence of them lacking a distinct germ line, and hence being subject to all the modifications that may occur in somatic cells, or it may be as an adaptation to their static form of life where, since it is not possible for them to escape an environmental change, they must have a capacity to change in response to the environment.

Rapid changes to the genome of somatic cells can be seen in plants. When the root tip meristems are cut from *Vicia faba*, and cell dedifferentiation is induced, rapid DNA replication is seen to occur. This results in the amplification of highly repetitive satellite DNA sequences in the nuclei of cells in the wound callus (Natali, et al., 1986). Whilst in this case the cells are unlikely to become the meiotic tissue of the reproductive organs the example serves to illustrate that the mechanisms must exist for selective DNA amplification. Such mechanisms may sometimes be expressed in the somatic tissue from which the germ cells are derived.

Numerous examples are to be found in the literature where rapid genome changes must have carried on to the germ cells of plants. Considering an evolutionary timescale one can find plentiful evidence for the divergence of the genomes of plants that once had a common ancestor. Comparison of the maize genome with that of its nearest relatives the teosintes shows quite pronounced differences in both the repetitive and single copy fractions (Hake & Walbot, 1980). Over a shorter timescale the amount of genome change that can be observed seems even more remarkable. At the University of Illinois there is a collection of maize lines that have been under selection since 1896 for changes in total protein or oil content. Amongst these lines is found a 2.5 fold variation in the ribosomal RNA gene (rDNA) content (Phillips, 1978). Thus, over less than a century great changes have occurred in the copy number of the rDNA repeat family in these lines. This is perhaps not surprising in
view of the notorious variability of rDNA sequences, however Rivin et al. (1986) have found a similarly high degree of variation in the copy numbers of other types of repeated sequence between different North American inbred lines and varieties of maize accompanying a high degree of unique sequence stability.

Genome fluidity is also manifest in certain sexual hybridisations both at the level of changes to chromosomes and at the level of changes to individual genes. In *Nicotiana tabacum* X *N. otophora* hybrids chromosomes up to 15 times their normal length can be found (Gerstel & Burns, 1976). Nothing is known of the amplification events that must give rise to these megachromosomes but it is possible that the mechanisms are active in circumstances other than those encountered in these interspecific hybridisations.

In intraspecific crosses of *Microseris douglassii*, plants with nuclear DNA contents varying by 10% can be obtained (Price et al., 1983). This difference in genome size is probably due to amplifications and deletions of repeated DNA components. Indeed most repeated DNA sequences in maize can show non-mendelian inheritance of copy number in intraspecific crosses between different inbred lines (Rivin & Cullis, 1983). The sequences found to change in copy number included rDNA and other repeats of unknown function present either in tandem arrays or dispersed throughout the genome.

An example of the unstable mutations seen following certain hybridisations is the so-called "rabbit ears" phenotype in peas (Pearson, 1968). It is also seen when maize is crossed with teosinte resulting in the appearance of both stable and unstable mutations at a high frequency; up to 2% of the F1 progeny showed visible signs of mutation (Manglesdorf, 1974). Since these mutations are stable, at least for a number of generations and sometimes permanently, some genetic change must have occurred. It
is tempting to draw comparisons with the well characterised phenomenon of hybrid dysgenesis in \textit{Drosophila}, a phenomenon related to transposable element activation (Fawcett, \textit{et al.}, 1986).

Paramutation is also worthy of mention in this context since it too has been shown to be an example of heritable genetic change related to transposable element interactions resulting from specific hybridisations (Krebbers \textit{et al.}, 1987). The unstable mutant allele \textit{niv} 53 of \textit{Antirrhinum majus} is an allele of the chalcone synthase gene that contains the transposable element Tam 1 which eliminates anthocyanin production except in cells where somatic reversions have resulted from excisions of the element. When \textit{niv} 53 is present in a heterozyote along with the chalcone synthase deficient allele \textit{niv} 44, itself containing a stable transposable element Tam 2, a change occurs to the unstable allele such that in future generations, including backcrosses when no \textit{niv} 44 allele is present, the allele no longer completely abolishes anthocyanin synthesis. The \textit{niv} 44 allele responsible for bringing about the change is said to be paramutagenic and such alleles have the capacity to alter the otherwise unchanging paramutable alleles such as \textit{niv} 53. A similar situation is seen in maize where alleles of the \textit{R} locus, which regulates anthocyanin synthesis in the aleurone layer, are found to be either paramutable or paramutagenic. When a hybrid is made containing both types of allele the level of expression of the paramutable allele is permanently changed (Kermicle, 1978).

The most striking example of a rapid genome change observed in a plant must, however, be the observation by Schaal and co-workers (cited by Walbot, 1985) of ribosomal RNA gene variation within a single plant. They studied the copy number and organisation of the rDNA in different apical meristems of the long-lived perennial \textit{Solidago} and found differences between DNA from different branches. Since all of these meristems can go on to produce the
meiotic tissue of the germ cells there must obviously be
differences in the ribosomal gene components of the progeny.
Whilst the other examples of rapid genome changes that can be
cited do perhaps represent rather unusual circumstances that are
perhaps only rarely encountered in nature the growth of a
perennial plant is perfectly normal. If rapid genome changes occur
here it is likely that they are very much the norm rather than an
exception.

A further, and extremely significant, observation of a rapid
genome change that can be observed at the molecular level in
plants is the example of gene amplification that is seen in the
acquisition of herbicide resistance. Amongst cultured alfalfa
cells it was possible to select a line showing an amplification of
the glutamine synthetase gene resulting in enhanced resistance to
the herbicide L-phosphinothricin (Donn et al., 1984). Similarly,
in *Petunia* resistance to the herbicide Glyphosate is achieved
through a 20 fold amplification in the genes encoding 5-
enolpyruvyl-shikimate-3-phosphate (EPSP) synthase (Shah, et al.,
1986). In mammalian systems similar gene amplification events can
be observed and Bostock and Tyler-Smith (1982) have estimated,
from a study of the acquisition of methotrexate resistance in mouse
cells, that about ten percent of all of the cells within a culture
may undergo a duplication of some DNA sequences over a period of
several weeks. With amplifications occurring at this frequency the
genome can only be considered fluid.

**STRESS AND GENOME CHANGE.**

Environmental and other stresses have been implicated in
triggering some of the rapid genetic changes seen in plants. One
of the most noteworthy examples is that of the flax genotrophs.
When flax plants are subjected to certain specific light and
nutrient regimes offspring with both large and small phenotypes
result. These phenotypes were found to be heritable over a number
of generations (Durrant, 1962) and were linked with changes in the nuclear DNA content, a 16% difference between large and small genotrophs being detected (Evans et al., 1966). In addition to the size differences other phenotypic changes were observed; hairs were lost from the false septae of the seed capsules in the large genotrophs whilst in the small genotrophs the peroxidase isozyme pattern had changed from the dominant to a recessive pattern. Cullis & Cleary (1986) have investigated specific repeated DNA sequences in flax genotrophs. The cloned DNA sequences that they used in their study included both highly- and moderately-repetitive sequences including the 25 S and 5 S rDNA sequences. All but one of the repeats analysed was found to vary in the genotrophs and earlier work (Goldsborough et al., 1981) had shown that specific subsets of each repeated DNA family were more prone to variation than other subsets of the same family. Evidence was also found of a moderately repeated sequence in the original flax line becoming a highly repeated sequence in a genotroph. Despite all of these genetic changes in the genotrophs no changes that can be causally linked to the phenotypic changes have been seen. Indeed, it has been suggested (Cullis & Charlton, 1981) that the phenotypic effects may result from changes in chromosome architecture reducing the expression of certain genes by bringing them into transcriptionally underexpressed regions of heterochromatin.

Flax genotrophs are not an isolated example of plant genome changes in response to stress. Similar phenomena can be seen in Nicotiana rustica (Hill, 1965) and pea (Highkin, 1958) though in this latter plant the changes are not as stable as those seen in flax. Equally, McClintock has drawn attention to the importance of stress in triggering another rapid genome change, namely transposable element activation (McClintock, 1978). The stress to the genome of chromosome breaks can result in the activation of the Ac / Ds transposable elements whilst the stress of virus infection is sufficient to activate the transposable element Bsl
in maize. The transposition of transposable elements can alter the expression of genes around their integration sites resulting in mutations. This alteration in expression can result either from the mobile element interrupting the coding sequence in an open reading frame or from disturbance of the control regions adjacent to the gene coding sequence (Sommer et al., 1988).

Clearly stress has an important effect on the genome as the great number of unrelated examples that can be cited implies. Indeed, Cullis and Charlton (1981) have suggested that the reason that there are not even more characterised examples of this phenomenon is that many plant systems' response to stress might be more constant meaning that any stably inherited environmentally induced changes are obscured by the constant environmentally induced variations that will occur in every successive generation.

That tissue culture might also impose a stress sufficient to activate the mechanisms responsible for the liberation of transposable elements, as well as bringing about the types of amplification / deletion events considered above, will be considered in a later section (Section 1.5).
1.3 CYTOSINE METHYLATION AND PLANT GENE EXPRESSION

SEQUENCE SPECIFICITY OF CYTOSINE METHYLATION.

Plant DNA differs from that of other organisms by virtue of its substantial content of the modified base 5-methylcytosine (5-MC), 23-29% of all cytosines having this modification in plants yet only 2-7% of the cytosine residues being affected in vertebrates (Shapiro, 1976). This base appears in the DNA by the post-replicative addition of a methyl group to cytosine residues by specific methylases such as that recently isolated by Theiss et al. (1987). The preferred substrate for these enzymes is hemi-methylated DNA, that is DNA that is methylated on one strand, and hence the very form of DNA that is generated by semi-conservative replication. Since the majority of 5-MCs, in plants, are present in the dinucleotide C-G (Gruenbaum, et al., 1981) and because a methyl group in this dinucleotide is complemented with one on the newly synthesised strand following replication, methylation patterns are maintained from one cell generation to the next. A consequence of this is that methylation patterns may be heritable both through mitosis and meiosis.

Gruenbaum et al. (1981) have studied the sequence distribution of 5-MCs in plant DNA. By using nearest neighbour analysis they have found that 82% of C-G dinucleotides in wheat germ DNA were methylated at the C position. When they investigated the situation in trinucleotides they found that more than 80% of C-A-G and C-T-G triplets were similarly modified. In contrast less than 20% of C-A and C-T dinucleotides and less than 4% of C-A-T trinucleotides contained 5-MC. The great majority of the 5-MCs seen can be explained in terms of methylation at C-G dinucleotides and C-N-G trinucleotides.

One consequence of C-G methylation, and something that should be remembered when considering genome change, is the under-
representation of this dinucleotide in plant DNA. This phenomenon, even more pronounced in vertebrates, is a consequence of the potentially mutagenic phenomenon of transition, where a spontaneous deamination of 5-MC causes the production of the similar base thymine in its place. Evidence from monkey cells (Brown and Jiricny, 1987) suggests that in the majority of cases the resulting G-T mismatch is repaired in favour of the guanine residue (i.e. the thymine is excised and replaced with a cytosine). In a low proportion (8%) of cases, however, repair is found to favour the thymine resulting in the replacement of the former C-G pair with a T-A pair. Thus transitions can bring about point mutations with inevitable effects on open reading frame codon sequence. The effect on the prevalence of the C-G dinucleotide and the C-N-G trinucleotide is obvious. Since it is only in these positions that 5-MC occurs, it will be in these sequences that the transition of cytosine to thymine residues occurs. Because the \textit{Msp} I and \textit{Hpa} II restriction endonucleases have a recognition sequence incorporating both of these potential cytosine methylation sites this recognition sequence is particularly underrepresented in plant DNA.

\textbf{THE RELATIONSHIP BETWEEN CYTOSINE METHYLATION AND GENE EXPRESSION.}

In vertebrates, where there is a much lower incidence of 5-MC compared to plants, there appears to be an inverse correlation between the degree of methylation of a sequence and its level of expression (Angelier \textit{et al.}, 1986). Similarly in plants, untranscribed satellite DNA's are found to be very highly methylated (Shengeliya \textit{et al.}, 1986). In lower copy number sequences in vertebrates cytosine methylation also appears to correlate with gene expression and may even be involved in the control of gene activity during development and cell differentiation. Whether such a function exists in plants is less clear. Whilst there is no correlation between methylation of and transcription of the alcohol dehydrogenase in maize (Nick, \textit{et al.},
1986) the ribosomal RNA genes of a number of plants show the opposite result.

There is strong evidence for a correlation between cytosine undermethylation and the developmental control of ribosomal RNA gene expression in *Pisum* (Watson et al., 1987). Methylation of the rDNA is found to be very high in bud tissue, to be very low in young seedlings and to progressively increase during development in contrast to ribosomal RNA transcription which is very low in buds, very high in young seedlings and which declines as development advances. The most pronounced changes in methylation state are observed at a *Hpa* II site 800 bp upstream from the end of the mature 18S rRNA in the intergenic spacer region (IGS). A hypomethylated site in the IGS region is also found in a constant number of the rDNA repeats in different flax lines where the gene copy numbers can vary from 300-800 per genome and the suggestion has been made that this is indicative of the expressed genes (Blundy et al., 1987). A similar inverse relationship between rDNA methylation and transcription is seen in the hierarchy of nucleolar organiser dominance in hexaploid wheat (Flavell et al., 1986). The size of a nucleolar organiser region, and hence the level of transcriptional activity shown by that nucleolus, is found to be inversely proportional to the prevalence of a 5-MC residue at a *Hpa* II site near the rRNA transcription promoter in the IGS region of the rDNA cistron. These examples provide evidence for an inverse correlation between methylation and transcription. They do not imply that the methylation state per se controls transcription. In rice the ribosomal RNA genes are very sensitive to *Hpa* II regardless of developmental state (Olmedilla, et al., 1984) even though the level of transcription may well be varying and, perhaps more significantly, *Xenopus* cocytes will transcribe very highly methylated rDNA from sperm (Macleod & Bird, 1983) implying that a hypomethylation of the IGS is not a requirement for rDNA transcription. It could be that undermethylation merely reflects, rather than causes, rDNA
transcription. Indeed, it has been proposed for the *Xenopus* system that transcription, facilitated by the binding of transcription factors, may itself lead to the reduction in methylation of the rDNA cistrons observed during development (Pennock & Reeder, 1984). However, extrapolation from *Xenopus* data should be treated with caution since it fails to explain the observation that treatment with 5-azacytidine, which results in the abolition of cytosine methylation, can activate previously silent T-DNA genes (van Slogteren et al., 1984, Hepburn et al., 1983). This finding suggests that cytosine methylation *per se.* is capable of suppressing transcription.

**RESTRICION ENDONUCLEASES TO MEASURE CYTOSINE METHYULATION.**

Methylation of cytosines in certain specific sequences can be studied through the use of methylation sensitive restriction endonucleases. *Hpa* II can be used to determine methylation at the internal C-G dinucleotide of the tetranucleotide recognition site C-C-G-G. *Hpa* II will not cut when this internal cytosine is methylated but it is insensitive to methylation of the external cytosine which can occur due to its position in a C-N-G trinucleotide. *Msp* I, on the other hand will cut if the internal cytosine is methylated but is sensitive to methylation of the external cytosine. These enzymes have proved to be very valuable in the study of plant DNA methylation particularly when combined with Southern hybridisation and the use of specific cloned DNA probes.

**1.4 PLANT TISSUE CULTURE**

**THE ORIGINS AND USES OF PLANT TISSUE CULTURE.**

Tissue culture is a process whereby small pieces of living plant tissue or individual cells are isolated from an organism and grown aseptically for an indefinite period. It is the main plant science
process that has paved the way for many of the manipulative techniques that the modern biologist can apply to plants and which has broadened the application of plants in biotechnology.

The theoretical advent of plant tissue culture could be attributed to Vochting who, in 1878, suggested that plant explants may well have the potential for totipotency (for review of the early history of plant tissue culture see Krikorian, 1982). Later, Haberlandt, in 1902, predicted that it would be possible to cultivate artificial embryos from cultured cells but it was not until 1922 that the first practical steps were taken. In that year Kotte was able to culture root tips, excised from pea and maize plants, for a limited period on a medium containing meat extract. By 1934 White had achieved the first indefinitely growing culture of roots. He had excised roots from tomato plants and had cultured them on a chemically defined medium. The culture of disorganised tissue, rather than merely the propagation of excised organs, required the use of exogenous plant growth regulators and in 1937 Gautheret was the first scientist to use indole acetic acid in a plant tissue culture medium. In the next two decades further experiments with growth regulators gradually led to the understanding, by Skoog and Miller (1957), of the ways by which organogenesis could be regulated. With regeneration possible through organogenesis it was only one year before Reinert (1958) showed that regeneration of carrot was possible by embryogenesis thus fulfilling the prophecy of Haberlandt. In 1962 Murashige and Skoog published details of a defined growth medium suitable for a wide range of applications and this medium (M&S) became central to much of the plant tissue culture work in the next two and a half decades.

The technology now exists to culture excised organs, disorganised callus tissue, suspensions of individual cells, single cells in the presence of a nurse callus, pollen cells, and even cells lacking a cell wall. Regeneration from these cultures can often be
achieved by either organogenesis, when adult plant organs such as shoots and roots differentiate, or by embryogenesis when structures analogous to zygotes differentiating after fertilisation appear. This technology makes possible applications in a number of areas. For the commercial production of plants, micropropagation allows for the rapid, asexual multiplication of improved varieties or for the rapid multiplication of plants such as orchids or trees whose reproductive cycles are long and hence slow (Jones, 1983). In plant breeding programmes the production of haploid plants by anther culture techniques facilitates the production of homozygous lines (Chu, 1982). Plant breeders can also take advantage of the embryo rescue techniques that facilitate crossing between distant species not normally amenable to crossing (Raghavan, 1977). Somatic hybridisation by protoplast fusion (Cocking, 1981) takes the potential for wide 'crosses' one step further whilst genetic transformation, by direct DNA uptake or Agrobacterium mediated DNA transfer, allows the introduction of specific genes from other plants, animals or even micro-organisms (Barton et al., 1987). Manipulation of the cytoplasmic makeup of plants can be achieved through 'cybridisation', the transfer of organelles from one plant to another through microplast/protoplast fusions (Lorz, et al., 1981). The resulting progeny from these experiments can, in theory, be preserved indefinitely by germplasm storage in the form of cryopreservation (Withers, 1983) whilst tissue culture produced material can be sown directly in the field through the production of artificial seed. Whilst not strictly breeding, plant improvers welcome the ability to eliminate viruses from plants by in vitro culture of their meristems (Walkey, 1978) since many plants contain apparently asymptomatic viruses which nevertheless have a negative effect on yield. In the more industrial field, use can now be made of plant tissue cultures for the production of plant secondary metabolite products in culture vessels analogous to microbial fermenters (Rhodes, et al., 1987). This can overcome problems with the supply of whole plants and can also allow for increased yields. Particularly important in the
development of such systems is the use of tissue culture for the 
in vitro induction of mutants and the selection of improved cell 
lines (Henke, 1981).

SOMACLONAL VARIATION: GENETIC VARIATION ARISING FROM PLANT TISSUE 
CULTURE.

The observation that clonally derived plants regenerated following 
a tissue culture phase can be found to be phenotypically distinct 
from each other, in one or a number of features, had been largely 
ignored in the literature until Larkin and Scowcroft (1981) 
brought it to attention and coined the term somaclonal variation 
to describe it. It appears that events occurring during tissue 
culture, and particularly during culture involving a callus phase, 
can bring about heritable changes to the phenotypes of the 
regenerated plants. This observation would seem to be a general 
one encompassing all callus systems regardless of whether 
regeneration is achieved by somatic embryogenesis or adventitious 
shoot formation and including situations where the callus is in 
fact tumour tissue (Peerbolte et al., 1987). Prolonging the callus 
phase prior to regeneration is found to increase the amount of 
heritable variation observed (Lorz and Scowcroft, 1983) and this 
correlates with an increase in the number of observable 
chromosomal rearrangements in the cells (Lee and Phillips, 1986). 
Additionally, the amount of variation resulting from callus 
culture is found to differ according to the precise culture 
conditions and the genotype of the plant material employed (Karp 
et al., 1987). However, in the vast majority of cases there is a 
sharp contrast to the situation observed when whole plant 
regeneration is achieved following meristem tip or axillary bud 
culture where the expected phenotypic and genetic uniformity is 
nearly always observed.

The phenomenon of somaclonal variation shows widespread occurrence 
affecting virtually every species where regeneration from a callus
phase is possible and affecting plants with every available ploidy level. The species in which somaclonal variation has been reported include major crop species, species normally propagated by asexual means and ornamental species including Petunia (Santos & Handro, 1983). Equally, somaclonal variation is not an infrequent occurrence amongst regenerants though precisely quantifying the extent of its occurrence is not easy. In a study of 65 potato plants regenerated from protoplasts every single plant varied with respect to at least one of the 22 characters tested and some individuals had 17 variant traits (Secor & Shepard, 1981). Indeed, not only may a number of individuals be affected but whole populations can be changed with regard to a particular trait since in sugar cane it was observed (Krishnamurthi, 1974) that the entire population of regenerated plants showed a shift towards improved resistance to Fiji disease.

When the extent of phenotypic variation resulting from callus culture is studied it is found that the traits affected include both quantitative traits, such as height, tiller number and heading date in wheat, as well as qualitative characters such as grain colour and gliadin seed proteins (Larkin, et al., 1984). Not all traits are seen to vary, though, and of the 22 characters studied by Secor and Shepard (1981) nine showed no variation. When the genetics of the variant traits is studied both homozygous and heterozygous traits are seen. Perhaps surprisingly, the appearance of homozygous variants is not a rare event, George and Rao (1982) finding 7 out of 85 regenerated mustard plants with the recessive yellow seed trait in the homozygous state. It has been suggested that the observed homozygous changes must have arisen as a consequence of mitotic crossing over (Evans and Sharp, 1983).

Many of the phenotypic changes induced by callus culture are heritable and must therefore result from genetic changes. The phenomenon could arise from the liberation of previously cryptic somatic mutations. However, if somatic mutations occurred at a
rate sufficient to generate variability at the frequency seen in somaclonal variation, there would be a requirement for some specific mechanism to prevent the appearance of such mutations in the meristematic tissues from which the germ cells are ultimately derived. If no such mechanism existed there would be a high incidence of sports and variant sexual progeny would result. Equally if pre-existing somatic cell variability was the sole cause, variation would not be seen between different plants derived from the same protoplast. It would be unreasonable, however, to claim that pre-existing genetic variability can play no part whatsoever in somaclonal variation since it is possible that chromosome number variations seen in some callus cultures may be related to the karyotype variation observed between different cells of the whole plant (D'Amato, 1952). Another trivial explanation of the cause of the phenomenon could be chemical mutagenesis induced by components of the culture medium. This is unlikely in that many of the culture medium constituents are common to both callus and organ culture media and in the latter instance no variation arises. Additionally, Dolezel & Novak (1984) were unable to observe any mutagenesis caused by medium components on a Tradescantia system which monitored changes in the expression of genes controlling pigmentation in the unicellular stamen hairs.

Karyotypic Variation.

The level where the genetic changes responsible for somaclonal variation may most easily be seen is at the level of chromosomes and so it is perhaps not surprising that there are numerous reports of aberrant karyotypes amongst callus regenerants. It should be noted, however, that somaclonal variation can be seen in chromosomally normal, euploid regenerants and so karyotype changes alone cannot be responsible for all of the events associated with the phenomenon (Karp et al., 1982).
Bayliss (1980) has reviewed the widely accepted connection between disorganised callus growth and chromosomal instability. The extent of chromosomal variation seen in callus cultures always exceeds that found in regenerated plants derived from them and a broad relationship can be shown between the morphogenic capacity of a callus and its cytological characteristics. Additionally, callus with a high mitotic index, low levels of polyploidy or aneuploidy and few chromosome breakages is likely to produce more phenotypically normal shoots than calli with a high frequency of cytological abnormalities, which will tend to produce more aberrant phenotypes. Some regeneration systems are more prone to chromosomal instability than others with protoplast regeneration being particularly susceptible (Karp, 1986). The extent of chromosome instability is also dependent on genotype (Bajaj et al., 1978). Chromosome instability can be manifest through changes in both chromosome numbers (ploidy) and chromosome architecture. Numerical changes to the chromosome complement include changes in the numbers of whole chromosome sets in the nucleus (euploidy) and changes in the numbers of part chromosome sets (aneuploidy). Structural changes affecting chromosome architecture include deletions, duplications, translocations and interchanges (Creissen & Karp, 1985).

Of the types of ploidy changes seen in callus regenerants, aneuploidy is the most detrimental to plant health through its pronounced effect on genic balance (Darlington, 1958). Aneuploidy is a common occurrence amongst the regenerants of polyploid plants but it is less frequent in plants regenerated from haploid or diploid material where increasing levels of euploidy are a more frequent event. High levels of aneuploidy are found to bring about a reduction in the morphogenic capacity of a callus (Murashige & Nakano, 1967). In a study of potato regenerants Karp et al. (1982) found most, though not all, of the plants with grossly abnormal phenotypes to be aneuploid, however not all of the aneuploids were grossly abnormal.
Like aneuploidy, changes in the level of euploidy can bring about reduced fertility. Increasing ploidy also brings about changes in plant size and shape (Santos and Handro, 1983) but very high ploidies are obviously detrimental and rarely found. In general, however, the effects of polyploidy on phenotype are limited.

Changes to chromosome architecture have an effect on fertility through interfering with normal bivalent pairing at meiosis (John & Lewis, 1966). However, effects on phenotype are not always found with chromosome structure changes (Ogihara, 1981). In the case of deletions and duplications, effects on phenotype can come about through changes in gene dosage. These effects will be most pronounced in diploids where there is not the buffering effect that can occur with higher ploidy levels. Heterozygous deletions might be expected to show more pronounced effects following selfing of the primary regenerants when, rather than simply modulating gene dosage, entire loss of gene function could occur. Translocations, interchanges and inversions are likely to have less of a pronounced effect than deletions and amplifications since no changes in gene dosage occur. They can influence phenotype, however, by bringing about position effects such as by moving active genes nearer to transcriptionally silent areas of heterochromatin where their expression may be suppressed. Equally genes that are normally silent could be removed from transcriptionally inactive regions and become active. There is evidence of position effects controlling plant gene expression (Catcheside, 1947) and changes in heterochromatin patterns can be seen in cultured cells and regenerants.

Somaclonal variation appears to result not only from changes to the nuclear genome but also from changes to the organelle genomes. Gengenbach et al. (1977) used maize cultures to select for resistance to the host specific toxin produced by Drechslera maydis race T, causative agent of Southern corn leaf blight. Sensitivity to this toxin is inherited tightly linked to the
cytoplasmic male sterility character that is known to be mitochondrial encoded. The workers were able to regenerate toxin resistant plants but these were all found to have reverted to male fertility. Thus there was good evidence for somaclonal variation resulting from mitochondrial changes. Chloroplast genome derived somaclonal variation has also been reported occasionally (see report in Miller, 1985) although it appears, from the literature, to be less prevalent than mitochondrial genome changes. This may be a consequence of less basic research being carried out on this organelle or it may be in some way related to the relative evolutionary stability that has resulted in the chloroplast genomes of widely divergent species having such a similarity in gross structure (Palmer and Thompson, 1982).

CONTROL AND EXPLOITATION OF VARIATION.

In many of the modern applications of plant cell culture genetic stability is important. The production of haploids by anther culture is rendered much less useful if in the process the genes of that haploid are changed. The transfer of a desirable genetic trait, by direct DNA uptake, to an otherwise near perfect crop variety is of greatly reduced value if, in the regeneration of whole plants, many of the genes in that variety are changed. An understanding of the causes of instability can be of value if it can lead, ultimately, to the control of somaclonal variation. As is obvious from the review above, at the present time our understanding of genetic instability in tissue cultured material is far from complete. To make the best use of modern plant manipulative techniques this must change. Whilst we are not yet in a position to control somaclonal variation in some instances at least we can take steps to minimise its effects. Though early plant genetic transformations relied almost entirely on protoplasts (Davey, et al., 1980), and even today this is often the norm (Bates, et al., 1988), it is now possible to avoid this highly unstable phase by the use of leaf disc transformation.
systems (Horsch, et al., 1985) where the callus phase is kept to a minimum. Direct transformation of plants by microinjection of DNA into developing embryos or floral primordia (de la Pena, et al., 1987) may be even better since this entirely circumvents the need for any in vitro culture phase.

Whilst there are steps that can be taken to minimise somaclonal variation during genetic transformation, in many other systems there is little that can be done. Since stress appears to be so important in bringing about genome reorganisations, it could be that taking steps to minimise the stress encountered by plant cells during the tissue culture process may help to minimise unwanted genome changes. Though Murashige and Skoog (1962) provided a great service to plant tissue culturalists through the development of a basic defined tissue culture medium with a wide range of applications perhaps they, inadvertently, did plant tissue culture a disservice. Evans and Gamborg (1982) have shown that if more effort is put into precise medium optimisation for the plant tissue culture system employed then genomic change can be reduced, presumably through a reduction in stress. Such precise optimisation would obviously be extremely laborious and it may be that a return to the use of chemically undefined media components, such as coconut milk, may provide a more rapid route to obtaining the ideal medium for each cell culture system.

Despite its disadvantages some good can come from somaclonal variation. It provides an apparently novel source of genetic variation from which plant breeders can select desirable traits. Whilst there is some dispute as to the extent to which really novel traits may occur some workers at least are convinced of the potential in this area (Miller, 1985). Certain improvements to crop varieties have been reported. Improved disease resistance has been observed, such as the increase in resistance to Fiji disease of culture derived sugar cane (Krishnamurthi, 1974) and an increased resistance to Fusarium wilt has been found in tomato
regenerants (Evans, et al., 1984). Additionally, the prospect of \textit{in vitro} selection for cell lines with improved disease resistance as a result of somaclonal variation means there is further potential (Brettell and Ingram, 1979). Apart from this area much appears to be promised (Millar, 1985, Evans and Sharp, 1986 and 1988) but few examples of positive benefits can be cited.

Work on tomato sponsored by the Campbell Soup Co. (Miller, 1985) has resulted in a range of fruit colour variants, male sterility and a jointless pedicel mutant which could aid mechanical harvesting. Similarly a lot of work has gone into assessing somaclonal variants of potato (Secor and Shepard, 1981) and there are reported instances of 'improvements' to ornamental species (Griesbach and Semenluk, 1987) but this list seems most inadequate when compared to the much flaunted potential. The principal stumbling block would appear to be the need for better selection procedures. Whilst the need for regeneration, selfing and extensive field trials remains, progress on the use of somaclonal variation to improve crop varieties will, of necessity, be slow. Major advances in the use of somaclonal variation in plant breeding can only come about when there is a better understanding of the correlation between whole plant performance and cell selection for a broad spectrum of traits.

In the meantime somaclonal variation may be of greatest use to the plant scientist interested in basic research studies into aspects of plant genetics, development and metabolism. Not only does it provide a useful pool of mutants for physiological studies but it can also result in the development of near isogenic lines with a single mutant trait. Isogenic lines can be particularly useful to the plant molecular biologist since cross hybridisation studies with messenger RNA molecules from the wild type and mutant variety may lead to the production of complimentary DNA clones of use in the isolation of the gene(s) controlling the mutant trait. Whilst
isogenic lines can be obtained through traditional breeding programmes somaclonal variation could speed the process.

1.5) MOLECULAR CHANGES DURING TISSUE CULTURE

DNA SEQUENCE CHANGES; AMPLIFICATIONS, DELETIONS, MUTATIONS.

When discussing the effect of stress on the genome, above, no real mention was made of tissue culture as a form of stress yet it must surely rank as one of the most extreme forms of environmental abuse that is survivable by plants. So, if callus culture imposes a stress could it activate transposable elements and bring about the types of amplification / deletion events associated with other plant stresses?

As recorded above, when a root tip is cut and callus forms, as part of the natural wound healing process, there is found to be a preferential replication of specific highly repeated DNA sequences (Natali, et al., 1986). Similarly when carrot explants are placed in culture, and callus proliferation occurs, a highly repeated DNA family is found to undergo early replication and hence become amplified (Kato & Tanifuji, 1986). The amplification of specific DNA sequences during the dedifferentiation that precedes callus proliferation would appears to be a very common occurrence (Zheng et al., 1987, Durrante et al., 1985) and contrasts with the under-replication of highly repetitive sequences that has been reported to accompany in vivo differentiation (Patankar Shubhada, and Rajekar, 1984 a & b). That some sequences are amplified during dedifferentiation and others during differentiation in different plants is hardly suprising in the light of the finding by Kikuchi et al. (1987) who observed both situations in the same plant. One rice DNA sequence was seen to amplify fifty fold during callus formation and then decrease in copy number during regeneration whilst another sequence appeared to do the opposite. This latter sequence was also of interest in that it was found to have
homology with chloroplast DNA sequences and hence qualify as thepromiscuous DNA of Ellis (1982). The sequence and behaviour ofthis element have led the workers to suggest that it mayconstitute a mobile genetic element. Whether or not theseparticular copy number changes observed during callusing areretained is largely of academic interest. What they serve toillustrate is that specific DNA replication systems must beactivated by the induction of callus and by regeneration fromcallus. That the activation of replication mechanisms can bebrought about in such a way shows how other processes resulting inthe reorganisation of DNA sequences may be induced by theinduction of random, disorganised callus growth.

Changes in the copy number of specific sequences, presumablyresulting from amplification and deletion events, appear to be acommon feature when tissues are subject to callus cultureconditions. Deletions occurring during the culture phase have beenfound to be responsible for the eventual inactivation of certainAgrobacterium sequences present in a tobacco tumour line(Peerbolte, et al., 1987). Similarly, in the wheat/rye hybridTriticale deletions have resulted in the almost completeelimination of a nucleolar organiser region (rDNA) from one ryechromosome (Brettell, et al., 1986b). The loss of rDNA repeats wasalso reported by Landsmann & Uhrig (1986) in their study of themolecular rearrangements resulting from the regeneration ofpotatoes from protoplasts.

The extent and results of putative amplification events aregraphically illustrated by the work of Cullis and Cleary (1986)where the copy numbers of several cloned repeated DNA sequenceswere estimated for DNA samples derived from a series of callusclones and from some clonally derived plants regenerated fromcallus culture. A range of repeated DNA sequence clones were used,including rDNA, and all but one sequence were found to vary incopy number both between different callus lines and between
different regenerant plants. This is an impressive illustration of the plasticity of the repeated DNA component of the plant genome and it serves to emphasise how active the mechanisms for genome rearrangement must be in cultured cells.

Although there is no published data from the laboratory of Williams and Widholm (1986) they are interested in the possible involvement of some of the large number of maize transposable elements in the somaclonal variation observed in that species and are involved in work to establish any connection. Tissue culture is known to induce transposon activation in *Drosophila* (Flavell and Ish-Horowicz, 1981) and Freeling (1984) has suggested that it may have a similar effect in plants.

Having discussed some of the processes by which DNA reorganisations can be manifest throughout the genome in numbers sufficient to explain the frequency of the genetic changes responsible for somaclonal variation, it must not be forgotten that a number of different mechanisms can be operating concurrently. Whilst point mutations alone are unlikely to be able to account for all somaclonal variation they can and do occur during callus culture. Brettell *et al.* (1986a) screened 645 maize regenerants for alterations to the electrophoretic mobility of alcohol dehydrogenase genes and identified one variant. Sequencing the cloned variant gene identified a single nucleotide substitution resulting in an amino acid substitution of valine for glutamic acid hence reducing the net negative charge of the resultant polypeptide. More recent work by the same laboratory (Dennis, *et al.*, 1987) using the same system has identified an altered codon in the *Adh 1* gene of a furthur maize regenerant. In this instance a null mutant resulted from the substitution of an adenine residue by a thymine, changing the A-A-G lysine encoding triplet to a T-A-G stop codon. In the course of their work the lab has screened the *Adh 1* gene in 1382 maize regenerants and identified two single base changes suggesting that point mutations
may occur in regenerants at a much higher frequency than would be accounted for by the normal rate of spontaneous mutation.

Before leaving a discussion of molecular rearrangements in the nucleus that might be responsible for the phenomenon of somaclonal variation, mention should be made of the fact that, as recorded above, some of the variation observed following regeneration from a callus culture is in characters controlled by cytoplasmic genes. The mitochondrial genomes of higher plants have the form of a basic circular molecule containing a large number of repeated sequences. Recombination between these repeats gives rise to the series of subgenomic circles that comprises the genome in some mitochondria (Palmer and Shields, 1984). Whilst homologous recombination occurs in the normal plant situation, and hence should not be considered unusual, its occurrence in tissue cultured cells may be even more common since the appearance of unusual mitochondrial bands following tissue culture is often seen (Chourey, et al., 1986). The variations seen in mitochondrial genome restriction patterns following tissue culture often take the form of differences in the stoichiometries of different bands (Borck and Walbot, 1982) but other changes, including the complete loss of part of the mitochondrial genome, have been observed (Rode, et al., 1987a). Additionally, there is evidence that some of the unusual mitochondrial restriction patterns seen following tissue culture may result from the formation of chimaeric genes. Boeshore, et al. (1985) have identified a chimaeric gene in Petunia mitochondria, following protoplast fusion, that consistently segregates with a cytoplasmic male sterility phenotype.

Having given the impression that the mitochondrial genome is extremely fluid in culture, and this is in part accurate, mention should be made of the large number of cases where no changes could be observed in mitochondrial organisation following tissue culture. Dale, et al. (1981) were unable to find differences
between young and old callus with respect to mitochondrial DNA organisation whilst Hanson (1984) found Petunia mitochondrial genomes to remain stable for over one year in culture. Indeed, Hartmann, et al. (1987) have suggested that the mitochondrial genome is primarily susceptible to change at the time of tissue culture initiation and that following callogenesis a comparative stability is observed.

DNA METHYLATION CHANGES.

That substantial changes in the overall level, and hence distribution, of DNA cytosine methylation can occur as a consequence of tissue culture has been graphically illustrated by the work of Brown & Lorz (1986). These workers have analysed DNA, extracted from the progeny of selfed plants regenerated from maize embryonic callus cultures, by digestion with the restriction endonucleases Msp I and Hpa II. Whilst DNA from one phenotypically normal plant showed the distribution of DNA fragment sizes expected for normal plants after digestion with these enzymes, namely predominantly large fragments resulting from Hpa II digestion and a range of smaller fragments resulting from Msp I digestion, in other plants the situation was very different. Another plant, phenotypically indistinguishable from the first, showed DNA restriction by Msp I to result in the largest fragments whilst digestion with Hpa II to give a range of smaller fragments. Assuming this result to be genuine, it indicates that there has been a shift from the normal situation, where C-G methylation is more common than C-N-G methylation, to a situation where C-N-G methylation is more prevalent than C-G methylation. In DNA from one phenotypically abnormal plant it was found that digestion with either enzyme gave a very limited degree of digestion, indicating extensive methylation of cytosines in both C-G and C-N-G configurations, whilst in another plant with disturbed phenotype both enzymes showed a high proportion of unmethylated sites.
These results indicate that there have been great changes in the pattern of cytosine methylation most likely as a consequence of callus culture. Whether they result from modifications to the level of expression of the enzymes concerned with cytosine methylation and whether they affect unique copy and highly repeated sequences equally is not shown by work of this type. Later, as yet unpublished, work by the same lab on the same system aims to determine which specific sequences are affected by these widespread changes and whether changes in methylation of these specific sequences are correlated with changes in their copy number. Whilst analysis of the affected sequences may well yield useful information, it is hard to credit their implied suggestion that these spectacular changes in the distribution of methylation result purely from attempts by the cells at copy number compensation.

Culture induced changes to the DNA cytosine methylation pattern of a specific DNA sequence have been published by another laboratory (Quemada et al., 1987). Working with a soybean tissue culture system these workers showed changes in the methylation of 5S RNA genes following induction of suspension cultures. In intact plants the 5S RNA genes can be partially digested by Msp I but are totally resistant to Hpa II digestion. Shortly after induction of cell cultures the genes become sensitive to hydrolysis by both enzymes indicating a reduction in cytosine methylation in this region of the genome. With prolonged culture, however, the Hpa II resistance was found to return indicating an increase in the frequency of 5-methyl cytosine in these genes. The paper does not make clear whether these changes were restricted to the 5S RNA gene sequences. It could be that the changes observed here resulted from changes in the prevalence of cytosine methylation in the whole genome, perhaps as a result of changes in the expression of DNA methylating enzymes, or they could reflect some specific requirement of the cells for elevated 5S RNA expression during culture initiation.
Because the literature provides only tantalising suggestions of methylation changes as a result of tissue culture, and because these few observations ask more questions than they answer, this is clearly an area where more work would be of value. Methylation changes obviously have the potential to provide one source of variation contributing to somaclonal variation but the extent to which they are important is yet to be determined.

THE ORIGINS OF SOMACLONAL VARIATION.

The available evidence shows that practically any component of the plant genome has the potential to change as a result of, or coincidently with, callus culture. The single base changes of the type observed in the maize alcohol dehydrogenase genes would easily pass undetected unless sequencing of large portions of the genomes of regenerated plants was attempted. Equally, changes of this type are unlikely to account for the broad spectrum of apparently unrelated changes observed in a single regenerant. Changes to the highly repeated fraction of the genome obviously could occur as the amplification of satellite DNA sequences shows (Zheng et al., 1987) and these may alter the expression of genes nearby. Whilst Hanson (1984) has found the mitochondrial DNA of Petunia to be stable for over one year in culture it is possible that mitochondrial DNA variation could occur in other cultures of Petunia and this could easily contribute to phenotypic variation whilst the significance of changes to DNA cytosine methylation is completely unknown. In truth somaclonal variation probably results from a range of genetic modifications brought about by a number of different processes. For the purposes of this project, with a limited time-span and limited resources, it seemed best to concentrate on a study of DNA methylation, since this could easily account for the wide variety of changes that are such a typical feature of somaclonal variation, and on the study of moderately repeated DNA sequences. These sequences have been shown to be highly variable in cultured cells and regenerants (Cullis and
Cleary, 1986). They are also the class of sequences that includes transposable elements which have been implicated in somaclonal variation and which are known to be present in *Petunia* (Gerats, et al., 1985). Equally, they are relatively easily screened by Southern hybridisation techniques (Southern, 1975) and, being scattered throughout the genome, they are ideally placed to influence the expression of a broad spectrum of unrelated genes.

1.6 PETUNIA AS A SYSTEM FOR THE MOLECULAR STUDY OF SOMACONAL VARIATION

*Petunia hybrida* has a number of attractions as a system to address questions concerning the nature of molecular changes that accompany and may be responsible for somaclonal variation. Principal amongst these is the comparative ease by which tissue culture manipulations, including callus culture, protoplast culture and plant regeneration, can be successfully applied (Frearson et al., 1973). Not only is regeneration possible but it has been shown that there can be karyotypic variation amongst the regenerants and that this can result in phenotypic changes (Santos and Handro, 1983). From the point of view of interpreting phenotypic variation arising during a callus phase it is important that the plant has been well characterised genetically. Not only are there numerous known mutants, many of which have been mapped to one of the seven linkage groups (Cornu, 1984), but in addition there has been much published concerning the effects, on phenotype, of monosomics and trisomics (Maizonnier, 1984).

Of equal importance to the cell culture and genetics, however, are a number of molecular considerations which also make *Petunia* attractive. At 1.6 pg per 1C haploid genome (Bennett and Smith, 1976), the genome of *Petunia hybrida* is of intermediate size by plant standards. This means that it is quite practicable to clone specific genes from the species and, indeed, a number of such clones are available (e.g. pCAB 146, Dunsmuir et al., 1983). Gene
cloning would be even easier in a plant with a very small genome but here there would be very little repetitive DNA. Since reorganisations of repetitive DNA are frequently reported amongst cultured cells it is possible that they might play some role in the occurrence of somaclonal variation and hence may be worthy of study. Molecular reorganisations rely on the presence of appropriate enzyme systems to perform, amongst other processes, the necessary DNA hydrolysis and ligation steps. In order to ensure that the plant species chosen had the molecular mechanisms necessary to reorganise DNA, a plant with a reasonable content of repetitive DNA was required since it is thought that this repetitive fraction arose through the action of such reorganisation mechanisms. Not only does the genome size of Petunia hybrida suggest that it is likely to capable of DNA amplifications, but work by Shah, et al. (1986) has shown that it is, indeed, capable of increasing the copy number of parts of its genome. The resistance to the herbicide Glyphosate that these workers were able to select arises due to the 20 fold amplification of the EPSP synthase genes. Also important in terms of the ability to show genome rearrangements is the fact that Petunia hybrida contains at least one transposable element system (Doodeman, et al., 1984).

Finally, being a member of the Solanaceae Petunia hybrida is related to a number of agronomically important species including potato, tomato and tobacco where knowledge of the mechanisms by which somaclonal variation may arise could be of practical benefit in years to come.
1.7 THE AIMS AND APPROACH OF THIS PROJECT.

The purpose of this project was to investigate molecular changes to the genome resulting from a cell culture phase. This investigation was intended to address two complimentary aims. The first was to exploit the phenomenon of somaclonal variation to learn more about the molecular processes by which genetic variation is generated in plants. It would appear that stress is an important factor in the induction of many genomic rearrangements in plants and it was hoped that the extreme stress provided by passage through a phase of disorganised callus growth would provide a means of generating genome rearrangements. Such rearrangements could be investigated at the molecular level and this may provide information concerning the mechanisms by which the genome changes necessary for evolution are brought about in plant cells.

The second aim was to attempt to gain some understanding of a number of the molecular processes which might be responsible for the appearance of somaclonal variation. Through the identification of some of the mechanisms by which the phenomenon arises it may become possible to suggest ways in which somaclonal variation may be controlled. Although karyotypic variation is known to account for some of the phenotypic differences seen amongst tissue culture regenerants, widespread changes can be observed in plants with a normal chromosome complement. Since such phenotypic differences must arise from molecular changes within the chromosomes, changes in DNA methylation or extensive sequence rearrangements are obvious candidates for the underlying causes. A molecular study of DNA from cultured cells and regenerated plants may help to show which types of molecular change might be particularly significant in this context.

To achieve the aims of this project, it was intended to study the copy numbers, organisation and methylation of a range of DNA
sequences in normal Petunia plants, in callus cultures of Petunia plants and in regenerated plants. Cloned sequences, for use as probes in Southern hybridisations, were available for a number of unique copy sequences, for the ribosomal RNA genes and it was intended to make a series of clones covering a range of other middle repetitive sequences. Callus was to be induced from Petunia explants, to be grown in culture for a period of time and then to be induced to cause plant regeneration. DNA collected at all of these stages would be used for analysis and any regenerants showing unusual phenotypes would be retained for further appropriate study. Somaclonal variation can provide for the production of a range of mutants that can be of use in studies of plant genetics, development or physiology.

In order to be able to establish any sort of correlation between molecular changes and somaclonal variation it would be necessary to determine that the system that produced the molecular changes during in vitro culture also showed somaclonal variation. Consequently, a phenotypic characterisation of plants regenerated from tissue culture was performed. Not only is variation amongst the primary regenerants necessary to establish the presence of somaclonal variation, heritability of the altered phenotypes is required too. Consequently, the phenotypes of the progeny of any regenerants were to be studied.
SECTI ON 2 MATERIALS & METHODS

2.1) MATERIALS.

PLANT MATERIAL.

Petunia hybrida cv. Rose of Heaven (ROH) seed was purchased from Charles Sharpe & Co. Ltd. (Sleaford, Lincs.). This inbred, and now discontinued, variety was maintained by repeated selfing under normal greenhouse conditions when no phenotypic variation was observed. The genotype of ROH is thought to be hf1, Hf2, Ht1, Rt, fl (A.G.M. Gerats, pers. comm. to S.M. Smith).

TISSUE CULTURE MATERIALS.

Media:

All media were based on the basic medium of Murashige and Skoog (1962) using Murashige and Skoog (M & S) salts (Flow Labs, Irvine) and Bacto-Agar (Difco, Detroit).

Medium 204: 4.7 g/l M&S salts, 30 g/l sucrose, 1% (w/v) agar, 2 g/l casein hydrolysate (Sigma, Poole), 0.1 mg/l 2,4-dichlorophenoxy-acetic acid (2,4-D), 3.0 mg/l kinetin. pH 5.8.

Medium JH: 4.7 g/l M&S salts, 30 g/l sucrose, 1% (w/v) agar, 1.0 mg/l zeatin. pH 5.8.

Medium JHA: 4.7 g/l M&S salts, 30 g/l sucrose, 1% (w/v) agar, 1.0 mg/l zeatin, 0.1 mg/l 2,4-D. pH 5.8.

Medium RM: 2.4 g/l M&S salts, 15 g/l sucrose, 1% (w/v) agar, 1.0 mg/l indole acetic acid (IAA).
All media were prepared using double distilled water and their pH was adjusted to 5.8 with 0.1 M NaOH prior to addition of the Bacto Agar. They were then autoclaved at 15 psi for 20 mins prior to being poured into 9 cm sterile packed petri dishes (Sterilin, Feltham) or, in the case of RM, into sterile 30 ml plastic tubes (Sterilin).

Surface Sterilant:

10% (v/v) solution of sodium hypochlorite (A.J.Beveridge, Edinburgh. 10-15% available chlorine) containing 15 drops per litre of By-Prox (B.P., Woking) detergent as a wetting agent.

MOLECULAR BIOLOGY MATERIALS.

Chemicals and Reagents:

All chemicals and reagents were analytical grade where possible. Solutions were prepared using double-distilled water.

Radiochemicals:

$\alpha^{[32P]}dCTP$ (410 Ci/mmol) in aqueous solution was purchased from Amersham International Plc. (Amersham, Bucks).

Enzymes:

Restriction endonucleases were purchased from Northumbria Biologicals Limited (Ashington), Anglian Biotech Limited (Colchester), Amersham International Plc., Boehringer Mannheim (Mannheim, W.Germany), Bethesda Research Limited (Paisley).

Klenow fragment of *Escherichia coli* DNA polymerase I was purchased from Amersham International Plc. T4 DNA ligase was obtained from Northumbria Biologicals Limited.
Gel Electrophoresis Materials:

Gel electrophoresis of DNA for Southern hybridisation (Southern, 1975) was performed using Sigma type II agarose.

Specific DNA fractions were isolated from gels using 0.045 µm DEAE membrane from Schleicher and Schuell (Dassel, W. Germany).

Autoradiography Film:

X-ray film was Cronex 4 (Dupont, Frankfurt) and Curix RP1 (Agfa).

Hybridisation Membrane:

Nitrocellulose was purchased from Schleicher and Schuell. Hybond N nylon membrane was from Amersham International.

Bacterial Strain and Plasmid:

E. coli. strain JM101 (Δ(lac pro), thi, SupE, F'traD36, proAB lac iαz) (Messing, 1979).

pUC 18 Amp' (Yanisch-Peron et al., 1985).

Bacteriological Media:

* Luria broth (LB): 10 g/l Bacto tryptone (Difco), 5 g/l Bacto yeast extract (Difco), 10 g/l NaCl. pH 7.2.

* LB Agar: 10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl, 1 % (w/v) Bacto Agar. pH 7.2.
Minimal Medium: 10 g/l Bacto Agar, 2 g/l \((\text{NH}_4)_2\text{SO}_4\), 6 g/l \(\text{K}_2\text{HPO}_4\), 1 g/l Na-citrate, 0.2 g/l Mg \(\text{SO}_4\), 0.2% (w/v) glucose, 1 mg/l thiamine hydrochloride.

Electrophoresis and Hybridisation Buffers and Solutions:

Tris Acetate Buffer: 40 mM TrisHCl, 2 mM ethylene diamine tetra-acetic acid (EDTA), 20mM Na acetate pH 8.

20 X SSC: 3 M NaCl, 0.3 M Na citrate, pH 7.

Southern Denaturing Solution: 0.5 M NaOH, 1.5 M NaCl.

Southern Neutralising Solution: 0.5 M Tris-Cl, 3 M NaCl, pH 7.

Prehybridisation Solution: 6X SSC, 100 ng/ml sonicated herring sperm DNA, 0.5% (v/v) sodium dodecyl sulphate (SDS), and 50 g/l powdered milk (Sainsbury's, London)

T.E. Buffer: 10 mM Tris-Cl, 1 mM EDTA, pH 8.0

Ebolgy: 125 mM EDTA, 50 % (v/v) glycerol, 0.1 % (w/v) SDS and a small amount of bromophenol blue

DNA Isolation Solutions:

Chopping Buffer: 84 g/l Sucrose, 2 mM Tris-Cl, 1 mM Mg Cl₂, 10mM β-mercaptoethanol, 20 g/l Ficoll 400 (Sigma), 20 g/l Dextran T40 (Sigma), 1% (v/v) Triton X100, pH 7.8

TMG: 140 g/l Sucrose, 50 mM Tris-Cl, 1 mM MgCl₂, 6 mM β-mercaptoethanol, 1% (v/v) Triton X100 pH 7.8

Sucrose Mixture: 25% (w/v) Sucrose, 40 mM EDTA, 50 mM Tris-Cl, pH 8.1
Triton mixture: 0.1% (v/v) Triton X100, 62.5 mM EDTA, 50 mM Tris-Cl pH 8.1

CYTOLOGICAL MATERIALS.

Pre-treatment Solution: Colchicine (Sigma) was used as a 0.05% (w/v) solution in water.

Fixative: 66% (v/v) ethanol, 14% (v/v) chloroform, 14% (v/v) acetic acid, 7% (v/v) formalin (40% formaldehyde in water).

Stain: 2% (w/v) natural orcein (George T. Gurr) 50% (v/v) lactic acid, 50% (v/v) propionic acid.

2.2) PROCEDURES.

PLANT GROWTH.

All plant material was grown in the greenhouses at Edinburgh under daylight conditions with supplementary lighting from mercury vapour bulbs maintaining a 16h daylength in the winter months. Plants for tissue culture were grown in Levington Universal (Fisons, Ipswich) potting compost in 3½" square pots. Plants regenerated from tissue culture were also grown in Universal compost in either 3½" square pots, in the first experiment, or 2" square pots, in the second experiment. Tissue culture regenerants were maintained by the propagation of cuttings. Cuttings were rooted in a peat:sand (1:1) mixture using Strike (May & Baker, Dagenham) rooting compound and a perspex propagator hood to maintain a high humidity.
SPECIFIC FERTILISATIONS.

Self fertilisations of callus regenerants and backcrosses to ROH plants were performed by S.M. Smith. Emasculated flowers left unattended on plants in the greenhouse were never found to set seed indicating that, under the conditions employed, unintentional cross pollination, by means of insects or air currents, was not a problem. Consequently, no special measures were necessary to prevent unwanted outbreeding other than the precautionary physical separation of the plants. Self fertilisations were performed, therefore, merely by manipulating the flowers such that pollen was transferred from the anthers to the surface of the stigmata and labels were tied to each pedicel indicating the nature of the fertilisation. After fruit ripening the seed was harvested in the intact capsule, removed from that capsule and stored at room temperature in labelled greaseproof paper bags.

Backcrosses were performed in a reciprocal manner with the regenerants and ROH plants acting as both male and female. The Regenerant x ROH cross was performed by emasculating the regenerant flower prior to anthesis by removal of the anthers. A dehisced anther from a ROH plant was used to apply pollen to the stigma surface. The pedicel was then labelled and fruit set and harvest occurred as described above. For the reciprocal cross, i.e., ROH x Regenerant, the ROH flower was emasculated in a similar manner and then pollinated using a dehisced anther from the regenerant.

PHENOTYPIC ANALYSIS AND STATISTICAL PROCEDURES.

All quantitative measurements of plant phenotype were performed on fresh plant material at the time of harvest. The resulting numerical data was analysed using the statistical techniques quoted in the "Results" section. The GENSTAT statistical package...
(Rothampstead Experimental Station) was used to perform the analysis of variance test.

PLANT TISSUE CULTURE.

All tissue culture procedures were performed under sterile conditions in laminar flow cabinets using standard aseptic technique. Cultures were grown in growth rooms with 24 h illumination from white fluorescent tubes (Thorn 3500) at a constant temperature of 25°C.

Callus Initiation, Culture and Plant Regeneration.

Young, fully expanded leaves were selected from greenhouse grown plants and surface sterilised by immersion in a 10% (v/v) solution of sodium hypochlorite containing a few drops per litre of By-Prox detergent as a wetting agent. After 30 minutes this was replaced by the first of five changes of sterile single-distilled water.

A 15mm diameter cork borer was used to punch lamina discs from the surface sterilised leaves. Care was taken to avoid including the midrib or other prominent veins in the discs produced. These discs were placed on plates of 204 medium, five per 9cm petri dish, which were then sealed with Parafilm (American Can Corp. Greenwich C.T.).

After 4 weeks, callus that had proliferated around the leaf disc margins was excised and transferred to fresh 204 medium. The resultant callus cultures were then subcultured by division of the callus clumps and transfer to fresh 204 medium every four weeks.

Regeneration from callus was achieved by transfer of callus pieces to JH medium and repeated subculture onto this same medium at 4 weekly intervals. When green shoots appeared these were excised and their bases inserted into a small volume of RM medium in the
bottom of 30ml tubes. The shoots and developing roots were cultured in these tubes until well established (≈ 6 cm) when the agar was washed from the roots under a running tap. The plantlets were then planted in *Universal* potting compost and kept at high humidity under perspex propagator hoods.

**MOLECULAR BIOLOGY TECHNIQUES.**

**Nuclear DNA Isolation from Leaves:**

Approximately 30g of leaf material was homogenised in 100ml Chopping Buffer using a Moulinex kitchen liquidiser. The resultant slurry was filtered through four layers of muslin and a single layer of 100 μm nylon mesh. The filtrate was centifuged at 400 x g_m for 4 min to sediment the nuclei and the resultant pellet was resuspended in TM3 buffer. Nuclei were again pelleted by centrifugation at 400 x g_m for 4 min and resuspended in 18 ml of T.E. After the addition of 2 ml 10% (w/v) sarcosyl (Sigma) the mixture was incubated at 65°C for 5 min prior to gentle agitation at 37°C for 3 hours. After the addition of 20 g of CsCl and 1.5 ml of 5 mg/ml ethidium bromide the sample was subjected to centrifugation for 48 h in a Beckman 70 Ti rotor at 1.1 x 10^5 x g_m. The DNA band was removed, rebanded on an identical CsCl gradient and then extracted with amy1 alcohol prior to dialysis against half strength T.E.

**Nuclear DNA Isolation from Callus:**

Callus from a single 9 cm. plate was homogenised in 100ml Chopping Buffer using a Moulinex kitchen liquidiser. The resultant slurry was filtered through four layers of muslin and a single layer of 100 μm nylon mesh. The filtrate was centifuged at 400 x g_m for 4 min to sediment the nuclei and the resultant pellet was resuspended in 15 ml of T.E. buffer. 1 ml of 20% (w/v) sodium dodecylsulphate was added and the resultant mixture was shaken and
then incubated at 65°C for 10 min. 5 ml of 5 M potassium acetate was added and, following agitation, the mixture was transferred to ice for 20 min. The insoluble potassium dodecylsulphate that forms was pelleted by centrifugation at 25,000 x g for 20 min. The supernatant was transferred to a sterile tube and 10 ml of isopropanol was added. After 30 min at -20°C the DNA in the solution was pelleted by centrifugation at 20,000 x g for 15 min. The pellet was air dried and then resuspended in T.E. DNA in the resultant solution was separated by ethidium bromide dye buoyant centrifugation in a caesium chloride density gradient as outlined above.

Restriction Endonuclease Digestion:

Plasmid DNA was digested using 2 units of enzyme per μg of DNA in the manufacturers recommended buffer at 37°C for three hours.

Nuclear DNA was digested using 5 units of enzyme per μg of DNA in the manufacturers recommended buffer overnight at 37°C.

DNA Ligation:

Restriction endonucleases were heat inactivated by heating for 10 min at 65°C. 100 ng of plasmid DNA was mixed with 500 ng of foreign DNA in 15 μl containing 1 mM ATP, 2 units of T4 Ligase and ligation buffer to the manufacturers recommendation. The resultant solution was incubated at 4°C overnight.

Transformation of Plasmid DNA into E. coli.:

A fresh colony of JM101 was picked from minimal medium plates into a 5 ml overnight culture of LB medium. 0.2 ml of this culture was used to inoculate a 50 ml culture of LB which was then grown at 37°C to an optical density at 600 nm of 0.2. The culture was stood on ice for 5 min prior to harvesting the cells by centrifugation
for 5 min at 2200 x g in an MSE LR4 centrifuge. The cells were resuspended in 20 ml of 0.1 M CaCl₂ and placed on ice for 20 min. The cells were again pelleted by the same procedure and resuspended in 0.5 ml of 0.1 M CaCl₂. These cells, competent for transformation, were then stored on ice for up to a few hours prior to transformation.

5 µl of the ligation mixture were mixed with 50 µl of competent cells and stood on ice for 15 min. After a heat shock at 37°C for 5 min the transformation mixture was returned to room temperature for 5 min. 1 ml of LB was added and the resultant culture incubated at 37°C for 60 min. 100 µl of this culture was mixed with 30 µl of a 20 mg/ml solution of X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 20 µl of a 24 mg/ml solution of IPTG (isopropyl-β-D-thiogalactopyranoside) prior to plating out on LB agar containing 50 µl/ml ampicillin.

Preparation of Plasmid DNA:

Plasmid DNA was isolated from E. coli cultures by the lysozyme/triton method of Katz et al. (1973). Colonies picked into 5 ml LB cultures containing 50 µg/ml ampicillin were grown overnight at 37°C and used to inoculate 500 ml cultures of the same medium. These were grown at 37°C until their optical density at 650 nm was 1.0. Addition of 150 µg/ml chloramphenicol allowed overnight amplification of the plasmid. Cells, harvested by centrifugation for 15 min at 2600 x g in a Sorval GSA rotor, were resuspended in 6 ml of sucrose mixture. 0.5 ml of 10 mg/ml lysozyme was added as well as 0.5 ml of 0.5 M EDTA and this was mixed by swirling on ice for 5 min. 6.5 ml of Triton mixture was added and the mixture stood on ice for 10 min. The resultant cell lysate was spun for 45 min in a Sorval SS34 rotor at 33,000 x g. The supernatant was removed, CsCl and ethidium bromide added to appropriate concentrations (Radloff et al., 1967) and the solution was centrifuged at 1.2 x 10³ x g in a Beckman 70 Ti rotor for 24
h. The lower band, of plasmid DNA, was removed, the ethidium bromide was extracted with amyl alcohol and the CsCl removed by dialysis into 1/2 strength T.E.

**Agarose Gel Electrophoresis of DNA fragments:**

DNA fragments were separated on the basis of their size by horizontal slab agarose gel electrophoresis using Tris acetate buffer and a 1% (w/v) gel of Sigma type II agarose. DNA samples were mixed with a 1/10th volume of EBOGLY and loaded into the gel wells. Gels were subject to electrophoresis with a current of 20 mA overnight. DNA fragments were visualised by staining with ethidium bromide solution (1 µg/ml) for 20 min. and photography under short wavelength U.V. radiation (254 nm, Ultraviolet Products Inc) using Ilford HP5 film.

**Transfer of DNA Fragments to Hybridisation Membrane:**

DNA fragments separated by gel electrophoresis were transferred to Hybond N membrane by a modification of the general method of Southern (1975). After ethidium bromide staining and photography, the gel was treated with 0.25 M HCl for 10 min to depurinate the DNA. The gel was then immersed in denaturing solution for 40 min followed by neutralising solution for the same period. The gel was then placed on a piece of 3MM paper (Whatman, Maidstone) fed with 20X SSC via a wick. A sheet of nylon membrane was then placed in close contact with the upper surface of the gel. Several layers of 3MM paper were placed on top of this topped by a stack of absorbant paper towels and and a weight of approximately 1 kg. DNA transfer was achieved overnight as a consequence of the 20X SSC flow resulting from capillary rise. The nylon membrane was then removed, dried in the atmosphere and subjected to 245nm irradiation for 10 min to bind the immobilised DNA.
Preparation of radioactively labelled DNA:

Specific DNA fragments for labelling were obtained from agarose gels by one of two techniques. At the start of the project the digested DNA was separated in a 1% (w/v) gel of Sigma Type VII (low gelling temperature) agarose, stained with ethidium bromide and the appropriate band excised in a small volume of gel. This gel piece was weighed and water was added at a ratio of 3 µg of gel. The gel piece was then boiled for 10 min prior to being stored at -20°C. Later in the project this technique was superseded by an improvement in which DNA fragments separated on Sigma type II agarose gels were transferred by continued electrophoresis onto a piece of DEAE membrane that had been prewetted in Tris acetate buffer and placed in a slot in the gel just in front of the desired band. After the top of the gel containing unwanted DNA had been removed, the electrophoresis was continued for 1/2 h at 60 mA. The DEAE membrane was then placed in elution buffer (Tris-Cl 10 mM, EDTA 1 mM, NaCl 1.5 M pH8) and heated to 65°C for 15 min. The membrane was then removed and the buffer extracted once with an equal volume of phenol and then twice with an equal volume of chloroform. DNA was precipitated from the buffer by addition of 1/10th volume 3M Na acetate and 2.5 volumes of ethanol at -20°C. After 30 min at -80°C the solution was centrifuged at full speed in an Eppendorf benchtop centrifuge for 15 min at 4°C. After washing the DNA pellet once by resuspension in 70% ethanol it was dried under vacuum prior to resuspension in T.E.

DNA fragments obtained by these means, or total nuclear DNA boiled for 10 mins, were labelled using α³²P(dCTP) by the random hexanucleotide primer method of Feinberg and Vogelstein (1984). DNA samples were denatured by boiling for 3 min then 50 µl of the following reaction mixture was incubated overnight at room temperature: 30 ng DNA, 50 mM Tris-Cl, 5 mM MgCl₂, 0.2 M Hepes, hexadeoxynucleotides 5.4 OD units/ml, 20 mM dATP, 20 mM dGTP, 20
nm dTTP. The reaction was stopped by the addition of 200 µl T.E. Unincorporated label was removed by centrifuging the probe through a column of Sephadex G50 suspended in T.E. (Maniatis et al., 1982).

Hybridisation of Nylon Membrane Bound DNA with Radiolabelled Probes and Autoradiography:

Hybond filters were placed in 50 ml prehybridisation solution and sealed in a plastic bag. The bag was then placed overnight in a plastic lunch box immersed in a shaking water bath at 65°C. The prehybridisation solution was replaced with 30 ml fresh prehybridisation solution and the appropriate labelled probe, boiled for 10 min and rapid cooled in ice, was added. The bag was then returned in a similar manner to the 65°C waterbath for overnight incubation. Following hybridisation the probe was retained for further use (needing only reboiling for 10 min and rapid cooling) and the filters washed in 2X SSC (a 1/10th dilution of the 20X SSC stock) for 15 min at 65°C in the shaking water bath. After a further 15 min wash in 2X SSC, a third 30 min wash in 2X SSC and a 10 min wash in 0.5X SSC the filters were air dried, taped onto 3MM paper (Whatman), covered with Saran Wrap (Dow Chemicals) and placed in contact with autoradiography film in a light-tight cassette. The film was exposed for approximately one week at -80°C prior to development.

Colony Hybridisation:

*E. coli* colonies picked from selective plates using sterile toothpicks were inoculated in a grid fashion onto LB plates containing 50 µg/ml ampicillin and in a replica manner onto squares of nitrocellulose placed on identical plates. The first plates were incubated overnight at 37°C and then stored at 4°C for subsequent colony retrieval. The plates bearing the nitrocellulose sheets were incubated for three hours at 37°C until the colonies had just
started to grow. The filters were then transferred to dishes containing 3MM paper soaked in Southern hybridisation denaturing solution. After 7 min in these dishes the nitrocellulose pieces were transferred to dishes where the 3MM paper was soaked in Southern hybridisation neutralising solution for 3 min. After 3 min in identical dishes of fresh neutralising solution the filters were rinsed in 2X SSC prior to air drying. Once dry the filters were wrapped in 3MM paper and baked for 90 min at 80°C in a vacuum oven. The filters were then incubated overnight in prehybridising solution prior to hybridisation to a radio-labelled total nuclear DNA probe in the manner outlined above.

CYTOLOGICAL TECHNIQUE.

Actively growing root tips from recent cuttings were treated for four hours at room temperature with a 0.05% (w/v) solution of colchicine after which fixation was achieved by transfer to fixation solution for five minutes. Maceration was achieved by a 5 minute treatment with 1M HCl at 60°C after which the root tips were transferred to water. Staining was achieved by disrupting a single root tip in a drop of 60% stain in water on a microscope slide. The cells were squashed by applying pressure to a glass coverslip and then viewed under a Vickers microscope using a 10X eyepiece and a 100X oil immersion objective. The chromosomes were counted in numerous mitotic cells in each root tip.
SECTION 3 RESULTS AND DISCUSSION

3.1) REGENERATION OF PLANTS FROM CALLUS CULTURE.

MEDIUM SELECTION.

Following preliminary experiments utilising plant tissue culture media containing a variety of plant growth regulators, callus cultures were initiated from leaf discs of RO1-I plants on a range of media based on the medium used by Frearson et al. (1973) for the initiation of Petunia leaf callus. In addition to M & S salts, sucrose, casein hydrolysate and agar these media contained the concentrations of 2,4-D and kinetin indicated in Table 1. A subjective assessment of callus performance was made for five cultures initiated on each of the medium types and medium 204 was selected as showing the best performance for callus initiation Figure 1.

CALLUS PHENOTYPE VARIATION.

Callus derived from the initial growth regulator concentration experiments was retained in culture, by monthly subculturing onto 204 medium, for a period in excess of one year. During that time a variety of different callus morphologies appeared amongst the cultures. Whilst the 'normal' phenotype was a relatively dense, pale green callus, the colour of that on different plates was seen to vary from white to dark brown whilst the degree of friability varied from a very loose 'fluffy' callus to a very compact, hard type. Additionally, the growth rates of different plates, as assessed subjectively, was seen to vary. Whilst some of this variation may reflect permanent genetic changes, much of it is probably due to epigenetic changes occurring in the callus. One particularly fast growing callus that was very pale green in colour and extremely friable was found by Stuart Lindsey (pers. comm.) to be habituated, a phenomenon resulting from epigenetic changes (Meins and Binns, 1978). One callus line, initiated from a single protoplast and cultured on 204 medium, produced a plate of callus that synthesised a coloured pigment in the cells on the surface of the callus mass. Prolonged culture of this callus showed that this ability was stably retained though that is no indication of a permanent genetic change. Similar pigment production has been observed in Petunia callus cultures by Colijn et al. (1981) who showed that the synthesis of anthocyanin in cultured cells is light inducible.

One observation suggesting that a genuine genetic change may have occurred at some stage during initiation or subculture of these callus cultures was made by Shirley Anderson (pers. comm.). Her recent work has shown that the callus culture RCal6, initiated from ROH leaf discs, has a reduced number of ribosomal RNA genes. Whilst this 60% reduction in copy number could have been a pre-
Table 1. Callus growth media hormone concentrations.

<table>
<thead>
<tr>
<th>2,4-D concentration (mg/l)</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>201(**)</td>
<td>202(**)</td>
<td>203(0)</td>
</tr>
<tr>
<td>Kinetin concentration (mg/l)</td>
<td>3.0</td>
<td>204(***)</td>
<td>205(**)</td>
</tr>
<tr>
<td>10.0</td>
<td>207(**)</td>
<td>208(**)</td>
<td>209(0)</td>
</tr>
</tbody>
</table>

The numbers 201-209 indicate the medium codes whilst the number of stars indicates the extent of callus growth one month after initiation of cultures from ROH leaf discs. The growth was assessed subjectively after a study of five replicates of each medium type scoring being on a scale of (0) for no callus growth to (*** for the maximum amount of growth observed.
FIGURE 1 Callus Growth on Different Culture Media.

Leaf discs from Petunia hybrida cv. Rose of Heaven plants six weeks after culture initiation on M&J media containing 2,4-D at 0.1, 0.3 and 1.0 mg/l and kinetin at 1.0, 3.0 and 10 mg/l. Three plates of each growth regulator combination are shown. (Magnification 0.25X)
existing change in one of the leaf mesophyll cells from which the callus was derived, it is not a phenomenon known to be associated with leaf differentiation and it probably arose during culture. Similar observations of rDNA copy number reductions have been made in a number of cell cultures and callus derived regenerants (see General Discussion).

One aspect of callus phenotype that was found to be highly variable between different plates of callus was the ease by which they could be regenerated. Some of this variability could well reflect underlying genetic changes since the extent of karyotypic abnormalities in callus is found to exceed that in regenerated plants (Hayashi and Nakajima, 1984) suggesting that some selection for genotypic normality occurs at this stage. Regeneration of plants from callus grown on 204 medium was attempted using a number of different growth regulators (6-benzly adenine, kinetin) at different concentrations with no success. Regeneration was eventually achieved using a medium containing 1mg/l zeatin (John Hamill, pers. comm.). This medium, termed JH, was used to bring about regeneration of the callus cultures initiated during the initial growth regulator concentration experiments. Whilst for many callus pieces the medium brought about no more than a slight greening, for some the effect of transfer to JH medium was the development of phenotypically abnormal shoots. The phenotypic abnormalities varied from the development of rosettes of tiny leaves with no real stem to the production of densely leafed shoots having fasciated stems. In a few, rare instances the callus was capable of regeneration of comparatively normal shoots. Attempts were made to root both the abnormal and normal shoots. Whilst a high proportion of the abnormal shoots failed to root and some of the normal shoots were similarly impaired, in five cases rooting was achieved on RM medium and whole plants were regenerated.
PLANT REGENERATION.

Two of these plants were regenerated after six months in culture as phenotypically 'normal' shoots and these were named CR,A and CR,B. Two further regenerants were obtained after about twelve months in culture. Once more these arose as relatively normal looking shoots and they were termed CR,C and CR,D. After nearly 18 months in culture a regenerant plant was obtained from one of the fasciated shoots this being termed CR,E.

These plants were not considered ideal for a study concerned with the causes of variation amongst regenerated plants since they were not derived from the same callus initiated from the same plant. As a consequence any differences noticed between them may represent genotypic differences between the plants from which the calli were initiated. Having emphasised this flaw it should be recorded that the plants used to establish the callus cultures cannot have escaped close scrutiny, during the process of leaf excision, and yet no differences were noted. Because of the apparent similarity amongst the starting plants it was considered worthwhile to proceed with further analysis of the regenerants since it is likely that any differences between them arose as a consequence of the callus culture process. To overcome this potential problem, however, a second series of regenerants was obtained.

This second series of regenerants were all obtained by regeneration on JH medium two months after the callus from which they were derived was initiated. This callus, which was initiated on 204 medium, was all derived from a single plant termed 4ROH. The seven plants obtained were all regenerated as normal looking shoots which rooted on RM medium. They were named 4R,1, 4R,2,.....4R,7.
KARYOTYPE ANALYSIS OF PRIMARY REGENERANTS

Using colchicine treatment followed by the orcein staining technique and light microscopy it was possible to count the number of metaphase chromosomes in the root tips of the primary callus regenerants obtained in both the first (CR,A-E) and second (4R,1-7) experiments.

Regenerants CR,A-E

Amongst the regenerants CR,A-E only one of the plants (CR,B) was found to have the normal diploid chromosome number of 14. Three out of five of the plants (CR,A, CR,C & CR,D) were found to be tetraploid or, at least to contain 28 chromosomes, and one (CR,E) was found to be aneuploid having 16 chromosomes. Because of the similarity between the 7 chromosomes of the haploid complement (Maizonnier, 1984) it is not possible to determine, by the technique used, if the plants with 28 chromosomes are genuine tetraploids or if they are aneuploids having more than four of a given chromosome and less than four of another chromosome. This possibility may be unlikely in that aneuploids, in the cell culture situation, most probably arise through the loss of chromosomes from a polyploid set. If this is the case, to obtain 28 chromosomes a hexaploid would have had to have lost fourteen chromosomes.

That numerical chromosome variation is seen is not suprising since Bayliss (1980) in a survey of 53 reports of cell culture karyotypes shows that only seven failed to detect numerical chromosome variation. Equally, in plants regenerated from mesophyll protoplasts of Nicotiana more than 50% were found to be tetraploid (Prat, 1983) perhaps indicating that tetraploidy may exist within some of the leaf cells in the starting material. In the experiment described here it is hard to conclude whether or
not tetraploidy in the starting material may be contributing to
the high frequency of tetraploidy seen since it is known that such
karyotypic abnormalities increase in frequency with the duration
of the callus phase (Lee and Phillips, 1986). All of this material
has been in culture for more than six months and therefore there
has been a long time for polyploidy to accumulate amongst the
cultured cells.

In view of the long time that cells giving rise to CR,E had been
in culture (nearly eighteen months) the finding that it is an
aneuploid with sixteen chromosomes is not surprising. Such
prolonged culture gives ample opportunity for the generation of
polyploidy followed by a breakdown to aneuploidy which is the
mechanism by which the karyotype of this plant was most likely to
have been derived. That it is trisomic for at least two
chromosomes may account for the fact that it does not have the
form of any of the primary trisomics described by Maizonnier
(1984) yet goes some way towards explaining its highly abnormal
phenotype. That the plant is viable is hardly surprising since
Petunia is renowned for its tolerance of aneuploidy (Rick, 1971).

Regenerants 4R,1-7

Karyotypic analysis of the seven regenerants showed two (4R,1 & 4)
to be diploid, four to be tetraploid (4R,2,3 5 & 6) and one (4R,7)
to be aneuploid having 27 (i.e. 4n - 1) chromosomes. The callus
from which these regenerants were derived had only been in culture
for two months and hence it is not surprising that two of the
plants had the normal ROH diploid chromosome complement of 14.
This would appear to indicate that even if the leaves were to
contain a proportion of tetraploid cells, as suggested above, some
leaf cells must remain diploid.

The finding that four of the plants were tetraploid again raises
this question of whether some of the leaf cells are already
tetraploid since the cultures had little time to accumulate karyotypic variants. An alternative suggestion, however, may be that the cultural conditions employed were very stressful and prone to induce karyotypic variation. Certainly there appears to be a link between the use of 2,4-D and chromosomal abnormalities (Bayliss, 1980) and the medium employed did contain this compound. One obvious experiment that would help to differentiate between these two possibilities would be to measure nuclear DNA contents in leaf cells by appropriate staining and microdensitometry.

The regenerant 4R,7 was found to be aneuploid containing 27 chromosomes presumably through chromosome loss following doubling of the diploid to give a tetraploid. That this plant is of comparatively normal morphology may indicate the buffering effect of higher levels of ploidy.

3.2) PHENOTYPIC VARIATION IN REGENERATED PLANTS.

PHENOTYPIC ANALYSIS OF PRIMARY REGENERANTS: EXPERIMENT 1.

A number of morphological abnormalities could be observed in the primary regenerants CR,A-E. The occurrence of these is recorded in Table 2. Growth habit was found to vary from the normal ROH type of erect plant through the more compact and densely leaved CR,E type with broad, flattened, fasciated stems, to the sprawling habit of CR,C and CR,D (Figure 2). Similarly, leaf shape appeared to be variable between plants. CR,A, CR,B and CR,E had leaves of the normal, flat, planar type whilst those of CR,C and CR,D were highly curled in both longitudinal and transverse directions. In addition the leaf shape, as measured by the ratio of leaf length/breadth, appeared to vary. Truly comparable quantitative data for leaf shapes cannot be obtained for the primary regenerants and for similar Rose of Heaven plants due to the fact that leaf shape varies with position on the plant (see below) and hence is likely to vary according to age and physiological
<table>
<thead>
<tr>
<th>Regenerant</th>
<th>Growth Habit</th>
<th>Leaf Shape Ratio*</th>
<th>Leaf Curling</th>
<th>Flower Pigmentation</th>
<th>Floral Abnormalities</th>
<th>Chromosome Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR,A</td>
<td>Normal</td>
<td>1.4</td>
<td>No</td>
<td>Normal</td>
<td>None</td>
<td>28</td>
</tr>
<tr>
<td>CR,B</td>
<td>Normal</td>
<td>2.2</td>
<td>No</td>
<td>Dark Tube Veination</td>
<td>None</td>
<td>14</td>
</tr>
<tr>
<td>CR,C</td>
<td>Sprawling</td>
<td>2.1</td>
<td>Yes</td>
<td>Blotchy</td>
<td>Anther/Petal Fusion</td>
<td>28</td>
</tr>
<tr>
<td>CR,D</td>
<td>Sprawling</td>
<td>1.6</td>
<td>Yes</td>
<td>Blotchy</td>
<td>Anther/Petal Fusion</td>
<td>28</td>
</tr>
<tr>
<td>CR,E</td>
<td>Compact</td>
<td>2.5</td>
<td>No</td>
<td>Pale Limb Pigmentation</td>
<td>Very Small</td>
<td>16</td>
</tr>
</tbody>
</table>

Note: * Leaf Shape Ratio values quoted are a mean of fifteen values for individual leaves of (leaf length/leaf breadth). Directly comparable ROH plants were not available but values for plants which have not been subject to tissue culture are within the range 1.5-1.9.

** ROH Chromosome Number is 14
FIGURE 2 Plant Growth Habit in Regenerants CR,A-E.

A typical *Petunia hybrida* cv. Rose of Heaven plant (extreme left) and (left to right) typical cuttings from the primary callus regenerants CR,A-E showing the unusual general growth habits of some of the regenerants. (Magnification 0.08X)
history. Nevertheless CR,A leaves appeared to be somewhat more circular than Rose of Heaven leaves, having a leaf shape ratio of about 1.4 compared with the typical Rose of Heaven leaf shape ratio which varies within the range 1.5-1.9. The other two tetraploid regenerants (CR,C and CR,D) had leaves with a more elongate outline than the diploid Rose of Heaven suggesting that ploidy alone cannot be responsible for the leaf shape variation seen amongst these tetraploid regenerants. Similarly, a change in genetic determinants other than ploidy might be responsible for the leaf shape seen in the diploid regenerant CR,B (Figure 3) which has particularly elongate leaves. The extremely unusual leaf shape seen in the regenerant CR,E may result from its aneuploid nature.

The flower pigmentation of CR,A was found to be similar to the normal ROH type suggesting that the more blotchy pigmentation seen in the other tetraploid regenerants, CR,C and CR,D, was probably not a consequence of their ploidy. Similarly ploidy must not have been responsible for the flower pigmentation in CR,B which had a rather greyish tinge to the pink colour of its flower limbs and dark purple/black flower tube veination (Figure 4). This dark tube pigmentation is probably due to the increased level of peonidin observed in this part of the CR,B flower (S. M. Smith and A. G. M. Gerats, pers. comm.). This finding resulted from analysis of the flower pigments by thin layer chromatography which also showed CR,E flowers to have reduced levels of quercitin in both limb and tube. The flowers of CR,C and CR,D showed a high incidence of the fusion of anther filaments with the adjacent parts of the petal tube giving the flowers a rather contorted appearance. According to the results of the second experiment with 4R,1-7 progeny, this may be a common feature of tetraploid plants of this cultivar.

All of the morphological abnormalities recorded above, apart from the leaf curling which is rarely observed in the ROH population, appear to be unique to these callus regenerants and have not been
FIGURE 3 Leaf Shape Variation in Primary Regenerants.

Three leaves of different sizes from the primary regenerant CR, A (upper left), showing their rhombic outline, from the primary regenerant CR, B (upper right), showing their more elongate form, and from a typical Petunia hybrida cv. Rose of Heaven plant (below) showing the normal leaf shape of diploid plants. (Magnification 0.8X)
FIGURE 4 Unusual Flower Pigmentation in Regenerants CR,B.

Normal *Petunia hybrida* cv. Rose of Heaven flower (above) showing typical pigmentation in contrast with the greyish-pink limb pigmentation and dark flower tube pigmentation of the primary regenerant CR,B (below). (Magnification Upper 1.5X, Lower 1.3X)
observed in the large number of ROH plants raised during the course of this work. Furthermore, the differences appear to show little correlation with ploidy implying that other genetic changes may have occurred. In consequence it seems reasonable to suggest that this variation results either from pre-existing genetic variation in the leaf mesophyll cells from which callus arises or, more likely, from the passage through, and/or regeneration from, the callus phase. Whist this is a significant result, suggesting that the process is having widespread effects on plant phenotype, it does not prove the existence of somaclonal variation in these plants since heritability must first be demonstrated. These data alone do not prove that these phenotypes are anything more than the plant’s response to the environmental stress experienced during culture and regeneration.

THE INHERITANCE OF VARIATION: PHENOTYPIC ANALYSIS EXPERIMENT 1.

The primary regenerants CR,A-D were self fertilised and also backcrossed to ROH in a pair of reciprocal crosses with the primary regenerants and ROH as both male and female parents. CR,E was found to be sterile, presumably a consequence of its aneuploid state, and hence was not used in this experiment. For comparison, crosses between different ROH plants were performed resulting in a progeny population designated ROH x ROH. The progeny of the self fertilisations and backcrosses of the primary regenerants were designated A Self, A x ROH, ROH x A, B Self, B x ROH,... etc. This experiment, where backcrosses were performed, was initiated before the chromosome complements of the regenerants were known when it was anticipated that many of them would be diploid. However, the tetraploid chromosome complement is generally quite stable, in plants, and is maintained during self fertilisation whilst producing triploid progeny in crosses with diploids. Steere (1932) has suggested that tetraploid nuclei of Petunia may be prone to a low percentage of unstable meioses in certain
circumstances and so the precise karyotypes of the progeny of the self fertilisations and backcrosses cannot be predicted with absolute certainty. However, despite the fact that some crosses involve both diploid and tetraploid parents, the results obtained provide valuable information which helps to establish that genetic variation exists amongst these regenerants.

Progeny from the selfing and backcrossing of CR,A-D were grown to flowering in the greenhouse. One aspect of variation amongst the progeny of these plants that was immediately obvious was the differences in seed viability. Seed from B selfings had an extremely low percentage of germination whilst seed from the crosses ROH X A, and C X ROH appeared to be much less viable than normal. The low viability of seed resulting from the self fertilisation of CR,B could be a consequence of that plant containing a homozygous recessive alteration affecting some aspect of seed development or physiology which may result in the production of seed prone to some failure in germination. This would only be likely if the homozygous alteration arose during the culture process. It would, however, suggest why normal viability is seen in the backcrosses since the potentially lethal mutation would then be in the heterozygous form. The low viability of ROH x A and C x ROH seed is not so easily explained.

A study was made in the regenerant progeny of the qualitative charaters found to be abnormal in the primary regenerants and, in addition, a systematic study of various numerical characters was performed. Fifteen leaves were picked at random from each plant, these comprising from about 50 to 100% of the leaves on the plants. Measurements were made of the lengths and breadths of each of these fifteen leaves and the values were used to calculate a leaf shape ratio (leaf length/leaf breadth) for each leaf. Additionally, the diameter of each flower on each plant was recorded and, following their dissection, the length of the longest anther and the length of the style was determined for each
flower. Statistical analyses were carried out using the resulting data in the manner outlined below.

Qualitative Characters.

The inheritance of leaf curling was found to be complex as the data (Table 3) indicates. No leaf curling was observed in the ROH x ROH or CR,B progeny and only one plant was produced from the C X ROH cross so there is no quantitative information here. That the curling is in some way genetically controlled is indicated by the fact that in virtually all cases the percentage of plants with curled leaves is higher in the progeny of self fertilisation than in the progeny of backcrosses. It should be noted that although no leaf curling was observed in CR,A it can be seen at high levels in its progeny. These results are difficult to explain in terms of the mendelian inheritance of alleles affecting leaf shape. It is possible that the phenomenon of curling might arise through changes in the balance of alleles of different interacting genes. Equally, linkage of genes controlling leaf curling with lethal genes may result in a deviation from normal mendelian behaviour. Knowledge of Petunia mutants shows that there are a number of genes controlling leaf curling and sometimes these mutants have linked effects on viability and fertility (Wiering and de Vlaming communicated by Cornu, 1984). Another alternative explanation may be that leaf curling is a consequence of abnormal chromosome behaviour with chromosome breaks or non-disjunctions contributing to the phenotype. Because of the differing patterns of inheritance shown by the progenies of the different primary regenerants it would seem likely that there may be different genetic causes of the phenomenon in each case.

Genetic analysis of the progeny of the diploid regenerant, CR,B, should be easier than that of the progeny of regenerants with the tetraploid chromosome number. The dark flower tube pigmentation of CR,B appeared to be retained in the progeny of its self-
Table 3. Leaf Curling in the Progeny of Primary Regenerants.

<table>
<thead>
<tr>
<th>PROGENY POPULATION:</th>
<th>A SELF</th>
<th>A x ROH</th>
<th>ROH x A</th>
<th>C SELF</th>
<th>ROH x C</th>
<th>D SELF</th>
<th>D x ROH</th>
<th>ROH x D</th>
</tr>
</thead>
<tbody>
<tr>
<td>% PLANTS WITH CURLY LEAVES:</td>
<td>56</td>
<td>20</td>
<td>20</td>
<td>65</td>
<td>42</td>
<td>29</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>POPULATION SIZE:</td>
<td>33</td>
<td>10</td>
<td>5</td>
<td>17</td>
<td>24</td>
<td>14</td>
<td>5</td>
<td>19</td>
</tr>
</tbody>
</table>
fertilisation along with the grey-tinged flower limb pigmentation, although only three flowering plants were obtained. The unusual limb pigmentation was not observed in any of the backcross progeny (fifteen plants from the ROH X B cross and seventeen plants from the B X ROH cross) but in these plants the dark vein pigmentation was observed at a frequency of about 50%. These results suggest that the greyish limb pigmentation is possibly a recessive character although it could equally show incomplete dominance since it might not be noticed in its heterozygous form as the normal balance of pigments produced by the ROH type alleles may well serve to mask the unusual balance of pigments produced by the CR,B type alleles. The increased production of the dark tube pigment, peonidin, would appear to result from a change from homozygous wild-type to heterozygous for increased pigment production in the CR,B genome. This would result in the 1:1 segregation observed in the backcross progeny and would be expected to give a 3:1 ratio of dark pigmentation to normal pigmentation in the progeny of self-fertilisation. Since there are too few progeny from the self fertilisation of CR,B to establish this ratio this explanation cannot be proven without analysis of the progeny of further crosses.

Leaf Shape Ratios.

For each plant fifteen values for the leaf shape ratio were obtained. As is usual when dealing with ratios, the values obtained from a given plant do not show normal distribution there being a slight tendency towards a skew (data not shown). This does not complicate the statistical analysis since the investigations are concerned with between-plant variation and require only one value per plant. A mean of the fifteen values for each plant was calculated and this is used as the mean leaf shape ratio for that plant in further analyses. The Central Limit Theorem states that even if individual values do not show a normal distribution the value of means calculated from them will get very close to having
a normal distribution as the number of values in the mean tends towards infinity. Fifteen values is quite enough to satisfy this theorem and so the distribution of the mean leaf shape ratios will be approximately normal allowing the application of standard statistical tests.

To compare the mean values of the mean leaf ratios for each progeny population (Table 4) one would normally employ the analysis of variance technique (In Parker, 1979). This test relies on the variances being approximately equal for each population. As shall be proven below, this is not the case for the C and D SELF progeny. Consequently, a t'-test (in Cochran and Cox, 1957) was used to compare these populations with the ROH x ROH population. This showed that while the mean of the C SELF population is not significantly different from the ROH x ROH mean, the mean of the D SELF population differs significantly at the 1% level. The means for the other progeny populations were compared using analysis of variance in conjunction with t-tests (in Parker, 1979) to show which populations are significantly different.

t-Tests show that, at the 0.1% level, the mean leaf shape ratio of the B SELF population is significantly different from that of the ROH X ROH population but that the A SELF mean is not. None of the backcross progeny have leaf shapes that are significantly different from the ROH x ROH population except for the backcross progeny of CR,B. In these crosses the mean leaf shape ratios are intermediate in value between the ROH x ROH value and that of the B SELF population from which they are also significantly different.

In some of the progeny populations, particularly those resulting from self fertilisations, the spread of the data for leaf shape ratios appears to be greater than in the ROH X ROH population. To test whether this is the case the Squared Ranks Test for Variances (In Conover, 1980) was employed. Normally it would be possible to
Table 4. Leaf Shape Ratios in the Progeny of Primary Regenerants.

<table>
<thead>
<tr>
<th>PROGENY POPULATION</th>
<th>MEAN OF INDIVIDUAL PLANT MEANS*</th>
<th>SIZE OF POPULATION</th>
<th>PROGENY POPULATION</th>
<th>MEAN OF INDIVIDUAL PLANT MEANS</th>
<th>SIZE OF POPULATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROH X ROH</td>
<td>1.52</td>
<td>18</td>
<td>C SELF</td>
<td>1.67</td>
<td>17</td>
</tr>
<tr>
<td>A SELF</td>
<td>1.51</td>
<td>33</td>
<td>C X ROH</td>
<td>1.53</td>
<td>1</td>
</tr>
<tr>
<td>A X ROH</td>
<td>1.57</td>
<td>10</td>
<td>ROH X C</td>
<td>1.56</td>
<td>24</td>
</tr>
<tr>
<td>ROH X A</td>
<td>1.56</td>
<td>5</td>
<td>D SELF</td>
<td>1.81</td>
<td>14</td>
</tr>
<tr>
<td>B SELF</td>
<td>2.08</td>
<td>5</td>
<td>D X ROH</td>
<td>1.69</td>
<td>5</td>
</tr>
<tr>
<td>B X ROH</td>
<td>1.88</td>
<td>17</td>
<td>ROH X D</td>
<td>1.48</td>
<td>19</td>
</tr>
<tr>
<td>ROH X B</td>
<td>1.84</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: * length/breadth measured on fifteen leaves on each plant in the population.
use the Bartlett test (In Wetherill, 1981) but this test is unreliable if the data for all of the populations does not show a normal distribution and the D SELF population appears not to satisfy this requirement (Figure 5). The test employed showed that, whilst the variances for A SELF and B SELF appear to be larger than for the ROH X ROH population, only the values for C SELF and D SELF are significantly greater. The backcross progeny populations have a variance similar to the ROH X ROH control.

The D SELF population appears to have a rather unusual spread of data (Figure 5) with half of the values clustered around a value similar to the control and the other half clustered around a much higher value. Ideally one would hope to test this suggestion of a bimodal distribution of the data, however there is insufficient data to do so in this case. The Shapiro-Wilk test (In Wetherill, 1981) does prove that the distribution differs from a normal distribution at the 0.1% level emphasising the unusual distribution of these values.

Hence there is a real difference between the leaf shapes of the diploid regenerant CR,B and the diploid control (ROH x ROH) population. The shape difference is clearly heritable and hence must result from a genetic difference in CR,B. Since the means of the progeny populations resulting from both self-fertilisations and backcrosses are different from the ROH x ROH population value it would appear that the change is not recessive. Equally, since there is no suggestion of bimodality in the distributions of the CR,B progeny populations (data not shown), and hence no evidence of segregation, it is suggested that the variant genetic determinant(s) probably exists in the homozygous state in the primary regenerant.

Explanation of the inheritance of leaf shape variation, in simple mendelian terms, amongst the progeny of the tetraploid regenerants cannot be made solely on the basis of the crosses performed.
FIGURE 5 Leaf Shape Ratio Frequency Plots.

Plots indicating the number of individual plants with the indicated plant mean leaf shape ratios (mean value of leaf length/leaf breadth for 15 leaves). The normal *Petunia hybrida* cv. Rose of Heaven population shows only a limited spread of values whilst the CR,C and CR,D callus regenerant progeny populations show a greater variance. The CR,D progeny population resulting from self-fertilisation (D SELF) shows what appears to be a bimodal distribution which is not present in the CR,D backcross progeny populations (D X ROH and ROH X D).
Nevertheless it is clear that there are real differences in both the mean leaf shape ratios and the degree of shape variation between the tetraploid regenerants. The results of Santos and Handro (1983) would suggest that both tetraploid plants and their triploid backcross progeny might be expected to have mean leaf shape ratios lower than that of the diploid population. In the unspecified cultivar used by these workers, ploidy levels higher than diploid resulted in leaves with a more rhombic outline than those of diploid plants. This is obviously not the case here and it is possible that either Rose of Heaven responds differently to the cultivar that they used, or that each of these tetraploids may have some other genetic determinant affecting leaf shape. Indeed there is a suggestion that there may be a contribution from both of these factors. Certainly there are other genetic determinants affecting leaf shape in the progeny of these three tetraploid regenerants since not all of them behave in the same way. Conversely though, higher levels of ploidy did appear to result in more elongate leaves in the tetraploid progeny of the second experiment (see below) suggesting that Rose of Heaven may, indeed, respond differently to the cultivar of Santos and Handro (1983).

That some genetic determinant other than ploidy is affecting the leaf shape in CR,D is implied by the apparent segregation of genetic determinants influencing leaf shape when it was self-fertilised (Figure 5). No bimodality in the distribution of leaf shape ratios is seen in the backcross progeny populations, where the means tend back towards normal ROH x ROH value, suggesting that the genetic difference may be recessive. Further work, such as the self-fertilisation of the elongate-leaved progeny of the initial self-fertilisation, would be necessary to substantiate this suggestion.

The effect of ploidy alone may explain the situation in the progeny of CR,C where the progeny of self-fertilisation, which are presumably tetraploid, have more elongate leaves than the
backcross progeny which are presumably triploid. It is hard to see, however, why the variance of the population of progeny resulting from self-fertilisation should be higher than that of backcross population. One possible explanation could be that the tetraploid primary regenerant shows a degree of meiotic instability similar to that reported by Steere (1932). The production of occasional haploid gametes would introduce a few diploids into the backcross populations resulting in variances barely greater than the control. Conversely, in the population of progeny resulting from self-fertilisation, since both of the gametes come from the potentially unstable meiosis, a greater range of karyotypic variation could occur with plants ranging from diploid through triploid to tetraploid being produced. Only an extensive study of karyotypes amongst the regenerant progeny could test this theory.

Flower Diameter

The values for the diameters of different flowers on each plant were used to calculate a mean flower diameter for each plant. These mean values, which appeared to have normal distribution and approximately equal variances (Table 5), were used as the data set for an analysis of variance to compare the mean flower diameters for each progeny population. This showed that there were significant differences between the means of some of the populations.

\[ t \text{ tests showed that the means of the B SELF, C SELF and B, C, and D backcross populations were not significantly different from the ROH X ROH control mean but that the mean of the D SELF population was significantly different at the 5% level. The mean values of the A SELF and A X ROH populations were significantly different from the control at the 0.1% level though only significantly different from each other at the 10% level. However there was no} \]
Table 5. Flower Component Dimensions in the Progeny of Primary Regenerants.

<table>
<thead>
<tr>
<th>PROGENY POPULATION</th>
<th>ANOTHER LENGTH (mm)</th>
<th>STYLE LENGTH (mm)</th>
<th>FLOWER DIAMETER (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>$\sigma_{n-1}$</td>
<td>n</td>
</tr>
<tr>
<td>ROH x ROH</td>
<td>15.45</td>
<td>1.133</td>
<td>15</td>
</tr>
<tr>
<td>A SELF</td>
<td>14.29</td>
<td>1.440</td>
<td>14</td>
</tr>
<tr>
<td>A x ROH</td>
<td>16.12</td>
<td>1.368</td>
<td>12</td>
</tr>
<tr>
<td>ROH x A</td>
<td>17.40</td>
<td>0.530</td>
<td>2</td>
</tr>
<tr>
<td>B SELF</td>
<td>12.00</td>
<td>0.957</td>
<td>3</td>
</tr>
<tr>
<td>B x ROH</td>
<td>13.77</td>
<td>1.135</td>
<td>15</td>
</tr>
<tr>
<td>ROH x B</td>
<td>14.01</td>
<td>1.721</td>
<td>16</td>
</tr>
<tr>
<td>C SELF</td>
<td>11.07</td>
<td>1.224</td>
<td>4</td>
</tr>
<tr>
<td>C x ROH</td>
<td>13.75</td>
<td>n/a</td>
<td>1</td>
</tr>
<tr>
<td>ROH x C</td>
<td>16.30</td>
<td>1.111</td>
<td>16</td>
</tr>
<tr>
<td>D SELF</td>
<td>12.66</td>
<td>1.084</td>
<td>7</td>
</tr>
<tr>
<td>D x ROH</td>
<td>12.07</td>
<td>1.930</td>
<td>3</td>
</tr>
<tr>
<td>ROH x D</td>
<td>14.33</td>
<td>0.918</td>
<td>10</td>
</tr>
</tbody>
</table>
significant difference between the ROH x A population mean and the control value.

The findings of Santos and Handro (1983), working with culture-derived plants of an unspecified Petunia hybrida cultivar, would suggest that tetraploid and triploid plants should have larger flowers than diploid controls. This is obviously not the case here and, once again, either cv. Rose of Heaven must behave differently from the cultivar used by those workers or somaclonal variation has introduced the different response.

The large mean flower size in the progeny population resulting from self-fertilisation of CR,D may be explained in terms of a recessive genetic difference affecting flower size. Since there is no suggestion of a bimodality of distribution for the data for the population of progeny resulting from self-fertilisation (data not shown), any difference would have to be homozygous in the primary regenerant. In the absence of further crosses and comparable quantitative information for the primary regenerant this suggestion will have to remain solely as speculation.

Likewise, the absence of comparable information for the primary regenerant and further crosses hampers explanation of the inheritance of flower size in the progeny of CR,A. That the flower size of A SELF and A X ROH progeny is significantly larger than that of the ROH x ROH and the ROH X A plants is remarkable. Whilst suggesting that there are some specific genetic determinants affecting flower size it also implies that there is a preferential inheritance of these determinants through the female.

Anther Length.

The anther length data (Table 5) show that anther size differs in almost all of the progeny when compared with normal ROH anther lengths. When analysis of variance is performed on this data, with
t-tests to show which populations vary significantly from the ROH x ROH population value, all progeny populations except for the CR,A backcross differ significantly from the ROH mean. There is no suggestion of karyotype influencing this character and it would appear that this, clearly heritable, variation must have some other genetic cause(s). Precise genetic interpretation is not possible due to the tetraploid nature of many of the primary regenerants however, that the patterns of inheritance differ in the progeny of the different regenerants implies that different genetic determinants may be affecting anther length in each regenerant. The one constant feature, implying that none of these genetic determinants are dominant, is that the progeny of backcrosses all have anther lengths that are closer to the ROH value than the progeny of self-fertilisation.

Style Length.

Analysis of variance performed on the data for style length variation amongst the regenerant progeny populations (Table 5) showed that the progeny of self-fertilisations all have styles that are significantly shorter than those of ROH plants whilst the style lengths in backcross progeny tend towards the ROH value although often remaining significantly shorter. On its own, interpretation of these data is difficult but an explanation appears when comparison is made with the style length data for the progeny of the primary regenerants 4R, 1-7 (Table 11). In that case there is clear evidence for a reduction in style length with increased ploidy. Here the populations of progeny resulting from self-fertilisation of the tetraploid regenerants CR,A,C & D would be expected to be tetraploid and thus have shorter styles than the triploid progenies of the backcrosses. CR,B is however diploid and it is perhaps significant that the progeny of self-fertilisation of this regenerant have the shortest style lengths of all. This may suggest that these shorter styles result from some distinct genetic difference in the primary regenerant CR,B. That the
backcross progeny populations tend back towards the control value suggests that this difference cannot be dominant and hence may have been homozygous in the primary regenerant although analysis of further crosses would be necessary to establish this.

Genetic Variation in the Primary Regenerants.

Although many of the characters studied are likely to be subject to polygenic control and many of the regenerants are tetraploid, further complicating genetic analysis, the data for phenotypic variation amongst these primary regenerant progeny populations suggest that there is widespread genetic variation between the primary regenerants. The plants used to initiate the cultures from which these regenerants were produced showed no such variation and hence it is likely that the differences recorded here reflect either pre-existing variation in the cells of the leaf or genetic changes that occurred during the culture process. Unlike the experiment with regenerants 4R,1-7, described below, where few characters are affected by changes other than ploidy, here specific genetic differences appeared to be a more significant source of variation than karyotypic variation. One principal difference between the two experiments is that in this first instance the callus cultures from which the regenerants were derived had been growing from three to nine times longer. It may be that prolonging the callus phase increases the amount of variation seen and this possibility shall be discussed further in the general discussion.

PHENOTYPIC ANALYSIS OF PRIMARY REGENERANTS: EXPERIMENT 2.

A limitation of the results from the experiment with callus regenerants CR,A-E was that not all of the regenerants were obtained from callus derived from a single plant. Therefore, a second experiment was performed using regenerants, 4R,1, 4R,2, ..., 4R,7, derived from just the single plant, 4ROH. A limited
analysis was performed on the primary regenerants with a more detailed analysis restricted to the progeny of self fertilisations of the primary regenerants. The data for the phenotypic analysis of the primary regenerants is shown in table 6. The leaf shape ratio value shown was obtained by measuring the dimensions of fifteen leaves taken at random positions from each of the plants. A leaf shape ratio (length/breadth) was calculated for each of the leaves and a mean value for each plant calculated. Since the mean leaf shape ratio in this analysis measures the average of the shapes of leaves from all regions of the plant, the values are not directly comparable with those quoted for their progeny which measure the leaf shape of specific leaves (see below). The values do, however, allow for comparison between the seven primary regenerants and appear to suggest that there is a link between ploidy and leaf shape since the plants with the lowest leaf shape ratios are the two diploid regenerants. This would appear to contradict the findings of Santos and Handro (1983), as discussed below.

Similarly, the flower diameter values quoted in table 6, which are the means of the diameters of five flowers from each plant, do not allow for direct comparison with the data for the progeny but do allow for comparison amongst the primary regenerants. Once more ploidy appears to be having an effect, since the two diploid regenerants have the largest flowers, and once again the result is at variance with published data (Santos and Handro, 1983) for other Petunia hybrida plants, see below.

THE INHERITANCE OF VARIATION: PHENOTYPIC ANALYSIS EXPERIMENT 2.

CR,1-7 are the progeny resulting from self-fertilisation of the seven primary regenerants obtained from a callus culture that was initiated from the single ROH plant, 4ROH. The progeny of 4R,1-7 were raised from seed under identical conditions and at the same time as progeny from 4ROH (termed ROH4). Phenotypic studies were
Table 6. Phenotypic Characters of the Primary Regenerants 4R,1-7.

<table>
<thead>
<tr>
<th>CHARACTER</th>
<th>CR,1</th>
<th>CR,2</th>
<th>CR,3</th>
<th>CR,4</th>
<th>CR,5</th>
<th>CR,6</th>
<th>CR,7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN FLOWER DIAMETER (mm)</td>
<td>77.4</td>
<td>58.6</td>
<td>68.8</td>
<td>78.8</td>
<td>65.8</td>
<td>65.2</td>
<td>60.0</td>
</tr>
<tr>
<td>MEAN LEAF SHAPE RATIO*</td>
<td>1.66</td>
<td>2.14</td>
<td>1.86</td>
<td>1.84</td>
<td>2.02</td>
<td>1.99</td>
<td>1.90</td>
</tr>
<tr>
<td>PLANT GROWTH HABIT</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>FLORAL ABNORMALITIES</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>CHROMOSOME NUMBER**</td>
<td>14</td>
<td>28</td>
<td>28</td>
<td>14</td>
<td>28</td>
<td>28</td>
<td>27</td>
</tr>
</tbody>
</table>

Note: * Leaf Shape Ratio = Leaf Length / Leaf Breadth. Directly comparable ROH plants were not available but values for plants which have not been subject to tissue culture are within the range 1.5-1.9.
** Normal ROH Diploid Complement = 14
performed on CR,1-7 and ROH4 plants and statistical analysis of
the quantitative data from ROH4 and CR,1-6 was performed. No
statistical analysis was possible with the data from the CR,7
population since only two plants were obtained from selfings of
4R,7. This resulted from low seed viability, since a normal number
of seeds were set, and was, presumably, a consequence of its
aneuploid state affecting meiosis. The characters studied included
leaf shape ratios (i.e. length/breadth values) for three leaves
from the centre of the plant, stamen length, internode lengths for
three internodes in the central region of the plant, and flower
diameters. The measurement of all of these quantitative characters
was performed on the date at which a plant first reached anthesis.
This date was recorded as were a number of qualitative characters
such as the relative positions of stigmata and anthers as well as
flower tube pigmentation.

Qualitative Characters.

The distribution of the characters affecting flower pigmentation
and the relative positions of floral organs is shown in Table 7.
There would appear to be a correlation between the tetraploid
state and the appearance of yellow flower tube pigmentation in a
proportion of the progeny. This yellow tube pigmentation may,
however, represent the phenotypic expression of a mutation that
only by chance correlates with the tetraploid state. It is
possible that all of the tetraploid regenerants were derived from
a single cell line in the callus and that this line aquired a
mutation in an allele affecting flower tube pigmentation. In
tetraploid plants it is hard to suggest the pattern of inheritance
that would be expected in the progeny of self-fertilisations
however it is reasonable to expect that such a mutation may only
be expressed in a proportion of the progeny so the observation
that only a small percentage of the progeny show this phenotype is
compatible with this suggestion.
Table 7. Qualitative Characters in Progeny of 4ROH and Regenerants 4R,1-7.

<table>
<thead>
<tr>
<th>Regenerant Progeny</th>
<th>ROH4</th>
<th>CR,1</th>
<th>CR,2</th>
<th>CR,3</th>
<th>CR,4</th>
<th>CR,5</th>
<th>CR,6</th>
<th>CR,7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants with Yellow Tube Pigmentation (%)</td>
<td>0</td>
<td>0</td>
<td>9.1</td>
<td>8.3</td>
<td>0</td>
<td>15.8</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>Plants with Anthers Longer than Styles (%)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Plants with Styles Longer than Anthers (%)</td>
<td>42</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>47</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plants with Styles Equal to Anthers (%)</td>
<td>34</td>
<td>58</td>
<td>100</td>
<td>98</td>
<td>53</td>
<td>88</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Plants with Anther-Petal Fusions (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.1</td>
<td>0</td>
<td>15.8</td>
<td>5.1</td>
<td>0</td>
</tr>
<tr>
<td>Population Size</td>
<td>35</td>
<td>106</td>
<td>22</td>
<td>48</td>
<td>89</td>
<td>19</td>
<td>58</td>
<td>2</td>
</tr>
</tbody>
</table>
The relative lengths of anthers and styles is found to vary amongst the regenerant progeny (Figure 6) and even amongst the progeny of 4ROH. In the 4ROH control population and in the diploid regenerants the stigmata can protrude beyond the anthers though in the tetraploid regenerants this never occurs presumably because of their relatively short styles (see below). When looking at the data for plants with anthers that protrude beyond the stigmata the same correlation with ploidy cannot be made since, though most of the tetraploid regenerant progeny populations showed a low level of flowers with protruding anthers, one (CR,2) did not. Whilst this break from the ploidy correlation may be explained in terms of the lower population size sampled for CR,2, the fact that one of the diploid regenerant progeny populations (CR,1) also shows a low percentage of plants with the protruding anthers cannot. Possibly some genetic change in CR,1 gives rise to its atypical behaviour but a much closer study of both anther length and the position of anther/petal tube attachment would be needed to make any meaningful comments.

With the exception of CR,2, where the sample size is small, there is a correlation between tetraploidy and the appearance of a low level of anther-petal fusions in the regenerant progeny populations. Once again it could be that this correlation is related to tetraploidy or it could be that all of the tetraploid regenerants were regenerated from a single callus line that underwent some genetic change that gives rise to these phenotypes amongst the regenerant progeny.

Leaf Shape Ratios.

The leaf shape ratios of the different leaves on a plant at first anthesis were studied by measuring the uppermost fifteen expanded leaves on five different plants. Plants at this stage have no more than 20 leaves and so these represent all but the senescing lower leaves. Numbering the leaves consecutively from apex to base, a
FIGURE 6 Relative Positions of Anthers and Styles.

Flowers from CR,1 plants showing (left) style longer than anther and (right) style and anthers of equal lengths. (Magnification 1.0X)
mean value of the leaf shape ratios for each of leaves 1-15 was obtained. These means are represented graphically in Figure 7 which shows how the leaves in the central region of the plant have a somewhat more rhombic profile than basal or apical leaves. This is also the region of the plant where adjacent leaves differ the least in leaf shape ratio and hence where the mean of three values has its greatest meaning. Accordingly, the three leaf shape ratios determined for each plant were those for leaves number 6, 7 and 8. The leaf shape ratio value used in statistical analysis was the mean of these three values, termed the mean leaf shape ratio for that plant.

These individual plant mean leaf shape ratios were used to calculate a mean ratio for each of the regenerant progeny populations and for the ROH4 population. These means and the standard deviation values for each population are shown in Table 8. The ratios show approximately normal distributions and have similar standard variation values so the data can be used for analysis of variation. This shows that there are significant differences between the mean ratio values of some of the populations. There are significant differences between the mean of the ROH4 population and the means of the CR,1,2,3,5 and 6. The mean values for the CR,2,3,5 and 6 populations are not significantly different from each other. Whilst there is no significant difference between the mean of the ROH4 population and the mean of the CR,4 and no significant difference between the means of the CR,4 and CR,1 populations, the CR,1 mean is significantly different from the ROH4 value.

There would appear to be a correlation between leaf shape ratio and ploidy. The progeny of tetraploid plants have more elongate leaves than the progeny of diploid plants. This is the opposite result to that obtained by Santos and Handro (1983) and suggests that the cultivar Rose of Heaven behaves differently to the unspecified cultivar used by those workers. There is, however,
Table 8. Leaf Shape Ratios in Progeny of 4ROH and Regenerants 4R, 1-7.

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</thead>
<tbody>
<tr>
<td>Leaf Shape Ratio Mean</td>
<td>1.52</td>
<td>1.44</td>
<td>1.63</td>
<td>1.63</td>
<td>1.47</td>
<td>1.63</td>
<td>1.67</td>
<td>1.66</td>
</tr>
<tr>
<td>Leaf Shape Ratio $\sigma_{n-1}$</td>
<td>0.180</td>
<td>0.231</td>
<td>0.147</td>
<td>0.154</td>
<td>0.133</td>
<td>0.133</td>
<td>0.198</td>
<td>0.325</td>
</tr>
<tr>
<td>Population Size</td>
<td>35</td>
<td>106</td>
<td>22</td>
<td>48</td>
<td>89</td>
<td>19</td>
<td>58</td>
<td>2</td>
</tr>
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</table>
FIGURE 7 Leaf Shape Ratios at Different Positions on the Plant.

The mean of the values for leaf shape ratio (leaf length / leaf breadth) for each of the expanded leaves, numbered 1-15 from apex to base, from five Petunia hybrida cv. Rose of Heaven plants. The error bars represent the mean ± one standard error.
evidence for a genetic change unrelated to ploidy in that CR,1 has more rhombic leaves than the control. This change is heritable and hence represents somaclonal variation.

Internode Lengths.

The three internodes immediately below the node from which the first pedicel arises were measured in each plant to give the plant mean internode length. The mean of these values was calculated for each progeny population and these are shown in Table 9. Because the standard deviations of these populations are all similar and the distributions of internode lengths are approximately normal it is possible to use analysis of variance and t-tests to determine any significant differences between these means.

The internodes of the CR,1 and CR,4 populations are not significantly different from the ROH4 population but the CR,2, 3, 5 and 6 populations have significantly shorter internodes than the control. There is no significant difference between these four shorter populations. Once again there is a direct correlation between the ploidy of the regenerant and the dimensions of its progeny. The progeny of tetraploid regenerants have shorter internodes. That the variances of both the internode length data and the leaf shape data are not higher in the tetraploid progeny than in the diploid progeny may suggest karyotypic uniformity in the tetraploid regenerant progeny populations. This would suggest that there are normal, stable meioses in the tetraploid regenerants giving diploid gametes.

Flower Diameter.

The flower diameter was recorded for each of the progeny plants and a mean value of the flower size was calculated for each of the progeny populations. These mean values are recorded in Table 10. Once again the populations show similar variances and
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</thead>
<tbody>
<tr>
<td>Internode Length</td>
<td>15.5</td>
<td>15.4</td>
<td>11.8</td>
<td>13.3</td>
<td>15.4</td>
<td>11.4</td>
<td>12.8</td>
<td>13.2</td>
</tr>
<tr>
<td>Mean (mm)</td>
<td>4.83</td>
<td>3.87</td>
<td>2.82</td>
<td>3.04</td>
<td>3.35</td>
<td>2.52</td>
<td>2.80</td>
<td>5.42</td>
</tr>
<tr>
<td>Internode Length $\sigma_{n-1}$</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Population Size</td>
<td>35</td>
<td>106</td>
<td>22</td>
<td>48</td>
<td>89</td>
<td>19</td>
<td>58</td>
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</tr>
<tr>
<td>Flower Diameter Mean (mm)</td>
<td>66.7</td>
<td>71.0</td>
<td>66.8</td>
<td>68.2</td>
<td>70.7</td>
<td>66.2</td>
<td>66.2</td>
<td>64.0</td>
</tr>
<tr>
<td>Flower Diameter $\sigma_{n-1}$</td>
<td>7.500</td>
<td>7.365</td>
<td>8.968</td>
<td>6.345</td>
<td>6.972</td>
<td>7.897</td>
<td>9.416</td>
<td>5.657</td>
</tr>
<tr>
<td>Population Size</td>
<td>35</td>
<td>106</td>
<td>22</td>
<td>48</td>
<td>89</td>
<td>19</td>
<td>58</td>
<td>2</td>
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</table>
approximately normal distributions and so analysis of variance and t-tests have been performed to show which populations have flowers which are significantly different from the ROH4 population in terms of flower diameter. Only the CR,1 and CR,4 populations had flowers that differed significantly, in terms of size, from the ROH4 control. These results suggests that ploidy has no effect on flower size in Rose of Heaven since the tetraploid regenerant progeny had flowers of the same size as the diploid ROH4 population. This again does not agree with the findings of Santos and Handro (1983) who found flower size to increase in tetraploids and presumably indicates a different response of the two Petunia hybrida cultivars. It would appear that some genetic change has occurred, however, in the cell line(s) that gave rise to 4R1 and 4. That their flower sizes should be the same suggests that the same change occurred in each regenerant and one explanation could be that these two regenerants were derived from cells that had, themselves, been derived from a single cell carrying this mutation.

**Style Length.**

The style length was recorded for the first flower on each plant and a mean of these values was calculated for each regenerant progeny population. These means are shown in Table 11 along with the values for standard deviations. ROH4, CR,1 and CR,4 populations have variances which appeared to differ from those for the other progeny populations. A Bartlett's Test showed that the variances were indeed different. In consequence it is not possible to perform a straightforward analysis of variance. Instead two separate analyses of variances have been performed, one on the ROH4, CR,1 and CR,4 populations and another on the other progeny populations. Both of these gave an F-ratio indicative of no significant variation in style length between the populations tested. A t'-test showed that there was a significant difference between the style lengths of the two groups. Thus again there is

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</tr>
</thead>
<tbody>
<tr>
<td>Style Length Mean (mm)</td>
<td>20.21</td>
<td>20.35</td>
<td>18.43</td>
<td>18.02</td>
<td>20.19</td>
<td>17.65</td>
<td>18.27</td>
<td>20.00</td>
</tr>
<tr>
<td>Style Length $\sigma_{n-1}$</td>
<td>1.269</td>
<td>1.507</td>
<td>2.428</td>
<td>2.445</td>
<td>1.343</td>
<td>2.465</td>
<td>2.177</td>
<td>2.000</td>
</tr>
<tr>
<td>Population Size</td>
<td>35</td>
<td>106</td>
<td>22</td>
<td>48</td>
<td>89</td>
<td>19</td>
<td>58</td>
<td>2</td>
</tr>
</tbody>
</table>
an apparent correlation of ploidy with the dimensions of the regenerant progeny. Here, however the case for this correlation being artifactual is strong. In other cases where a correlation was proposed there was no more variation in the tetraploid regenerant progeny populations than in the control implying a karyotypic uniformity in these populations. In this case the variances of the tetraploid regenerant progeny populations are higher than in the control and diploid regenerant progeny populations. Such variation is unlikely to result from karyotypic variation since this has not been recorded for the other phenotypes and it could result from segregation at meiosis. If a single cell line gave rise to regenerants 4R, 2, 3, 5, 6, and 7 and carried a heterozygous mutation resulting in reduced style lengths this would segregate in the progeny to give a range of style lengths and accounting for the high variances seen.

Flowering Dates.

The date of first anthesis was recorded for each plant and a frequency plot was made for each progeny population indicating what percentage of the plants in that population had flowered by a particular date. These data are shown in Figure 8. It would appear from the plots that the progeny populations fall into two classes with respect to flowering date and a $\chi^2$-test (In Conover, 1980) shows that this is a significant result. Once again phenotype correlates with ploidy, the tetraploid regenerant progeny having delayed flowering. This probably reflects slower overall growth in these, presumably, tetraploid plants which may be related to lengthened cell division cycles resulting from the longer S phase necessary to replicate the doubled genome.

Genetic Variation in the Primary Regenerants.

Whilst most of the phenotypic variation seen amongst the cell culture regenerants in this instance correlates with ploidy there
FIGURE 8 Flowering Profiles for Regenerant Progeny Populations.

Curves showing the percentage of plants in the population which have reached, or passed, first anthesis by the times shown. Populations shown represent the progeny of self-fertilisation of the primary regenerants 4C,1-6 (CR,1-6) and the progeny of self-fertilisation of 4ROH (ROH4). Populations CR,1 and CR,4 behave as the ROH4 population with respect to flowering date (see CR,1 on lower right graph). Populations CR,2, 3, 5 and 6 show delayed flowering (see CR,3 on upper right graph).
The graphs illustrate the percentage of plants past first anthesis over days after sowing for different cultivars:

- **ROH4**
- **CR1**
- **CR2**
- **CR3**
- **CR4**
- **CR5**
- **CR6**

The x-axis represents days after sowing, ranging from 60 to 120 days, and the y-axis represents the percentage of plants past first anthesis, ranging from 0 to 100%. The graphs show the progression of anthesis across different days for each cultivar.
is evidence for other genetic differences. Something would appear to be affecting the relative positions of anthers and styles in the CR,1 population and this must result from some genetic change. Equally the leaves of the CR,1, and possibly the CR,4, populations are more rhombic than the control. Once again, the CR,1 and CR,4 populations showed variation, compared with the control, in terms of their increased flower size. As far as the tetraploid regenerants are concerned, there is the suggestion that they may all carry genetic determinants resulting in reduced style lengths. These results indicate two things. Firstly they prove heritable variation amongst the callus regenerants although they do not differentiate between pre-existing variation amongst the leaf cells or genetic changes during culture as the cause of this somaclonal variation. Further experimentation with callus derived from a single protoplast would be necessary for this.

Secondly these results may suggest a close relationship between all of the tetraploid and all of the diploid regenerants. For the proposed genetic change affecting style length to have occurred independently in all of the tetraploid regenerants would be a remarkable coincidence. It is more likely that it occurred once in a single cell that gave rise, ultimately, to all of the tetraploid regenerants. Similarly it would seem reasonable to suppose that the genetic changes that affected both the CR,1 and CR,4 populations in the same way, namely the flower size and leaf shape changes, were unlikely to have occurred independently suggesting that the two diploid regenerants may have been derived from a single cell carrying these two mutations. The regenerant shoots, however, arose at different positions on different plates of callus even though these different plates were all derived from the same initial plate. The inference must be, therefore, that these changes occurred early in the history of the callus or were pre-existing variants within the leaf. After initiation the callus must have grown and been subcultured with few further changes. The significance of this result, suggesting that variation occurred early in the history of the callus, is discussed in the general discussion later.
3.3) MOLECULAR ANALYSIS OF PETUNIA HYBRIDA, CULTURED CELLS AND REGENERATED PLANTS.

Despite the fact that the callus regenerants CR,A,C & D were found to be tetraploid, numerous phenotypic differences were observed between them. Equally differences were seen between the diploid CR,B and the ROH control. Those differences that are heritable would be classed as somaclonal variation and, clearly, some are. Additionally, heritable differences can be detected between the different tetraploid and the different diploid regenerants of the 4R, 1-7 series. Such variation can only have arisen through molecular rearrangements in either the nuclear or cytoplasmic genomes of these plants. It is not possible to determine, in this situation, if the observed variation arose during culture or merely represents the liberation of pre-existing variation amongst the cells of the leaf mesophyll, however a molecular analysis of these plants might show genome differences that may correlate with the appearance of phenotypic variation. Using the regenerants CR,A-E, since they were the only regenerants available at that time, an attempt has been made to identify the types of molecular reorganisations that accompany somaclonal variation. Candidates for molecular reorganisations that may be responsible for the phenomenon include changes in the organisation and distribution of repeated sequences and changes in cytosine methylation.

CHARACTERISATION OF REPEATED SEQUENCES IN PETUNIA HYBRIDA.

Very little is known about the molecular organisation of the Petunia nuclear genome. No C\textsubscript{ot} analysis has been performed and so there is not much information available concerning sequence repetition although there has been some characterisation of the ribosomal RNA genes in cv. Mitchell. As far as information about single copy sequences is concerned, only a few protein coding genes have been cloned, providing information about a small number of gene families, so more work is required here too. Consequently,
in addition to attempting to gain an insight into the processes by which plant genomes are reorganised, it was hoped that this work could provide some preliminary analysis of the organisation of repeated sequences in the *Petunia* genome.

**Isolation of Repeated Sequences.**

To study both the organisation of repeated sequences in *Petunia hybrida* and molecular reorganisations in callus regenerants, a small collection of cloned repeated sequences was prepared from nuclear DNA from cv. Rose of Heaven. Repeated sequence rearrangements have been shown to occur in cultured cells and so this class of sequence seemed particularly suitable for a study of this type. Fragments of ROH DNA were cloned at random into a series of plasmids and then the resultant bank of plasmids was screened to identify sequences of different frequency classes. This screening was achieved by colony hybridisation using total nuclear DNA as a probe.

Following ligation of *Mbo* I digested nuclear DNA fragments, from the ROH genome, into the *Bam* HI site of pUC 18, transformation into *E.coli* strain JM101 and selection on medium containing ampicillin and X-GAL, some 400 white colonies were obtained. When these colonies were subjected to the colony hybridisation procedure, using $^{32}$P-labelled *Mbo* I digested nuclear DNA as a hybridisation probe, apparent positive hybridisation signals were obtained for 32 of the colonies. These 32 colonies were designated pROH 1-32 and were subjected to the same colony hybridisation procedure two further times when the intensity of the hybridisation signal was recorded using an arbitrary scale of 0-3 where 0 indicates no observable hybridisation, 1 indicates the lowest level of visible hybridisation, 2 indicates an hybridisation intensity intermediate between that categorised as 1 and the very intense hybridisation recorded for the colonies classified as 3 (Table 12).
Table 12. Hybridisation Signal Scores For pROH 1-32.

<table>
<thead>
<tr>
<th>pROH No.</th>
<th>Hybridisations:</th>
<th>pROH No.</th>
<th>Hybridisations:</th>
<th>pROH No.</th>
<th>Hybridisations:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>12</td>
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<td>2</td>
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<td>13</td>
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<td>1</td>
</tr>
<tr>
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<td>16</td>
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<td>6</td>
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<td>9</td>
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<tr>
<td>10</td>
<td>0</td>
<td>1</td>
<td>21</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>1</td>
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</tbody>
</table>
The precise stringency of hybridisation and the degree of growth of particular colonies must have varied sufficiently between the three hybridisations to produce the hybridisation intensity differences observed for some of the colonies in the different screenings.

For a sequence to show hybridisation to the total genomic DNA probe, used in these colony hybridisation screens, it is likely to have a higher abundance in the genome than the unique copy sequences comprising the majority of protein coding genes. All of the clones selected were anticipated to represent sequences of at least moderate repetition, an expectation confirmed by study of their sequence organisation in the genome.

**Analysis of Repeated Sequences in *Petunia hybrida***.

Cloned ROH DNA sequences, isolated from plasmids by double digestion with *Eco* RI and *Hind* III, were used to prepare hybridisation probes which were employed to study the organisation of the cloned sequences in the ROH nuclear genome. Nuclear DNA from ROH was digested with a range of different restriction endonucleases and subject to agarose gel electrophoresis. The DNA fragments separated in this manner were transferred to nylon membrane by the general principle of Southern (1975). DNA immobilised on the nylon was subjected to DNA-DNA hybridisation using $^{32}$P-labelled DNA obtained from the plasmid clones. The resultant filters were subject to autoradiography when the distribution of hybridisation to different DNA fragments could be observed.

**pROH 29**

Clone pROH 29, which was a sequence of approximately 1.5 kb (data not shown), was one of the clones that showed a consistently intense hybridisation signal in the colony hybridisation
FIGURE 9 Hybridisation of ROH DNA with Repeated DNA Probe pROH 29.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of *Petunia hybrida* cv. Rose of Heaven and hybridised with DNA from sequence clone pROH 29. The DNA has been digested with *Eco* RI (left), *Bam* HI (centre) and *Hind* III (right).
screens. This suggested that the sequence represents a highly repeated DNA element and pROH 29 was found to result in an intense smear of hybridisation on Eco RI, Hind III and Bam HI tracks when used as a hybridisation probe in genomic Southern hybridisations (Figure 9). The intensity was consistent with a very high copy number and the range of fragment sizes to which the probe hybridised can be explained in terms of relatively short sequences dispersed throughout the genome. It is possible that the 1.5 kb fragment cloned contains more than one type of sequence particularly if the repeats are short. If the repeat sequences represented by the pROH 29 clone were long it would be likely that they would contain a pair of internal sites for one of the three restriction enzymes used in this analysis. This would result in at least one intense hybridisation band that should visible above any background smear. Likewise if the clone represents a member of a family of short repeats arranged in tandem arrays there would, most probably, be either a site for one of the three enzymes within the repeat, hence resulting in the appearance of a band, or the enzymes would cut the whole tandem array out as a single unit which would hybridise to the probe near the top of each track. The results obtained here, where only three hexanucleotide-cutting restriction enzymes were used, cannot differentiate between the possibility that the repeats are very short, lacking internal sites for the three enzymes, and dispersed throughout the genome in fairly short tandem arrays of widely differing length, and the situation where they are short repeats which are dispersed singly.

Because no discrete bands are observed in the hybridisation patterns, this probe is not suitable for studying sequence reorganisations in cultured cells or regenerated plants. If time had permitted it could have been used in copy number determinations in both Petunia hybrida callus cultures and regenerated plants.
Clone pROH 25, which was a sequence of approximately 650 bp (data not shown), was one of the clones that showed a very low hybridisation intensity in the colony hybridisation screens and was therefore anticipated to represent a moderately repeated sequence. The finding by Ailsa Shepherd (pers. comm.) that this sequence is present in the ROH genome at about 150 copies is in agreement with this suggestion. When used as a probe in Southern hybridisations with Eco RI, Hind III and Bam HI digested ROH DNA its hybridisation pattern is comparatively simple (Figure 10). In Hind III digests a single intense low molecular weight band is seen along with a number of faint bands in the 2-15 kb region. In Eco RI digests three bands (approximately 3.7, 3.4 & 2.0 kb) are seen all having equal stoichiometries whilst in Bam HI digests two bands (approximately 6.7 & 2.4 kb) are seen. The significance of these bands is unknown but the relatively simple hybridisation pattern obtained suggests that the clone may represent a single type of repetitive element. Further work would be required to determine the precise nature and organisation of this sequence though, without this information, it is still of value as a sequence with which to screen the genomes of cell culture-derived material for genomic rearrangements.

pROH 15

pROH 15 is a cloned sequence of approximately 1.5 kb (data not shown) which Ailsa Shepherd (pers. comm.) has estimated to be present at about 250 copies per ROH genome. The patterns of hybridisation obtained with different enzyme digests when pROH 15 is used as a probe (Figure 11) are more complex than those obtained with pROH 25 and resemble those obtained when the chlorophyll a/b binding protein gene probe (pCAB 146 of Dunsmuir et al., 1983) is used (Figure 12). This gene probe represents one member of a small gene family and gives a range of bands in each
FIGURE 10 Hybridisation of ROH DNA with Repeated DNA Probe pROH 25.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of Petunia hybrida cv. Rose of Heaven and hybridised with DNA from clone pROH 25. The DNA has been digested with Eco RI (left), Hind III (centre) and Bam HI (right).
FIGURE 11 Hybridisation of ROH DNA with Repeated DNA Probe pROH 15.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of *Petunia hybrida* cv. Rose of Heaven and hybridised with DNA from clone pROH 15. The DNA has been digested with (left to right) *Eco* RI, *Hind* III, *Bam* HI, *Alu* I and *Sau* 3A.
pROH 15

Eco RI  Hind III  Bam HI  Alu I  Sau 3A

kb
24
9.5
6.7
4.3
2.25
1.96
FIGURE 12 Hybridisation of ROH DNA with the Petunia Chlorophyll a/b Binding Protein Gene Clone.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of Petunia hybrida cv. Rose of Heaven and hybridised with the clone pCAB 146 (Dunsmuir, et al., 1983). The DNA has been digested with (left to right) Eco RI, Bam HI, Hind III and Pst I.
pCAB 146

kb

24
9.5
6.7
4.3
2.25
1.96

Eco RI  Bam HI  Hind III  Pst I
digest. Some intense bands represent fragments found in all copies of the gene others represent boundary fragments and sequences of only limited homology. The patterns obtained with pROH 15 are more intense than the single copy patterns would be, for the same loading of DNA, indicating the higher copy number of the pROH 15 sequence and it is possible that the sequence cloned in pROH 15 might be one example of a series of related sequences dispersed singly throughout the genome.

**Highly Repeated DNA Sequences.**

The use of specific cloned sequences to study the copy number and organisation of repeated sequences in the nuclear genome is, as indicated above, a powerful technique. It permits an assessment to be made of the relative abundance of that sequence within a particular genome by comparison of Southern blots of that genome with those of other genomes and with those hybridised with different probes of known copy number. The technique is limited however in that it can only provide information about sequences for which cloned probes are available and it is very laborious if a significant proportion of the repeated sequences are to be surveyed.

A simple and rapid assessment of the relative abundances of the major highly repeated DNA sequences in different genomes can be made using a "genome fingerprint" method for which a preliminary study has been made. In this technique a Southern hybridisation is performed using a total nuclear genomic DNA hybridisation probe and nuclear DNA digested with a range of restriction endonucleases. The result obtained for ROH DNA (Figure 13) shows bands, representing highly repeated homogeneous sequences, standing out from a background smear, resulting from a heterogeneous collection of related, dispersed repeats. In the example shown only bands at the bottom of the tracks are visible with a 'doublet' of Alu I repeats being particularly prominent.
FIGURE 13 Hybridisation of ROH DNA with a ROH Nuclear DNA Probe.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from the leaves of Petunia hybrida cv. Rose of Heaven plants and hybridised with $^{32}$P-labelled nuclear DNA from similar plants. The DNA has been digested with (left to right) Eco RI, Hind III, Bam HI, Xba I, Sau 3A and Alu I. The digestion in the Sau 3A track has not been to completion.
Control of the amount of DNA loaded on the gel, the stringency of washing following hybridisation and the length of exposure of the autoradiograph will permit improved resolution of bands further up the lanes.

**Ribosomal RNA Genes.**

When the 8 kb ribosomal RNA gene from wheat (pTA 71, Gerlach & Bedbrook, 1979) was used as a hybridisation probe in Southern hybridisations of *Hind III* digested nuclear DNA obtained from a population of ROH plants, a prominent broad band of approx 9 kb was observed (Figure 14). This band is thought to represent the major repeat unit of the ribosomal RNA genes in ROH and is similar in size to the main repeats reported for other species (Table 13). The pronounced breadth of this band is thought to indicate a range of repeat length heterogeneity similar to that observed in many other species including the Mitchell petunia genome (Waldron et al., 1983). In one particular DNA preparation from a collection of ROH plants the *Hind III* hybridisation pattern appears as two distinct but closely adjacent bands (Figure 15) suggesting two main repeat lengths in equal stoichiometries. This DNA preparation appears to have sampled an atypical subset of the population and was not repeatable. When the same probe is used in Southern hybridisations to nuclear DNA extracted from individual ROH plants (Figure 16) a narrower band is obtained suggesting that the heterogeneity in repeat length observed in the population reflects differences between different individuals rather than different repeat lengths within a single genome such as has been reported in the Mitchell *Petunia* genome (Waldron et al., 1983) and a number of species including pea (Watson et al., 1987). This would agree with the findings of Rogers and Bendich (1987a) who report a high degree of repeat length variability between different individuals of *Vicia faba*. It should be noted, however, that the Southern hybridisations of DNA samples from individual plants may not have given sufficient resolution to differentiate between repeat units.
### 13. Ribosomal RNA Gene Repeat Length in Different Plant Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Repeat Length(s) (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>9.5-10</td>
<td>Gerlach and Bedbrook (1979)</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>9.5</td>
<td>Gerlach and Bedbrook (1979)</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>8</td>
<td>Fredrich, <em>et al.</em> (1979)</td>
</tr>
</tbody>
</table>
FIGURE 14 Hybridisation of ROH DNA with the Ribosomal RNA Gene Clone.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from the leaves of a population of *Petunia hybrida* cv. Rose of Heaven plants and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has been digested with (left) *Eco* RI and (right) *Hind* III.
FIGURE 15 Hybridisation of ROH DNA with the Ribosomal RNA Gene Clone.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from the leaves of an atypical population of Petunia hybrida cv. Rose of Heaven plants and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has been digested with (left) Eco RI and (right) Hind III.
FIGURE 16 Hybridisation of DNA from Individual ROH Plants with the Ribosomal RNA Gene Clone.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from the leaves of a range of individual Petunia hybrida cv. Rose of Heaven plants and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has in each case been digested with Hind III.
5 Individual ROH Plants

Hind III
differing in length by only two hundred base pairs and so it is conceivable that there could be undetected within-plant heterogeneity of rDNA repeat length.

When studying Eco RI digests of DNA from the atypical subset of the population of ROH plants that gave the Hind III 'doublet' when probed with pTA 71, five bands are observed (Figure 15). Two of these are present as a 'doublet' of approximately 2.5 kb. In the Eco RI patterns obtained using DNA samples from single plants only one band of this approximate size was obtained suggesting that this is the region where the repeat unit length heterogeneity arises and hence, presumably, the intergenic spacer region of the repeat. The probe used for all of these rDNA investigations is a sequence derived from wheat (Gerlach and Bedbrook, 1979) and it is unlikely that there should be sufficient homology between the intergenic spacers of the two species for hybridisation to result. The probe is presumably hybridising to gene sequence adjoining the proposed intergenic spacer in these Southern hybridisations.

In Southern hybridisations using the pTA 71 probe on Hind III digests of nuclear DNA obtained from a population of ROH plants a faint band with a mobility lower than that of the main 9 kb band is observed (Figures 14, 20). This band is of approximately 18 kb and may represent dimers of the basic repeat where a Hind III site has been lost. That the bands are not merely indicative of partial digestion of the DNA was illustrated by their persistance when the enzyme concentration used in the digestions was increased from 5 units/μg of DNA to five times that level (Figure 20). When DNA prepared from a single plant is used in the digestions a similar faint band is observed in digestes of DNA from all of the separate plants tested (Figure 16). Perhaps not suprisingly, the relative stoichiometries of the main 9 kb bands and the minor 18 kb bands vary slightly between individuals as the relative copy numbers of the normal and Hind III deficient repeats differ. This is consistent with the presence of a low but variable number of the
Hind III deficient sequences dispersed throughout tandem arrays of normal repeats however it does not exclude the possibility that the dimers are concentrated together in a single tandem array with a copy number that differs in different individuals. Indeed this situation could be the most likely since the frequency of Hind III deficient dimers appears to be rather high (about 1%) suggesting that the mechanisms of unequal crossing over and gene conversion that may maintain the homogeneity of the ribosomal repeats are not acting to remove these variants at the rate at which chance mutations might create them. As discussed later, this suggestion is furthur favoured by the situation observed in the callus regenerant CR,B. The presence of Hind III deficient repeats is in agreement with the findings of Waldron et al. (1983) who cloned repeats both containing and lacking this site from the Mitchell Petunia genome. These workers did not comment on the arrangement of the different repeat types in that genome.

One rDNA variant observed in Hind III digests of DNA from a population of plants (Figure 20), but not visible in DNA samples from any of the individual plants screened (Figure 16), resulted in the appearance of a band of much higher mobility. Such a band could occur if a proportion of the repeats contained an additional Hind III site but this would then generate a furthur band that is not seen. It is possible that this band represents the duplication of a short sequence at the IGS-coding sequence border that duplicates the Hind III site and extends to one side of it since this would not change the lengths of the adjacent repeat units. The duplication would have to contain coding sequence since the probe used is from wheat and it is unlikely that there would be any cross hybridisation between the IGS of this probe and Petunia IGS sequences. Such duplications of IGS and adjacent coding sequence have been recorded in cucumber (Ganal, et al., 1988).
ORGANISATION AND COPY NUMBER OF REPEATED SEQUENCES IN CALLUS REGENERANTS.

ANALYSIS OF REPEATED SEQUENCES IN REGENERANTS.

DNA was isolated from the callus regenerants CR,A-E and subjected to enzyme digestion, electrophoresis, Southern hybridisation and autoradiography in the same manner as the ROH DNA described above for DNA samples from Petunia hybrida plants. Clones that gave hybridisation patterns with a number of clearly defined bands in ROH DNA were used for this study since such patterns would clearly show any differences in mobility or intensity that might result from sequence reorganisation during the callus phase.

pROH 25

No changes could be observed in the mobility or stoichiometry of the hybridisation bands when pROH 25 was used as a probe in Southern hybridisations with DNA samples extracted from CR,A-E (Figure 17). Small changes in copy number would go undetected in this hybridisation but there is no evidence of any changes in copy number of the extent seen by Landsmann & Uhrig (1986) for rDNA repeats. Changes in stoichiometry would have indicated either copy number changes of portions of the repeat family or internal reorganisation of some of the repeats. If this sequence represented a transposable element the result obtained here would not permit any conclusions to be made concerning transposition within the genome. The bands showing strong hybridisation would have to represent fragments internal to the mobile element and these would not be expected to change in mobility as a result of transposition. Sequences around the site of insertion would produce much fainter hybridisation bands and it would be only these that would change with renewed transpositional activity. Consequently all that can be said concerning the pROH 25 type of
FIGURE 17 Hybridisation of DNA from Primary Regenerants CR,A-E with the Repeated DNA Clone pROH 25.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of the primary callus regenerants CR,A-E and hybridised with the moderately repeated sequence clone pROH 25. The DNA has been digested with (left five tracks) Eco RI and (right five tracks) Hind III.
sequences in the callus regenerants is that there have been no major changes in copy number or internal reorganisations of the repeats.

pROH 15

As with the pROH 25 type sequences, no large scale changes in the overall copy numbers of pROH 15 type sequences can be observed in Southern hybridisations using DNA samples from the callus regenerants CR,A-E (Figure 18). Equally, no changes in the relative stoichiometries of different hybridisation bands can be observed suggesting that there has been no internal reorganisation of the vast majority of the repeats. It should be noted that changes in the organisation of a small fraction of the repeats would pass undetected in a screen of this type.

pCAS 13

This clone, a 400 bp fragment of ROH DNA, was a gift from Ailsa Shepherd in the same laboratory who obtained it by a similar screening technique. It was used to screen the callus regenerants since it too had shown potential for the observation of any changes in band mobility or intensity amongst the regenerants. In ROH DNA this sequence is found in approximately 100 copies per genome. This copy number is probably similar to that seen in the callus regenerants and suggests that there has been no major amplification or deletion of this sequence during the culture process. In DNA samples from the callus regenerants CR,A-E a major band and two bands of lower intensity are seen in the Eco RI digests whilst a set of bands of different intensities with a range of sizes are seen in the Hind III digests (Figure 19). As with the other middle repetitive clones, precise interpretation of this banding pattern is not possible without further experimentation. Consequently, the true significance of the observation that there are no major differences in the relative
FIGURE 18 Hybridisation of DNA from Primary Regenerants CR, A-E with the Repeated DNA Clone pROH 15.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of the primary callus regenerants CR, A-E and hybridised with the moderately repeated sequence clone pROH 15. The DNA has been digested with (left five tracks) Eco RI and (right five tracks) Hind III.
FIGURE 19 Hybridisation of DNA from Primary Regenerants CR,A-E with the Repeated DNA Clone pCAS 13.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves of the primary callus regenerants CR,A-E and hybridised with the moderately repeated sequence clone pCAS 13. The DNA has been digested with (left five tracks) Eco RI and (right five tracks) Hind III.
stoichiometries of the bands in digests from the different regenerants, cannot be appreciated. What the result does suggest, however, is that there has been no extensive reorganisation of these repeats in the callus regenerants.

Highly Repeated Sequences

It was hoped to use the "genome fingerprint" method, outlined above, to screen for changes in the organisation or copy number of the major homogeneous repeat families in the genomes of the regenerants CR,A-E. This technique might have shown the enhancement or diminution of the hybridisation intensities of different bands but time did not allow for this experiment and so the true potential of this technique remains unknown.

rDNA

The hybridisation patterns obtained using the pTA 71 ribosomal RNA gene probe on Hind III and Eco RI digested DNA samples from the callus regenerants CR,A, CR,C, CR,D and CR,E were indistinguishable from those of individual ROH plants (data not shown). Thus there appeared to have been no major reorganisation of these sequences in these plants. Similarly in all of the regenerants CR,A-E no large differences in the copy numbers of these sequences were detected (Figure 32). In the majority of callus DNA samples no major differences in the copy number of rDNA sequences were detectable (Figures 27, 28, 29) although Anderson and Smith (pers. comm.) have detected one ROH callus line with a marked reduction in rDNA copy number. This finding shows that changes of the type reported by Landsmann and Uhrig (1986) in potato can occur in this Petunia system.

In the regenerant CR,B, however, whilst rDNA copy number appeared to be normal, the hybridisation pattern produced by the pTA 71 probe with Hind III digested DNA showed the main band to have a
FIGURE 20 Hybridisation of DNA from ROH and CR,B with the Ribosomal RNA Gene Clone.

Autoradiograph of a Southern hybridisation of nuclear DNA isolated from leaves from a population of Petunia hybrida cv. Rose of Heaven plants (left two tracks) and from leaves of the primary callus regenerant CR,B (right two tracks) then hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has been digested with Hind III using enzyme concentrations of both 5 and 25 units / μg DNA.
slightly lower molecular weight than the normal ROH band (Figure 20). Such length variation is not unexpected and may be of the type that generates the broad main band in the hybridisations with DNA from the ROH population (Figure 14). This particular length variant could possibly have been generated in culture and propagated throughout the entire population of rDNA cistrons in a manner similar to that reported by Rode et al (1987b). However it may merely represent a pre-existing length variant carried by the plant used to initiate the culture from which CR,B was derived since Anderson and Smith (pers. comm.) have observed rDNA repeat length variation when examining Hind III-digested DNA isolated from individual ROH plants.

Perhaps more interesting than this main band length variation is the mobility of the minor band of higher molecular weight. This band is larger than the 18 kb Hind III deficient putative dimer observed in all of the other DNA samples examined and could represent a trimer of the main repeat in this plant. Regardless of whether this is the case, no dimers are present in this plant. If the dimers observed in the other DNA samples studied were dispersed throughout the tandem arrays of normal repeats, having arisen by chance mutations eliminating the occasional Hind III site, then similar dimers would be expected in DNA from CR,B. The absence of such dimers suggests that they probably exist clustered in a separate tandem array of their own which in the CR,B plant has been replaced by a tandem array of the larger size, and possibly trimeric, repeat units. This would be a situation not dissimilar to that in pea where short and long repeats are present in discrete tandem arrays (Watson, et al., 1987). Unlike some other species, all of the rDNA genes in Petunia are present at a single chromosomal locus (D. Maizonnier, pers. comm. to S. M. Smith).
3.4) DNA METHYLATION IN *PETUNIA HYBRIDA* PLANTS, CULTURED CELLS AND CALLUS REGENERANTS

GROSS GENOME METHYLATION.

As discussed in the introduction, the CCGG tetranucleotide recognition sequence of *Msp* I and *Hpa* II, which contains both C-G and C-N-G elements, is underrepresented in wheat DNA (Gruenbaum *et al.*, 1981). Additionally, through possession of both the C-G dinucleotide and the C-N-G trinucleotide, the sequence is often methylated with the frequency of methylation of the internal cytosine, in the dinucleotide, being higher than the frequency of methylation of the external cytosine. To confirm that a similar situation is found in *Petunia hybrida*, nuclear DNA from ROH was digested with a range of tetranucleotide cutting enzymes, subject to agarose gel electrophoresis, stained with ethidium bromide and then observed paying particular attention to the size distribution of the DNA fragments resulting from the different digestions (Figure 21). Restriction enzymes that cut a tetranucleotide recognition sequence would, in random sequence DNA, be expected to cleave DNA molecules into pieces with an average length of about 260 bp. *Alu* I and *Sau* 3A cut recognition sequences containing four nucleotides that are not normally underrepresented in eukaryote DNA. Consequently they might be expected to fragment the DNA into pieces with approximately this average length. The result obtained with these enzymes, where *Alu* I and *Sau* 3A do indeed result in fragments with an average size that approaches 260 bp, indicates that ROH nuclear DNA does not have an unusual nucleotide composition. The results of *Msp* I and *Hpa* II digestion indicate that the distribution of cytosine methylation in the ROH genome is also in agreement with published data for plants (Gruenbaum, *et al.*, 1981). Because of the underrepresentation of CCGG sites and because they are often methylated *Hpa* II and *Msp* I cut the DNA into much larger fragments than the other tetranucleotide cutting
FIGURE 21 ROH DNA Digested with a Range of Restriction Endonucleases.

Ethidium bromide stained agarose electrophoresis gel containing DNA isolated from the leaves of *Petunia hybrida* cv. Rose of Heaven plants and digested with the restriction endonucleases (left to right) *Hpa II*, *Msp I*, *Sau 3A*, and *Alu I* all of which cleave a tetranucleotide recognition sequence. The track on the extreme left contains DNA from bacteriophage λ digested with *Hind III*. 
enzymes. ROH DNA must also have a similar proportion of methylated cytosines in C-G and C-N-G sequences to the published data since the average fragment size obtained with Hpa II digestion is greater than that obtained with Msp I digestion.

Much of this cytosine methylation may be concentrated in repeated DNA's and hybridisation of Msp I and Hpa II digested ROH nuclear DNA with a total nuclear DNA probe (Figure 22) showed how many of the major repeated DNA's are insensitive to digestion by these enzymes. The smaller fragments that do hybridise in this experiment, causing the lower molecular weight region of the smear, could result from hybridisation to small dispersed repeats situated amongst regions of unmethylated DNA.

METHYLATION OF SPECIFIC SEQUENCES IN PETUNIA HYBRIDA.

Highly Repeated Sequences.

When pROH 29 was used as a probe in Southern hybridisations of Msp I and Hpa II digested ROH nuclear DNA (Figure 23) a smear of hybridisation was seen at the very top of both tracks in the region representing very high molecular weight fragments. This is consistent with the high incidence of methylation of cytosine residues reported for highly repeated sequences, and presumably reflects their transcriptional inactivity. If, as suggested, these elements are short repeats dispersed throughout the genome they are unlikely to contain CCGG tetranucleotides and hence Msp I or Hpa II recognition sites. The hybridisation patterns obtained using pROH 29 and Msp I and Hpa II digested ROH DNA really monitor the methylation of DNA sequences around the sites of the repeats. That such high molecular weight molecules hybridise will result from a combination of an under-representation of cytosine residues due to transitions accumulated during sequence evolution and a near 100% methylation of both C-G dinucleotides and C-N-G trinucleotides in and around these sequences. Were they to be
FIGURE 22 ROH and Callus DNA Hybridised with ROH Total Nuclear DNA.

Autoradiograph of a Southern hybridisation using nuclear DNA from the leaves of *Petunia hybrida* cv. Rose of Heaven plants (two left-hand tracks) and from callus cells (two right-hand tracks) that has been hybridised with $^{32}$P-labelled nuclear DNA from Rose of Heaven leaves. The left-hand track of each DNA sample is digested with *Hpa* II and the right hand tracks with *Msp* I.
FIGURE 23 ROH Nuclear DNA Hybridised with the pROH 29 Repeated Sequence Clone.

Autoradiograph of a Southern hybridisation using nuclear DNA isolated from the leaves of *Petunia hybrida* cv. Rose of Heaven plants and hybridised to the pROH 29 moderately repeated sequence clone. The DNA has been digested with *Hpa* II (left) and *Msp* I (right).
dispersed through regions containing active genes, the CCGG sites around them would be likely to be undermethylated, and hence Msp I or Hpa II sensitive, resulting in hybridisation to comparatively short fragments in the Southern hybridisation tracks. This is obviously not the case and so it must be concluded that the repeats are either present in tandem arrays lacking both partially and completely unmethylated CCGG tetranucleotides and bounded by similarly highly methylated DNA, which is inconsistent with the Eco RI, Hind III and Bam HI results discussed above, or, more likely, dispersed singly throughout regions of methylated DNA.

Moderately Repetitive Sequences.

No data is available concerning the methylation of pROH 25 and pCAS 13 sequences. When the pROH 15 probe is used in Southern hybridisations with Msp I and Hpa II digested ROH nuclear DNA (Figure 24) a pattern is found that is very similar to that obtained with the highly repeated DNA probe. All hybridisation is to molecules of very high molecular weight. It seems likely, therefore, that, as in the case of pROH 29, these repeats are dispersed through highly methylated regions of the genome rather than in close proximity to active genes. Thus, even if there are undetected amplifications or deletions of some of the copies of this sequence class they are unlikely to have a pronounced effect on gene expression and hence may not contribute to the appearance of somaclonal variation.

rDNA.

When ROH nuclear DNA was extracted from leaves, digested with Msp I and Hpa II and subjected to Southern hybridisation with the pTA 71 ribosomal gene probe a large number of fragments were found to hybridise (Figure 25). This results from cleavage by these enzymes at a large number of CCGG sites in the ribosomal gene tandem arrays. Most of the bands can be identified in both Msp I and Hpa
FIGURE 24 ROH Nuclear DNA Hybridised with the pROH 15 Repeated Sequence Clone.

Autoradiograph of a Southern hybridisation using nuclear DNA isolated from the leaves of *Petunia hybrida* cv. Rose of Heaven plants and hybridised with the moderately repeated sequence clone pROH 15. The DNA has been digested with *Msp* I (left) and *Hpa* II (right).
pROH 15

Msp I  Hpa II

24  9.5  6.7  4.3  2.25  1.96
FIGURE 25 Nuclear DNA Hybridised with the Ribosomal RNA Gene Clone.

Autoradiograph of a Southern hybridisation using nuclear DNA isolated from the leaves of Petunia hybrida cv. Rose of Heaven plants and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has been digested with Hpa II (left) and Msp I (right).
II tracks showing that the sites that are in some instances unmethylated at the internal C-G of the recognition sequence are in other repeats unmethylated at the external C, or at both C's. In the \textit{Hpa} II digests, however, most of the hybridisation is localised in fragments of larger sizes whilst in \textit{Msp} I digests the hybridisation is distributed throughout smaller fragments. This must indicate a higher prevalence of C-G methylation over C-N-G methylation in these sequences. That some fragments in both digests exceed 9kb suggests that some repeats have all of their CCGG sequences methylated. Whilst the frequent presence of a 9 kb band in \textit{Hpa} II digests of many DNA samples may indicate that a single hypomethylated \textit{Hpa} II site exists in the \textit{Petunia} repeat as has been found in the ribosomal genes of pea (Watson \textit{et al.}, 1987).

The precise distribution of hybridisation between the ribosomal bands of different sizes was seen to vary slightly in different leaf DNA preparations (Figures 25, 27, 28, 29) suggesting changes in the overall degree of methylation of these sequences. These differences may reflect differences in the general physiological state of the plants used for the DNA extractions. The age or the precise growth conditions under which the plants had been raised may have an influence on the level of ribosomal gene expression in the leaves and hence on their degree of methylation. To establish whether leaf age had an effect on ribosomal gene methylation leaves of different ages were removed from a batch of ROH plants and DNA extracted from them. No differences could be observed in the distribution of hybridisation between the different ribosomal DNA bands in \textit{Msp} I and \textit{Hpa} II digests of DNA from leaf numbers 1-3, 5-7, and 9-11 ,numbering expanded leaves consecutively from the apex to the base of the plant, (Figure 26). This would suggest that differences in leaf age are unlikely to account for the slight differences in digestion pattern recorded for different bulk leaf DNA preparations. It would therefore appear that environmental differences between the different plant batches are
FIGURE 26 Ribosomal RNA Gene Methylation in Different Parts of the Plant.

Autoradiograph of a Southern hybridisation using DNA isolated from different leaves of *Petunia hybrida* cv. Rose of Heaven and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA has been digested with *Hpa* II (three left-hand tracks) and *Msp* I (three right-hand tracks). The DNA samples were isolated from leaves numbered 1-3 (left track of each digestion), 5-7 (centre track of each digestion) and 9-11 (right track of each digestion), numbering being of expanded leaves from the apex to the base of the plant.
Hpa II  pTA 71  Msp I

<table>
<thead>
<tr>
<th>LEAF NUMBERS</th>
<th>1-3</th>
<th>5-7</th>
<th>9-11</th>
</tr>
</thead>
</table>

- 24
- 9.5
- 6.7
- 4.3
- 2.25
- 1.96

kb
the most probable cause of the phenomenon however it is possible that the variation in repeat length of the rDNA that has been observed (Figure 20) may be affecting methylation.

Unique Sequence Methylation.

It was hoped to study the patterns of 5-MC distribution around protein coding genes, such as the chlorophyll a/b binding protein gene (Figure 12), in normal ROH plants through the use of Msp I and Hpa II. This would have provided information concerning the extent of methylation of these sequences in the normal plant situation which would have provided a comparison for similar analysis in callus and regenerant-derived DNA samples. Unfortunately due to problems with the Southern hybridisation technique and constraints on time this was not possible.

METHYLATION OF SPECIFIC SEQUENCES IN CULTURED CELLS AND REGENERATED PLANTS.

Highly Repeated DNA.

The massive changes in the extent of cytosine methylation observed in the progeny of different callus regenerants by Brown and Lorz (1986) could probably only occur if the extent of methylation of highly repeated sequences was to change. Whilst this change to highly repeated sequences may well have no direct effect on phenotype, since the highly repeated sequences are often present in heterochromatic blocks well away from any potentially active genes, changes in methylation of this extent are likely to affect transcribed sequences and hence gene expression. The technique of using a total nuclear DNA probe to determine the extent of highly repeated sequence methylation failed to show any significant difference between DNA from leaves and from a callus culture (Figure 22). This does not indicate that such differences never occur in this Petunia system since Brown and Lorz did obtain
plants from callus cultures where the extent of methylation was normal. Hence, it is possible that, were the technique to be applied to a range of DNA samples from different callus preparations, differences may be observed. The technique would not be sufficiently sensitive to show small overall changes in the extent of methylation to highly repeated sequences or specific changes affecting only a few of the repeated DNA families in the genome and so nothing can be concluded about these possibilities.

*rDNA*

Early observations of the hybridisation patterns showed a striking difference between the distribution of hybridisation of the pTA 71 probe to *Hpa* II and *Msp* I digests of DNA from leaf and callus sources. DNA extracted from callus that had been in culture for several months (>6) but which had not been subcultured for more than six weeks showed a distribution of hybridisation centred on the larger fragments in both *Hpa* II and *Msp* I digests (Figure 27). This indicates a much greater proportion of methylated CCGG sites in the callus than in leaf DNA. Indeed, in the callus many of the repeats appear to be methylated at all of their CCGG sites. Additionally, the proportion of methylated C-N-G trinucleotides is approaching the proportion of methylated CG dinucleotides, perhaps not surprising as both tend towards 100% methylation. However, in the face of this massive increase in the overall methylation of the ribosomal gene sequences in this callus DNA preparation, one feature stood out that has subsequently been shown to be a extremely common feature in callus DNA samples. A pair of small, similar sized fragments was clearly observed in both the *Hpa* II and *Msp* I lanes of the callus DNA digests whilst the same pair was only prominent in the *Msp* I track of leaf DNA digests. Thus, in the callus some specific CCGG sequences appear to become less methylated at the internal C-G than in leaf tissue.
FIGURE 27 Ribosomal Gene Methylation in Leaf and Callus DNA Samples.

Autoradiograph of a Southern hybridisation using DNA isolated from Petunia hybrida cv. Rose of Heaven leaves (two left-hand tracks) and from a ROH callus culture that had not been subject to regular subculture (two right-hand tracks), hybridisation being with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples were digested with Hpa II (left-hand track of each sample) and Msp I (right-hand track of each sample).
When the distribution of hybridisation to $Msp$ I and $Hpa$ II digested DNA from a well established (> 6 months) and regularly subcultured (once every four weeks) callus sample was studied, the contrast to the previous observation was very marked (Figure 28). Once again the small $Hpa$ II derived bands were prominent in the callus DNA sample but much less distinct in the leaf DNA sample and once again there was little difference between the distribution of hybridisation in $Hpa$ II and $Msp$ I tracks. In this sample, however, the centres of the distributions were of much lower molecular weight. The ribosomal RNA genes in the callus DNA had many fewer methylated CCGG sequences than the leaf DNA. Once again some of the ribosomal RNA gene repeats had no unmethylated CCGG sequences but a high proportion of the repeats had unmethylated sites. Viewed alongside the first result (Figure 27) the differences in rDNA methylation are striking. Differences in the growth conditions of the two callus samples were considered to be a possible cause of these methylation differences with actively growing callus having less methylation, and presumably more transcription of the ribosomal RNA genes, than callus that was either in a stationary phase or growing only slowly.

A third result eliminated this suggestion (Figure 29). Once again DNA was extracted from a well established and regularly subcultured line and once again the prominent small $Hpa$ II fragments were seen but in this case the overall level of ribosomal gene methylation was higher in the callus DNA than in leaf DNA. Once again the proportion of $Msp$ I insensitive sites approached the proportion of $Hpa$ II insensitive sites. There was a greatly increased number of methylated CCGG sequences and whilst the level of ribosomal gene methylation was perhaps not quite as high as in the first callus sample the difference was not great.

To investigate further the suggestion that ribosomal gene methylation in callus DNA correlates with callus growth state a more controlled experiment was performed. Leaf discs, excised from
FIGURE 28 Ribosomal Gene Methylation in Leaf and Callus DNA Samples.

Autoradiograph of a Southern hybridisation using DNA isolated from the leaves of *Petunia hybrida* cv. Rose of Heaven plants (two left-hand tracks) and from a ROH callus culture that had been subject to regular subculture (two right-hand tracks), hybridisation being with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples have been digested with *Hpa* II (left-hand track of each sample) and *Msp* I (right-hand track of each sample).
FIGURE 29 Ribosomal Gene Methylation in Leaf and Callus DNA Samples.

Autoradiograph of a Southern hybridisation using DNA isolated from the leaves of Petunia hybrida cv. Rose of Heaven plants (two left-hand tracks) and from ROH callus that had been subject to regular subculture (two right-hand tracks), hybridisation being with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples have been digested with Msp I (left-hand track of each sample) and Hpa II (right-hand track of each sample).
a batch of young ROH plants of identical age and raised under the same conditions, were used to initiate a large number of callus cultures on 204 medium. The resultant plates were grown under identical conditions and callus was harvested by excision from the margins of the leaf pieces on a proportion of the plates once a week from the end of week one to the end of week eight. After week four the leaf pieces and associated calli were transferred to fresh medium in the manner of a normal subculture. DNA was isolated from the callus samples and digested with Msp I and Hpa II prior to Southern hybridisation using the pTA 71 probe.

Although problems with the Southern hybridisation technique resulted in very poor autoradiographs it is still possible to see that the distribution of hybridisation amongst the different fragments in a given track is indistinguishable from the distribution in any other track (Figure 30). There would appear to be no changes in the level of methylation over the time of the experiment when the growth state of the callus might well be expected to have changed. In all cases the ribosomal genes of the callus DNA samples show a high proportion of methylated CCGG sites with both Msp I and Hpa II sites showing slightly higher levels of insensitivity to digestion than would be expected in leaf DNA.

If the timepoint of a callus in the monthly subculture cycle does not affect the level of ribosomal gene methylation, yet the degree of methylation in different callus samples can vary, perhaps some other physiological difference can bring about changes in the extent of rDNA methylation. To examine whether the different physiological states that must exist between normal callus and that moving towards regeneration can affect ribosomal gene methylation a longer term study was performed. Callus was initiated on 204 medium from leaf discs from a single plant of the same batch and age as the plants used to initiate the callus cultures in the previous experiment. This callus was subcultured at monthly intervals and multiplied for 6 weeks when a sample of
FIGURE 30 Ribosomal Gene Methylation During the Callus Subculture Cycle.

Autoradiographs of Southern hybridisations using DNA samples isolated from different time points throughout two callus subculture cycles and hybridised with the ribosomal RNA gene clone pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples in the upper autoradiograph have been digested with Hpa II whilst those in the lower autoradiograph have been digested with Msp I. The callus was initiated from a range of Petunia hybrida cv. Rose of Heaven plants onto a series of plates containing medium 204. At weekly intervals, from the end of the first week to the end of the eighth week, a proportion of the plates were harvested for DNA isolation. At the end of the fourth week callus on all of the remaining plates was subcultured onto fresh 204 medium. The DNA samples represented on each autoradiograph were harvested (left to right) at the end of the first - eighth weeks.
callus was used to prepare DNA. After two months one half of the callus was subcultured onto 204 medium and the other half was transferred to JH regeneration medium where it had died within a month. After 10 weeks a further callus DNA preparation was made from the callus grown on 204 medium and after three months half of the callus stock was transferred to JHA medium where it was seen to proliferate as a dark green, dense callus of the type that may give rise to regenerants. Calli on both JHA and 204 media were maintained by monthly subculture until 6 months had elapsed with harvesting of monthly samples, for DNA preparation, from each medium in the middle of the subculture cycle. After 6 months no further subculturing was performed though the calli were maintained on the same plates until 10 months when they were harvested for DNA preparations.

Msp I and Hpa II digests of callus from the different media at the different timepoints were used in Southern hybridisations with the pTA 71 probe to assess the distribution of hybridisation in the different lanes (Figure 31). Although problems with the Southern hybridisation procedure have resulted in poor autoradiographs it is still possible to compare the results in the different tracks. No differences can be detected in the distribution of hybridisation between the ribosomal gene bands in the different DNA preparations. It would appear that in all cases the ribosomal genes have a high proportion of unmethylated CCGG sequences though a few repeats still have all of their sites Msp I and Hpa II insensitive. With such a low level of methylation there appears to be little difference in the proportions of methylated C-G dinucleotides and C-N-G trinucleotides in the ribosomal genes.

This result suggests that the physiological state of the callus does not affect the degree of ribosomal gene methylation. Equally it suggests that the period of time that a callus line has been in culture does not affect the extent of ribosomal gene methylation. Finally it implies that it is immaterial, as far as the extent of
FIGURE 31 Ribosomal Gene Methylation During Prolonged Callus Culture.

Autoradiographs of Southern hybridisations of DNA samples harvested from a range of *Petunia hybrida* cv. Rose of Heaven callus cultures and hybridised with the ribosomal RNA gene probe pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples have been digested with *Msp I* (upper autoradiograph) and *Hpa II* (lower autoradiograph). Callus cultures were initiated from the leaves of a single plant onto a series of plates of 204 medium. Callus from these plates was subcultured onto fresh 204 medium at monthly intervals until six months had passed when it was left, without further subculture, until ten months after the date of callus initiation. After 3 months half of the callus was transferred from 204 medium to JHA medium and then treated in exactly the same way as that remaining on 204 medium. DNA was harvested at timepoints in the middle of each of the second, third, fourth, fifth and sixth months and at the end of the tenth month (left to right on each autoradiograph). DNA obtained from callus harvested from plates of 204 medium during the fourth, fifth, sixth and tenth months is indicated by the letter C. DNA obtained from callus harvested from plates of JHA medium at the same times is indicated by the letter R. During the second and third months DNA was harvested only from plates of 204 medium.
ribosomal gene methylation is concerned, whether a callus is actively growing or approaching senescense which must have been the case in the callus that has not been subcultured for four months.

The great difference in the distribution of hybridisation between the two experiments just discussed (Figures 30, 31) is striking. In the first the ribosomal genes were very extensively methylated. In the second very few of the CCGG sequences were methylated. The difference is as pronounced as that shown by the first two experiments yet in this case the plant used to initiate the callus for the experiment using regeneration medium was of the same age and the same batch as the plants used to initiate the calli for the eight week subculture cycle study. Additionally the calli were initiated on the same batch of medium on two consecutive days and cultured simultaneously in the same growth room. If the batch of plants used to initiate the eight week subculture cycle study were sufficiently uniform to give rise to cultures that gave such a consistant methylation pattern why should the one plant from the same batch give rise to callus with such a different ribosomal gene methylation pattern? It is not inconcievable that by chance the one plant used to initiate the callus for the longer term experiment could be genetically different, although viewed in the light of the initial observations of callus DNA which showed the similar result of widely divergent callus rDNA methylation patterns, this would seem unlikely. Rather it would appear that something that happens at the time of callus initiation, perhaps the precise time between excision of the leaves and initiation of the culture, fixes a ribosomal gene methylation pattern on that callus which it retains throughout the culture phase.

The overall distribution of hybridisation to \textit{Msp} I and \textit{Hpa} II digests of DNA from leaves of the five callus regenerants is remarkable by its similarity in all cases although the relative intensities of all the individual bands in DNA from CR, A and CR, B
FIGURE 32 Ribosomal DNA Methylation in Regenerated Plants.

Autoradiograph of a Southern hybridisation of DNA isolated from the leaves of primary callus regenerants CR,A-D (left to right of each digest type) and hybridised with the ribosomal RNA gene probe pTA 71 (Gerlach and Bedbrook, 1979). The DNA samples have been digested with Msp I (four left-hand tracks) and Hpa II (four right-hand tracks).
plants is not the same (Figure 32). These small differences may represent changes in the IGS region (CR,B is known to have a shorter repeat length), which may or may not include CCGG sites, or differences in the susceptibility of a particular CCGG site to methylation. Differences in the susceptibility of a site to methylation would not be unexpected since something possibly related to the DNA conformation or protein binding around the prominent Hpa II site in the IGS region of the rDNA repeats in pea means that it behaves differently from other CCGG sequences in the same repeat with respect to methylation (Watson, et al., 1987). These small differences in individual bands could have arisen during the culture phase but equally they could represent pre-existing differences amongst individuals. The leaf DNA preparations that have been studied before were harvests of DNA from a population of individuals so small differences between individuals would have been masked although they did perhaps contribute to the slight differences in the distribution of hybridisation between different DNA preparations.

The significance of the overall similarity in the distribution of hybridisation amongst the different fragments between regenerants cannot be over-emphasised. The four plants for which there is data on this point were all obtained from callus initiated at different occasions, from different batches of plants grown at different times of year in the greenhouse and hence under different environmental conditions. The callus lines had been in culture for different lengths of time with different subculturing histories and not always on the same medium yet despite all of these differences the ribosomal gene methylation patterns were remarkably similar. At a simplistic level it would be easy to suggest that the pattern seen in leaves is a leaf-specific methylation pattern and that a randomly fixed methylation pattern seen in callus reverts to the normal leaf pattern upon regeneration. Such an explanation takes little account of the
function of methylation and also overlooks a number of other important points.

One thing that the regenerants CR,A-E have in common is that they had all been regenerated; they were all derived from callus capable of regeneration. The callus in the long term regeneration medium experiment that had very under-methylated ribosomal genes proved incapable of regeneration. The regeneration capability of the callus with highly methylated DNA is unknown. It may be that only callus with a normal, leaf type ribosomal gene methylation pattern is capable of regeneration. The information necessary to conclude on this point is not available though some form of test could be devised.

The results here suggest a number of explanations for differences in rDNA methylation and these shall be discussed in the general discussion below. Whatever the causes, the effects of methylation differences may have important implications for somaclonal variation.

Other Sequences.

Since there is evidence that in plants cytosine methylation may influence gene activity (Hepburn, et al., 1987), it was hoped to study the methylation of single copy sequences in DNA from callus and callus regenerants to see if changes occur that may be sufficient to influence expression. Due to problems with the Southern hybridisation technique, that resulted in its sensitivity being greatly reduced, no success was had when attempting this important experiment which would have provided for an interesting comparison with the results obtained with rDNA. Likewise it was hoped to study the methylation of 5S ribosomal genes to confirm the findings of Quemada, et al. (1987) who found changes to the methylation of these sequences on culture induction in soybean. With this Petunia system no results were obtainable because Msp I
and \textit{Hpa} II digests of ROH DNA subject to Southern hybridisation with a 5S ribosomal gene clone showed hybridisation in a dense band at the top of the autoradiograph (results not shown). This must result from either the 5S genes of \textit{Petunia} lacking CCGG sites or through the complete methylation of all such sequences in these genes. Cloning and sequencing of the genes would be necessary to differentiate between these alternative explanations.

3.5) \textbf{GENERAL DISCUSSION.}

\textbf{REORGANISATION OF RIBOSOMAL RNA GENES.}

The observation of differences in the organisation of ribosomal RNA gene sequences in the callus regenerant CR,B would appear to be consistent with the highly variable nature of these sequences. It has often been reported that rDNA sequences have been subject to rearrangement or copy number changes as a result of cell culture. The results of the studies with CR,B may also provide information concerning the processes by which rDNA sequences can be changed.

\textbf{Copy Number.}

Ribosomal RNA genes are found in very high numbers in plants when compared to other eukaryotes (Gerlach and Bedbrook, 1979) and the number of copies per genome shows a high degree of variation between different species (Sorensen, 1984). In animal systems there is reported to be a marked homogeneity of the rDNA repeat unit within species contrasting with pronounced variation between species (Tautz \textit{et al.}, 1987) and suggestive of concerted evolution occurring through recombinational processes (Gerbi, 1985). In plants the situation would appear to be much more complex.

The results presented here suggest no extreme differences in the copy number of rDNA repeats between different \textit{Petunia} individuals.
Whilst the Southern hybridisation methods used would be too insensitive to show up small copy number differences they should have shown up any differences of the type seen between different individuals of barley by (Zhang, et al., cited by Rogers and Bendich, 1987a). These workers found a six fold variation in rDNA content between individuals whilst Rogers and Bendich (1987b) showed a 95 fold variation between individual plants of *Vicia faba*. Lesser differences in rDNA copy number between individuals have also been reported by Cullis (1981) and it is possible that changes of a similar magnitude may be occurring in the *Petunia* system. Whilst all of these differences may have little functional effect, due to the apparent redundancy of most plant rDNA repeats, they do serve to indicate the ability of these sequences to be amplified and deleted from the genome under normal circumstances.

In addition to rDNA copy number differences between normal sexual progeny, the literature contains ample evidence of quantitative rDNA changes following cell culture (Brettell, et al., 1986b, Landsman and Uhrig, 1986, Blundy, et al., 1987). Presumably the mechanisms that operate under normal growth circumstances also operate in culture although the great magnitude of the changes shown in the work of Brettell et al., where virtually all of the rDNA repeats at a single nucleolar organiser were lost, suggests that they may be more extreme in their effect in cell culture. The observation by Anderson and Smith (pers. comm.) of a reduction in rDNA copy number in one Petunia callus confirms that this species must have molecular mechanisms that can cause rDNA deletions.

Repeat Length.

Considering the organisation of rDNA, the results presented here would tend to suggest that there is a general homogeneity of rDNA repeat unit length within individuals but that there can be quite pronounced variations between individuals. This would tend to agree with the published information concerning animal cells and
with several other plant systems where only a very limited number of length variants are found in an individual (Waldron, et al., 1983, Ganal and Hemleben, 1986, Jorgensen, et al., 1987). This observation, however, contrasts with the situation in *Vicia faba* where there are differences in repeat length between adjacent rDNA units in the same tandem array.

**Mechanisms of Repeat Length Change.**

When considering coding sequence and intergenic spacer (IGS) sequence evolution in animal systems (Tautz, et al, 1987), the concerted evolution shown by the repeats is suggested to arise by a gradual process whereby novel repeat units generated by mutation are propagated through the population of rDNA repeats by processes of unequal crossing over and gene conversion occurring during both meiotic and mitotic recombination. Theoretical work involving the use of computer simulations has shown that repeated rounds of unequal crossing over alone can lead to all repeat units having the same altered sequence (Ohta, 1980). Considering animal cells with 200 rDNA repeats and a rate of unequal exchange of $3 \times 10^{-4}$ per generation, in the absence of selection one variant could be spread to all copies of the rDNA cluster in $10^7$ generations (Dover, et al., 1982). Gene conversion, either by mismatch repair within a heteroduplex formed during recombination (Holliday, 1964) or by some other mechanism (Szostak, et al., 1983), may speed the process. So too should selection, though negative selection is reported to be more frequent in this situation than positive selection (Kimura, 1983). One consequence of this gradual change in the repeat populations would be that intermediates would be seen. When studying sequence microheterogeneity arising from point mutations, intermediates in this process can be observed in both plant and animal systems (Tautz, et al., 1987, Ganal and Hemleben, 1986).
In contrast, the repeat unit length homogeneity observed within but not between individuals in both animals and a number of plants, including this Petunia system, is not easily explained in the same terms due to an apparent absence of intermediates. Whilst the within plant heterogeneity of repeat unit length shown by Vicia could be explained, simplistically, as an intermediate stage in a gradual process of concerted evolution in repeat size, why is no similar heterogeneity observed amongst the other species examined where repeat unit length is known to change?

The repeat unit length differences that have been recorded in both animal and plant systems occur primarily through changes in the numbers of subrepeats in the IGS region of the repeat unit (Lassner and Dvorak, 1986). Whilst the sequences of different subrepeats have diverged between and within species (Jorgensen, et al., 1987) their presence is apparently universal and it is assumed that they play some role in the control of transcription. Indeed there is evidence that, in wheat, rDNA units with higher numbers of subrepeats are transcribed at a higher rate than repeats containing fewer subrepeat sequences. Although there is a suggestion that these sequences might have arisen from transposable sequences (Rogers and Bendich, 1987a), there seems little dispute that the changes in copy numbers of these subrepeats within the IGS region occurs through unequal crossing over during recombination at the time of either meiosis or mitosis (Rogers, et al., 1986). Such unequal cross-overs could also generate some of the observed repeat unit copy number variation (Gerbi, 1985). Whilst it may be the case that in Vicia it is possible to observe the propagation of novel length variants, resulting from unequal crossing over, through the repeat population, the same cannot be said of this Petunia or many other systems.

Rode et al. (1987b) have detected changed repeat unit lengths in the rDNA of anther-derived dihaploids of spring wheat. In a single
generation a 4.4 kb fragment from the rDNA family was seen to be replaced by a 4.55 kb fragment. If this change had occurred through unequal crossing over a 4.25 kb fragment would have been generated at the same time. Consequently an explanation of the cause that invoked merely recombinational processes would seem unreasonable since both the 4.4 kb and the 4.25 kb repeat fragments would have to be eliminated and replaced by the 4.55 kb fragment in a very short timespan. The authors have proposed that the rearrangement must have occurred by an unequal crossing over event followed by the deletion of all but the altered repeat unit which was then amplified to generate the new repeat family. Such an occurrence results in the propagation of a new 'master' repeat throughout the repeat unit population, a situation reminiscent of the master-slave proposal of Callan (1967) who, ambitiously, predicted that a similar correction event may occur every cell generation to maintain the homogeneity of all multigene families and other repeated sequences. The suggestion of deletion/amplification events affecting rDNA units is of interest when considering the situation found in in the callus primary regenerant CRB.

Regardless of whether the rDNA changes seen in CRB arose during culture or whether they arose during normal sexual propagation, the result obtained with this plant appears to be significant in the context of rDNA rearrangement processes. No intermediates in the formation of the novel proposed trimeric repeats were seen in any of the numerous plants examined during the course of this work. The result may be explained in terms of unequal crossing over amongst the IGS regions of the dimeric repeats generating a trimeric repeat. Deletion of all other dimeric repeat units would be followed by amplification of the trimeric 'master repeat'. The monomeric repeats also in this nucleus must have remained unaffected by this process suggesting two distinct populations with two distinct master repeats. In species with more than one nucleolar organiser region it is possible that there might be only
one master repeat per nucleolar organiser. In the *Triticale* somaclonal variant of Brettell et al. (1986b) the disappearance of almost all the rDNA repeats at one nucleolar organiser could be considered as an intermediate in this process. Since Petunia has only one nucleolar organiser region it is perhaps advantageous to have two repeat families controlled by different master copies at that site.

It would be most obvious to envisage these two repeat families existing in two distinct but adjacent tandem arrays otherwise the process of deletion that eliminated the dimeric repeats would have to be extremely complex. There is no evidence for this, however, and complex molecular mechanisms could exist. Equally, the same arrangement would not necessarily be found in all species and it is possible that the *Vicia* situation could represent the interspersion of repeats generated by a number of distinct master copies at the single nucleolar organiser locus in that species. It is not easy to suggest how this interspersion would arise but given that the whole area is one of unknown mechanisms this should not be seen as a barrier.

Whilst the suggestion by Rode et al. (1987b) of this mechanism, for the propagation of novel repeat unit length variants through the tandem arrays of the ribosomal RNA genes, would appear to agree with the results obtained in this study, it fails to consider the observed point mutation heterogeneity seen amongst repeats in an individual. This is obviously an area that requires further attention. It needs to be established if such sequence heterogeneity is seen in species where repeat length changes are frequently observed. In *Cucumis sativus* Ganal and Hemleben (1986) have observed sequence differences between repeats as well as length variation. It is not clear from their work, however, if the sequence variation occurs within units of a given length or between them. It is possible that the sequence heterogeneity occurs between three 'master' repeats and that all repeats of a
given length would be homogeneous with respect to sequence. This would fit easily with the proposed model. Equally, however, the sequence heterogeneity could occur within a size class. This could still fit with the proposed model if unequal crossovers were rare amongst the rDNA IGS subrepeats in this species and if deletion/amplification events were also infrequent since the point mutations could have arisen after the last repeat length change occurred. The differences seen between species could arise due to different periodicities of action of the various molecular changes that are proposed to occur. *Vicia*, for example, may have a high frequency of recombinational mismatches combined with a very low frequency of deletion/amplification events.

Further Investigation of rDNA Changes.

To determine precisely what is causing the rDNA homogeneities and heterogeneities that are seen further experimentation is required. Much of the published work, and indeed the CRB result, is devalued through the unknown relationship between different individuals examined. A cell culture system using material derived from a single plant would perhaps be a good system with which to study this phenomenon. However, if the rather ambiguous statement of Walbot (1985) concerning the *Solidago* system of Schaal means what it appears to, an even better system may be available. Changes in the copy number and, apparently, organisation of the rDNA at the different branch meristems of this long-lived perennial plant appear to have occurred. It would be interesting to know if the organisational changes were gradual, as would be expected if they were propagated by unequal cross-overs, or of the more dramatic type reported for the wheat gametoclone (Rode, et al., 1987b). When a system can be found that can produce these dramatic appearances of new length variants in plants with a precisely established relationship, it must then be established whether there is sequence variation amongst the repeats. If there is not
the proposed model may still remain valid however a more radical reappraisal of the situation may yet be required.

REPEATED SEQUENCE REORGANISATION.

The result that there were no major changes in the organisation or copy number of the cloned repeated sequences studied in the regenerated plants is of comparatively minor significance. Given the size and copy number of the repeats, the proportion of the genome screened by them was very limited. Additionally, this screening would only have detected major changes. Whilst these statements would appear to suggest that there was little value in a screen of this type, comparison with the situation seen in rDNA might suggest otherwise. Equally, the findings of other workers concerning rDNA might suggest that the 'genomic fingerprint' technique discussed above could be of value.

Whilst rDNA sequences are found in tandem arrays and dramatic changes of the type reported by Rode et al. (1987b) may only occur in a single array, the rapid change of repeated sequences is an area where little is known. Cullis and Cleary (1986) have used cloned repeats to study copy number changes in cell cultures and regenerated plants. All but one of the repeats that they screened was found to vary in copy number. Such variation could arise either through deletion of some copies, amplification of some copies or a combination of the two. It is unclear whether the sequences exist as dispersed single repeats, dispersed tandem arrays or in single tandem arrays but the extent of the changes is such that more than a single copy is affected. It is possible that all of the variant sequences have some tandem arrays which may be deleted or amplified to give changes of the magnitude seen and that the reason for the invariant repeat being constant in copy number was that it lacked these arrays. Equally, it is possible that there is some mechanism that results in the elimination of single copies of a dispersed repeat.
DNA amplification of portions of the genome appears to be an extremely prevalent phenomenon in eukaryotes (Bostock and Tyler-Smith, 1982, Shah, et al., 1986) and the finding, by Van't Hoff et al. (1987), that different parts of the genome are replicated at different times during the S phase of the cell division cycle shows how the phenomenon could quite easily arise. There is a suggestion that sequence amplification is often sequence specific (Kato and Tanifuji, 1986) although this could arise due to the tandem organisation of these sequences. What appears to be unknown is whether specific mechanisms can cause dispersed repeats to be amplified or under-replicated in a concerted fashion. The dedifferentiation and differentiation associated with passage through a callus phase seem particularly strong inducers of the early replication, and hence possible amplification, of specific portions of the genome (Natali, et al., 1986, Hendre, et al., 1986) making tissue culture systems ideal for studying such reorganisations. Consequently, a study of any quantitative changes in dispersed repeated sequences within such a system would be of value.

CHANGES IN DNA METHYLATION IN CULTURED CELLS.

The observations concerning methylation of the ribosomal RNA genes in callus cultures are, potentially, extremely important and can only furthur our knowledge of the effect of cell culture on methylation patterns. Results on intact plants (Watson, et al., 1987) and crown gall tumours (Blundy, et al., 1987) imply that cytosine methylation may play some role in the control of ribosomal RNA gene expression. A reduction in the extent of methylation of these sequences correlates with the developmentally regulated increase in rDNA transcription. Additionally, in a comparison between a flax genotroph and a crown gall tumour with different rDNA copy numbers, a similar number of rDNA cistrons with a specific hypomethylated CCGG site were observed and this was taken to indicate a similar number of active genes. Because
nothing is known of the position of CCGG sequences in the *Petunia hybrida* rDNA repeat unit; the results presented here cannot be interpreted in terms of the frequency of methylation at a specific site and so no particular hypomethylated sites can be proposed. The overall level of methylation can be measured, however, and the results obtained raise further questions.

**Methylation Patterns and Transcription.**

Interpretation of the results obtained in this experiment in the light of published results suggests a number of possible explanations for the differing levels of rDNA methylation found in DNA preparations from leaf and callus material. One explanation of the results obtained could be that the methylation differences between the leaves and the different callus preparations could represent different levels of rDNA expression. No measurements were made of the relative growth rates in the different callus preparations and so the suggestion that the calli with undermethylated rDNA sequences might be growing faster than those with the same sequences extensively methylated cannot be eliminated. Perhaps, in the comparatively uniform conditions of the greenhouse, the ribosomal RNA gene activity of regenerant and normal ROH plants are similar resulting in a similar degree of rDNA methylation. It is hard to believe, however, that the ribosomal gene activity of a callus after four months without subculture is the same as that of recently initiated callus. Equally it is hard to conceive that the level of rDNA expression in expanding leaves should be the same as that in leaves that have been fully expanded for some weeks.

Alternatively, an explanation of the observations made in this study could be that methylation plays no role in the control of rDNA expression in *Petunia*. In rice this may, indeed, be the case since the genes show a consistently low degree of cytosine methylation (Olmedilla, *et al.*, 1984) although there are only
about 850 copies per genome in this plant. It is probably significant that the rDNA sequences in rice are undermethylated rather than heavily methylated since it could well be that undermethylation is merely necessary rather than sufficient for expression. Because high levels of methylation tend only to correlate with unexpressed sequences (Shengeliya, et al., 1986) it would appear that extensive methylation may prevent transcription. If methylation were playing no role in this *Petunia* system it would seem unlikely that the methylation patterns observed in the leaves of regenerated plants would be so similar to those of normal plant leaves whilst callus culture patterns could be so different. Such a situation could arise, however, through regeneration from a small number of cells where the methylation pattern has not been changed as a consequence of culture.

A third possibility is that rDNA expression could be controlled by some factor other than methylation but that changes in methylation resulting from cell culture cycles are imposing an upper limit on the level of rDNA expression that can be achieved. It may be that the level of rDNA methylation seen in *Petunia* leaves results from methylation of all of the ribosomal RNA genes that are never going to be required in the normal whole-plant situation. The superfluous rDNA is, perhaps, methylated whilst the rDNA cistrons that may be expressed, when maximal ribosome synthesis is required, remain unmethylated. The callus with extensive rDNA methylation may be able to synthesise enough rRNA to survive *in vitro* but may not have the capacity for the highest levels of rRNA synthesis that are seen *in vivo*. If this were the case it might not be unexpected if the callus with extensive methylation was found to be incapable of regeneration which would explain why regenerants with extensive rDNA methylation are not found.

If the proposal that methylation is restricted to the superfluous repeats was to be accurate and some other factor is modulating rDNA expression why are rDNA sequences methylated at all? There
would appear to be some advantage to be gained from having unexpressed sequences methylated otherwise the highly repeated sequences, that are unlikely to have the signals for start of transcription, need not be methylated. Unknown though it may be, perhaps the need for methylation is such that calli with undermethylated rDNA sequences are not capable of regeneration and that is why regenerants with undermethylated ribosomal RNA genes are not found.

**Methylation and Regeneration.**

If callus with highly methylated rDNA and callus with the same sequences undermethylated are both incapable of regeneration perhaps it is possible to predict whether a callus has the potential for regeneration by study of its rDNA methylation. In broad terms this may be possible since callus with an intermediate degree of methylation may be highly regenerable. It would be unlikely, however, that the presence of abnormal methylation patterns completely eliminated the possibility of regeneration from a callus. This is because callus is composed of a population of individual cells. When a DNA preparation is made from a callus it contains DNA from a very large number of individual cells. Though the vast majority of cells in a callus may have an abnormal rDNA methylation pattern it is possible that a small proportion of the cells that comprise that callus may have a perfectly normal rDNA methylation pattern. These cells might be the ones that would regenerate.

Because of this problem experimentation in this area is extremely difficult and it is not easy to suggest which experiments should be attempted. It would seem obvious to test whether highly methylated or undermethylated calli were capable of regeneration and then to study the methylation of the regenerants. At a simplistic level, if they were found capable of regeneration and if the regenerants had normal leaf type patterns of rDNA
methylation then it may appear that rDNA methylation patterns merely reflected either the type of "organ" (leaf or callus) containing the repeats or the level of expression in that organ, a suggestion made earlier. Such a conclusion would be invalid since nothing is known of the methylation pattern of the single cell from which the regenerants arose. That is not to say that the experiment is not worthwhile. It could be that callus with highly methylated rDNA gives rise to regenerants with the same sequences highly methylated. Although the observations that the regenerants CRA-E had normal rDNA methylation patterns and that all of the callus preparations tested had abnormal rDNA methylation patterns suggest that there is going to be a tendency for normal methylation patterns amongst regenerants this is far from proven. Until this question is resolved little can be concluded on the effect of rDNA methylation on regeneration.

The Origin of Methylation Changes.

The results of this experiment raise a question concerning the mechanisms by which these methylation changes arise which in turn may have consequences for the methylation of other types of sequence. It would appear that the rDNA methylation pattern of a callus is established at the time of callus initiation and remains fixed after that time (Section 3.4). That explants initiated from similar material at a similar time can have such differing rDNA methylation patterns would appear to imply that the pattern imposed is random. Conversely, however, that all the explants initiated on a single occasion should have the same pattern implies that the pattern that is fixed does not appear at random. Something seems to be ensuring that all callus induced on a particular occasion has the same pattern of rDNA methylation but the pattern on different occasions would appear to be fixed either at random or by some unknown variable factor.
If at the time of callus initiation the expression of the methylase enzymes, responsible for the maintenance of methylation patterns, was impaired yet cell division proceeded, a low level of methylation in all types of sequence would result. If during callus initiation the same modification methylases were over-expressed, because of their suggested preference for hemi-methylated DNA as a substrate, overmethylation would not be expected. It is possible, however, that such an abundance of methylase activity could see a proportion of the enzymes acting on unmethylated DNA resulting in an increase in gross DNA methylation and hence an increase in rDNA methylation. Changes in the level of gross genome methylation have been reported by Brown and Lorz (1986), although only in a preliminary communication. They were not observed, however, in this experiment. Consequently, it may be that a more specific mechanism is involved. Before sensible speculation can be made a survey of the types of sequence affected by methylation changes during callus initiation is required. It was hoped to study the methylation of single copy sequences in this work and this is something that requires urgent attention. An analysis of the methylation of rDNA and other expressed sequences in different callus preparations combined with a quantification of 5-MC levels in the genomes as a whole is a necessary first step in this area.

The Significance of Methylation Changes and Somaclonal Variation.

Until more information is available on the sequence specificity and functional significance of culture-induced methylation changes little can be concluded concerning its potential role in the appearance of somaclonal variation. If, however, methylation differences arising through culture can be shown to affect the expression of ribosomal and other genes in regenerated plants a major breakthrough in the understanding of somaclonal variation would have occurred. With the long term view of controlling variation in mind, further work on the activity and specificity of
the enzymes responsible for causing methylation differences would be invaluable.

THE TIMING OF GENETIC CHANGES IN CALLUS CULTURES.

Variation in Long Term Cultures.

The results of the two studies of phenotypic variation between callus regenerants suggest two different aspects of the timing of the genetic changes responsible for the phenomenon of somaclonal variation. The experiment with callus regenerants CRA-E showed the effect of long-term culture on regenerants and their phenotypes. The first thing that was apparent concerning regeneration from these cultures, that had been growing for periods of up to 18 months, was the extreme difficulty with which shooting could be induced. Only five regenerants were obtained during 12 months on regeneration medium in contrast to the second (CR,1-?) experiment where seven regenerants were obtained, from the much younger callus, in the space of only a month. The difficulty with which regeneration was achieved in the older callus presumably, reflected the fact that a high proportion of the cells in that callus must have had genomes that had been subject to such extensive changes as to make them incapable of regeneration. Only those cells with all the genes necessary for organised, phototrophic growth in an unaltered form would be able to regenerate and in the 18 month old callus these would appear to be very much in the minority.

The widespread genome change, suggested by this reluctance to regenerate, was also seen in respect to the phenotypes of the plants that did eventually regenerate since most of the phenotypic characters scored were found to be variable amongst the regenerants and different from the normal Petunia control. Many of the phenotypic parameters measured were likely to be influenced by a number of genes and so it is not possible to suggest that most
of the genes screened, in this indirect manner, were subject to alteration. Nevertheless, it is evident that in these regenerants a substantial proportion of the genome would appear to have been affected by some sort of heritable change.

The karyotypes of the regenerants CRA-E show a tendency towards numerical change. As has been discussed, it is not known if the prevalence of the tetraploid condition reflects pre-existing karyotypic variation between leaf cell nuclei. Whilst this may be one component of the variation, the fact that only one regenerant had the normal diploid complement of chromosomes suggests that karyotypic instability may arise in culture. Such instability is well documented and the degree of karyotypic variation seen amongst callus regenerants has been shown to be related to the length of time that they were grown as callus cultures (Lee and Phillips, 1986). Whilst some of the altered phenotypes may have resulted from changes in karyotype, the fact that there was widespread variation between the different tetraploid regenerants suggests that this cannot be the sole cause.

One aspect of karyotypic variation that was not studied in the work reported here but which could have been involved was that of structural chromosome alteration. As recorded in the introduction, duplications, inversions and translocations are widely recorded amongst callus regenerants and have been suggested as possible contributors to somaclonal variation. The detailed chromosome banding analysis necessary to conclude on this point was not considered appropriate to this project and in any case Karp and Bright (1985) have suggested that, in potato, this type of variation is not responsible for phenotypic differences.

Hence it appears likely, though far from proven, that phenotypic variation resulting from molecular changes accumulates during prolonged callus culture. The mechanisms responsible for the amplifications, deletions, mutations and/or methylation changes
that may give rise to this variation must be active during normal callus growth. That is not to say, however, that they show the peak of their activity at this time.

Genetic Changes at the Time of Callus Induction.

The evidence from the experiment with regenerants 4R,1-7 would seem to suggest that the maximum activities of the mechanisms responsible for the appearance of heritable phenotypic variation may occur around the time of callus induction. The observation that suggests this is that a number of regenerants from different parts of the callus show the same phenotypic differences. Whilst it is remotely possible that they could have, independently, acquired the same genetic changes, it is most likely that they had a common cell ancestry and that the changes occurred before the callus multiplied and was subcultured onto the different plates.

That regenerants from different parts of the callus could have a common cell ancestry may seem remarkable since callus was induced from a large number of leaf discs and around each leaf disc numerous cells must have contributed to the callus proliferation. There are, however, two principal explanations of this apparent phenomenon. Firstly the few cell lines from which the regenerants arose could have been ideally suited to callus growth and could have outgrown and outcompeted the other cell lines present at the time of callus initiation. Secondly, it could be that the few cell lines from which regenerants arose were the only cell lines that had not undergone some alteration that made them incapable, or at least less capable, of regeneration. Of the two, the latter suggestion would appear to be the most likely since, upon induction, shoots arose at a limited number of positions on the callus. If the majority of the callus was composed of closely related cells, with most of their genetic changes in common, then many of the callus cells could be assumed to have the same regeneration capability. Upon induction, therefore, there might be
expected to be a dense crop of regenerating shoots rather than the
more dispersed individual shoots that are seen.

The suggestion that the greatest activity of the mechanisms
responsible for genome rearrangement may occur at the time of
callus initiation is in agreement with some of the published
information concerning somaclonal variation. Considering the
timing of changes in the mitochondrial genome, Hartmann et al.
(1987) found variation to occur primarily at the time of callus
induction and Hanson (1984) found stability thereafter. In the
nucleus the amplification of certain specific sequences appears to
accompany callus induction (Kato and Tanifuji, 1986) and the
results presented above suggest that methylation changes also
occur principally during callogenesis. Why is this early stage in
callus growth so critical?

In broadest terms the phenomenon can perhaps be best explained in
terms of adaptation to stress. Walbot and Cullis (1983) have
suggested that the extreme fluidity of the plant genome is part of
a plant's adaptation to its static lifestyle. Whilst an animal can
sometimes move away from stress, a plant cannot move and has to
adapt to, or succumb to, that stress. Natural selection may,
therefore, favour a genome that can change rapidly in response to
stress and hence generate the variation that allows for the
alleviation of the stress. It would seem quite likely that the
stress imposed by cell culture may be at its greatest at the time
of callus induction when the physiology of the tissue would be
having to change and switch from photo-autotrophy to heterotrophy.
When the stress is greatest so the response might be greatest and
the mechanisms for genetic change might be at their most active.

Plants have not been selected to cope with cell culture so how
could this apparent adaptation have arisen? The explanation is
probably that the change from photo-autotrophy to heterotrophy is
of little importance and what the response is really concerned
with is surviving injury. All plants must be subject to some form of mechanical injury either through grazing, insect damage or fungal infection. Aside from these biologically-derived damages many plants will suffer physical damage through wind, water and ice. The selection pressures favouring mechanisms that allow plants to survive mechanical damage must be enormous.

The wound response of a plant sees massive changes in the biochemistry of its cells. Enzyme induction results in the synthesis of compounds concerned with self defence whilst the induction of cell division brings about the proliferation of a wound callus around the site of attack. The induction of replication from specific origins of replication in the nuclear genome is seen to accompany the wound response and results in the amplification of specific repeated sequences (Natali, et al., 1986). Similar genome changes could generate the molecular diversity necessary for the evolution of wound responses and so may be favoured by natural selection. Hence, somaclonal variation may be, in part, a by-product of the plant's natural response to physical damage.

Control and Exploitation of Genetic Changes.

With two apparent components to the generation of somaclonal variation how can steps be taken to overcome the problem or to exploit it where desired? As discussed in the introduction, it may be possible to reduce the degree of stress encountered in long-term culture by precise medium optimisation and this may, in turn, reduce the genetic variability that gradually accumulates during prolonged culture. In the converse situation, where genetic change is required it may be possible to increase stress-induced genome change by utilising less than optimal media.

As far as overcoming the initial burst of genetic change, that appears to accompany callus induction, is concerned a different
approach may be required. In the culture methods currently employed, at the time of callus induction the initial wounding response occurs at the same time as the switch to disorganised growth. It might be possible to separate these two temporally and this may have the effect of reducing variation. The simplest way of achieving this might be to delay the onset of callus proliferation which would allow the wound response to diminish before cell division commences. Precisely how this may be achieved would have to be established. It may be that a low temperature treatment at the time of callus induction may delay the onset of cell division or that sub-lethal doses of antibiotics that interfere with DNA replication could achieve this aim. A more elaborate phased introduction to culture may also bring about this temporal separation of wound response and cell division.

If meristems, either shoot or root tips, were introduced to culture as organs with the points of their excision being some distance from them then the wound response at the cut edges may have little effect on the meristematic tissue. Continued culture of these meristems might allow for the wound response to have diminished before the medium employed is switched to one inducing callus formation. Callus may then be able to proliferate from the meristem producing cells that show little genetic variation. Further experimentation would be necessary to test the feasibility of obtaining a switch from intact organ growth to disorganised callus growth in the absence of a recent cut surface. Even if it were found to be impractical, it might be that the initial establishment of an organ culture, prior to excision of the small piece of tissue necessary to induce callus formation, may reduce the initial stress encountered during callus induction and this, alone, may help to reduce genetic variability in the resulting culture. A similar reduction in additional stress may be brought about if cultures were established initially on regeneration medium for a brief period of "culture acclimatisation" prior to transfer to callus induction medium.
In the absence of either a gradual introduction to culture or a separation of wound response and callus initiation phases, the principal alternative approach to reducing the generation of excessive variability at the time of culture induction would be to avoid unnecessary wounding. Protoplasts could be considered as the most damaged cells that remain viable and, viewed in this light, their reputation for producing enhanced somaclonal variation is not unexpected (Karp, 1986). Whilst they might appear to offer an ideal route for many plant genetic manipulations (Cocking, 1981) perhaps alternative techniques are worthy of more study.

GENETIC VARIATION AND PLANT CELL TRANSFORMATION.

The relevance of the results of this study to the origins of somaclonal variation have been discussed, in length, above but little mention has been made of the significance of this experiment to genetic transformation studies. This is an area of expanding interest in plant molecular biology, where it promises to provide answers to a great number of questions, and of increasing importance to the plant breeder, for whom it promises to expand the available gene pool. Whilst the plant breeder is liable to study the resultant plants in great detail and involve them in many further crosses, for the plant biologist the transformant is but a tool allowing him study the expression of modified genes in a heterologous plant system. The scientist must use this new technique with great care if meaningful results are to be obtained.

This study has shown that there appears to be a period of genetic instability at the time of callus induction and hence one which cannot be easily avoided if a callus culture is to be used. Ploidy changes and genetic variations would appear to occur at this time and these changes are not necessarily accompanied by obvious phenotypic differences. Consequently, the fact that a transgenic plant recovered from a protoplast, or even leaf disc,
transformation system might appear to have a normal phenotype can be misleading. Before any other studies are performed on such a plant its karyotype should be determined. Once the normal diploid karyotype has been established certain experiments could be performed however, in the ideal world, backcrossing with the original cultivar should be performed first. Genetic variation unrelated to karyotype changes can occur in the initial phase of callus induction and such changes may have a bearing on the activity of the transgene. Repeated crossing with a non-culture derived plant would serve to reduce this variation and hence eliminate somaclonal variation as the cause of any observed changes. Finally, caution should be exercised in the interpretation of expression levels. Methylation patterns are potentially heritable and any methylation pattern conferred on the transgene at the time of transformation may not be eliminated through backcrossing and might influence the expression of the novel gene. Only a direct comparison of methylation patterns between the transgene in the transformant and in its native state could confirm conclusively that the behaviour of the introduced gene is not being influenced by an atypical distribution of cytosine methylation.

SUMMARY AND FUTURE DIRECTIONS.

Plants regenerated from callus cultures of *Petunia hybrida* were found to show phenotypic variation much of which was heritable. Many of these regenerated plants were found to have the tetraploid chromosome number with only three out of the twelve retaining the normal diploid state. Two of the plants were aneuploid having more than the diploid complement of chromosomes.

In plants regenerated from callus that had been in culture for only two months the recorded variation was subtle being restricted, primarily, to quantitative traits which would be easily overlooked. Even the aneuploid regenerant from this group
had an apparently normal gross morphology. Most of the heritable differences seen in these plants, derived from short term callus cultures, were related to ploidy variation. The changes that were probably not related to ploidy (e.g. style length changes, anther-petal fusions and flower diameter changes) were found in in the majority of plants of a given ploidy indicating that they may have arisen very early in the callus phase. It is suggested that the genome might be particularly susceptible to rearrangement at the time of callus initiation maybe, in part, a consequence of the plant wound response.

Plants regenerated from callus that had been in culture for prolonged periods (>1yr) showed more pronounced variation with gross morphology being affected in every case. Most of the traits studied in these plants were found to show heritable variation little of which was related solely to karyotypic variation. This high frequency of variation was taken to indicate that a progressive accumulation of genetic changes must have occurred throughout the callus phase. Both dominant and recessive traits were observed amongst the regenerant progeny.

The organisation and methylation of a range of repeated DNA sequences has been studied in normal Petunia hybrida plants. A dispersed highly repeated sequence and a moderately repeated sequence were found to show extensive cytosine methylation in contrast to the moderately repeated rDNA genes which showed numerous unmethylated CCGG sequences. The organisation of a number of moderately repetitive sequences in DNA obtained from callus regenerants was found to be indistinguishable from that seen in normal Petunia plants and there was no evidence for large scale copy number changes. In one callus regenerant, however, the repeat length of the ribosomal RNA genes was found to vary from the normal 9 kb value. Alongside the observation that rDNA copy numbers can vary in Petunia callus, the fact that there is no observable within-plant variation in rDNA copy numbers may suggest
that changes in rDNA repeat length may be propagated through the population of repeats by a process of deletion and re-amplification.

The extent of cytosine methylation in ribosomal RNA genes was found to differ between different callus cultures. There was no evidence that this variation was related to different degrees of physiological activity since the pattern did not change during the callus subculture cycle and did not alter during callus senescence. It is likely, therefore, that the variation is not related to different levels of rDNA transcription. Equally, the methylation pattern of rDNA sequences in a callus did not change with prolonged culture but rather appeared to be fixed at the time of callus initiation by some unknown factor.

It is not known whether some callus cultures with extensive or little rDNA methylation are capable of regeneration, however all of the regenerants showed normal patterns of leaf rDNA methylation. This may imply that methylation patterns change during regeneration or that there is some form of selection whereby only cells with normal patterns of rDNA methylation are capable of regeneration. It is not known if the variation in methylation patterns seen in rDNA sequences occurs in other sequence types but, if protein coding genes showed the same degree of variability, it is possible that alterations to DNA methylation patterns may contribute to somaclonal variation.

Further experiments that address specific questions about cytosine methylation and rDNA reorganisation have been discussed above but no mention has been made of the overall strategy that should now be adopted to study somaclonal variation. In the light of the findings of this study several recommendations can be made.

The role of repeated DNA sequences in somaclonal variation has yet to be determined and requires further work. Whilst the analysis
the organisation of specific cloned repeats in regenerants would be the most satisfactory approach it is probably unrealistic due to the time consuming nature of such studies. This approach should probably be confined to the study of known transposable elements, since such sequences have now been identified, and changes to other repeats could be studied by the "genomic fingerprint" technique.

More important, perhaps, than the study of specific repeats might be the study of DNA synthesis in callus cultures since this may suggest how frequent and extensive specific amplifications and deletions may be. The finding by Van't Hof et al. (1987) of differences in the timing of replication of different sequences promises an explanation of how amplifications could occur merely through a change in the sequence of normal replication events.

Methylation remains a potential contributor to somaclonal variation. The methylation patterns of a range of expressed sequences need analysis in regenerated plants and the activities of methylase enzymes in initiating callus needs investigating.

The most important approach, however, may be the study of known genes in regenerated plants. Somaclonal variation arises through changes in the expression of coding sequences so perhaps a detailed analysis of as many different coding sequences as possible in many different regenerants may provide the most rapid means of determining what types of changes affect these sequences. This is a potentially enormous job but perhaps steps can be taken to maximise the chances of success. Whilst small changes in the expression of "housekeeping" genes, arising from slight changes in promoter regions or methylation patterns, may well contribute to somaclonal variation the extent to which changes can be tolerated in these genes must be limited. Large scale rearrangements to such essential genes would presumably prevent regeneration and so would not be found in regenerants. Similar large scale rearrangements to
other genes, either members of multigene families or genes whose products are less essential, may contribute to somaclonal variation and hence are worthy of study. So it is perhaps to the non-essential genes that attention should be directed. Both large scale and small scale reorganisations should affect floral pigment synthesis genes just as much as they affect housekeeping genes yet in the former case viability should not be affected. Equally it is easy to screen regenerants for alterations in the activity of genes affecting flower colour and hence eliminate much unnecessary work. So it may be through the study of genes affecting anthocyanin synthesis that some molecular explanations of somaclonal variation are to be revealed. *Petunia* provides an ideal system on which to conduct such a study given the wealth of information on anthocyanin synthesis in this plant (Mol, et al., 1983) and the availability of cloned genes from it encoding enzymes of the anthocyanin pathway (Reif, et al., 1985).
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