GENE EXPRESSION IN CULTURED CELLS

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A thesis presented for the degree of Doctor of Philosophy in the University of Leicester

June, 1989
A Papà
AKNOWLEDGEMENTS

My thanks to J. Draper for supervision and corrections to the manuscript, and to K. Harikrishna for constant input of valuable ideas and indispensable technical help. I am also grateful to E. Paul, K. Harikrishna and H. Bailey for allowing me to quote their results, thus presenting a clearer picture of the subject.

I thank F. Robson, K. Harikrishna, G. Dury and the other members of the Botany Department for an unforgettable time, and D. Ellis for his friendship, numerous hot dinners and unyielding support. Finally, I thank my parents for moral and financial support throughout the years.
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<th>Full Form</th>
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<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>pb</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTAB</td>
<td>hexadecyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl fluoride</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphonyl oxide</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis-((\beta)-aminoethyl ether) (N,N',N'')-tetraacetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>((N)-1-hydroxyethylpiperazine-(N')-2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>hnRNA</td>
<td>unspliced, nuclear RNA</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-(\beta)-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases (1000 bases)</td>
</tr>
<tr>
<td>kD</td>
<td>kilo daltons</td>
</tr>
<tr>
<td>Mol. Wt.</td>
<td>molecular weight</td>
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<tr>
<td>MOPS</td>
<td>3-((N)-morpholino) propane-sulphonic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-(N,N')-bis(2-ethane-sulphonic acid)</td>
</tr>
<tr>
<td>poly A(^+) RNA</td>
<td>RNA with poly(A) tails</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-Di-2-(5-phenyloxazolyl)-benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>PR</td>
<td>pathogenesis-related protein</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N',N'')-tetramethylethylenediamine</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>X-gal.</td>
<td>5-bromo-4-chloro-3-indolyl-(\beta)-D-galactopyranose</td>
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CHAPTER ONE

GENERAL INTRODUCTION
1.1 Plastic Responses in Plants

The plant kingdom, as compared to the animal kingdom, is characterised by a greater capacity to survive extreme atmospheric conditions; lack of mobility necessitates that poor growth conditions must be tolerated rather than avoided. Both physiological and morphological adaptations occur in response to stressful conditions. The process of photosynthesis and respiration can adapt, and this is frequently coupled with changes in stem and leaf morphology. In addition most plants can generate lost organs (roots, buds or flowers) and virtually all plant tissues, except lignified vascular tissue or hairs, can be induced to divide and subsequently form callus.

This aspect of the adaptive armoury of plants is termed phenotypic plasticity. Examples of such plastic responses can be found in natural growth responses such as the alterations in size of annual ring in perennials, leaf size and variegation. There are also more extreme responses such as those generated by grazing and experimental decapitation, wounding, shoot formation from axillary buds and reestablishment of severed stems via the production of adventitious roots.

The leaf is a typical example of an organ that undergoes structural and biochemical modifications in response to external stimuli. For example, leaf size may be affected by temperature, photoperiod, light quality and quantity, mineral nutrition and water stress; these changes result from either cell number modifications, or mean cell volume alterations. Variegated leaves also show plasticity. In these leaves, cells (other than epidermal and stomatal cells) are derived from two layers of leaf primordia, in which the chloroplasts are either present or absent. Coloration of the leaf depends upon the composition of the two types of cells; while the extent of variegation does not affect leaf shape, the contribution of the layers is extremely plastic, varying between and within leaves in an apparently non predictable manner. This will greatly affect the metabolic activities at different parts of the leaf.
Decapitation may also affect the leaves of a plant. Decapitation of *Phaseolus* plantlets when the first trifoliate leaf begins to unfold and the primary leaves have virtually completed expansion results, after a short lag period, in a very large increase in area and thickness of the primary leaves (Carmin and Van Staden, 1983). This is accompanied by an increase in chlorophyll and soluble protein content per unit area, suggesting an increase in the chloroplast number.

In extreme cases plastic responses may be consolidated into the genotype. An example of this is the substantial differences in shape and size of leaves of plants of *Geranium sanguineum* (Lewis, 1969), depending on the provenience of the plants. Here, the morphological differences seen in the wild are maintained when the lines are cultivated side by side, showing that "the phenotypic expression of leaf dissection is under relatively direct genotypic control".

Another process which is mediated by plastic responses is that of flowering. Flowering usually occurs as a direct response to favourable daylength, but has been shown to be mediated by temperature conditions (Bernier, 1986), or inter-organ correlations, such as the distance between the flowering buds and the root system (McDaniel, 1980).

Perhaps the most striking plastic responses are those arising as a result of wounding. The wound response in plants is very complex, as it has to serve several functions: wound healing, prevention of pathogen entry and prevention of dessication. Analysis of cell size and cell number (Wilson and Grange, 1984) has been used to divide the wound healing process into three phases: a lag phase characterised by metabolic reactivation of cells and no cell enlargement, a division phase characterised by cell enlargement and division, and a differentiation phase characterised by the formation of differentiated structures such as vascular and cork cambium (Rittinger et al., 1987).

One of the most striking processes to occur as a result of wounding is that of dedifferentiation. This is the process by which specialised cells, that are division cycle arrested, give rise to heterotrophic, dividing cells. Many events occur during dedifferentiation, including DNA synthesis, cell division, changes in the nuclear morphology, and an increase in cell wall synthesis (Paul et al., 1989). Cellular development
during these changes must be controlled by alterations in the amounts and activities of various enzymes and structural proteins; these may be mirrored by an increase in rRNA and qualitative changes in the mRNA populations of the cells.

1.2 Gene Expression in Wounded Tissues

During wounding several biochemical and developmental changes occur, for example cell cycle reactivation leading to cell proliferation. Wounding results in a massive transient fall in overall protein synthesis (Davies et al., 1986), coupled to formation of new polysomes and increased synthesis of particular wound related mRNAs and proteins. For example, cell disruption brings about the activation of hydrolase activity (Thompson et al., 1987), resulting in the production of free radicals, some of which are utilised in the formation of lignin. These activated oxygen species can damage lipid membranes, and the plant dilutes this threat by an increase in catalase (Esaka et al., 1983) and peroxidase (Thompson et al., 1987) synthesis. Other genes actively expressed as a result of wounding have been identified. They include PAL (Borchert, 1978), 4-coumarate:CoA ligase (Fritzemeier et al., 1987), proteinase inhibitors (Graham et al., 1986), thionins (Bohlemann et al., 1988), pathogenesis related proteins (Van Loon, 1985), hydroxyproline-rich glycoproteins (Chen and Varner, 1985), chalcone synthase (Cramer et al., 1985), chalcone isomerase (Mehdy and Lamb, 1987), stilbene synthase (Vornam et al., 1988), peroxidase (Espelie et al., 1986) and chorismate mutase (Kuroki and Conn, 1988).

There are two main types of responses associated with wounding, the localised response and the so called systemic reaction. Responses in the locality of the wound site include the production of aromatic amino acids, via the phenylpropanoid pathway, as precursors for the synthesis of lignin, flavonoid pigments and isoflavonoid phytoalexins (Lamb et al., 1989). One of the enzymes involved in the biosynthesis of phytoalexins is stilbene synthase (Vornam et al., 1988). As with other proteins in the phenylpropanoid pathway, stilbene synthase is not an enzyme specifically
induced by wounding alone, but its synthesis may be activated by fungal cell fragments, or subculturing in suspension cultures (Vornam et al., 1988).

Another example of proteins synthesised as a response to general stress stimuli is the so called pathogenesis related (PR) proteins. PR proteins are characterised by their low molecular weight (10-40kD), solubility at low pH, resistance to protease action, extracellular localisation and serological similarity (Van Loon, 1985). These proteins have been identified, mainly in dicots, as having 1,3-β-glucanase or endochitinase activity (Kombrink et al., 1988). Glucanases have also been identified in maize (Nasser et al., 1988), and a chitinase in barley (Swegle et al., 1989). PR proteins can be induced by plasmolysis, senescence, ethylene, high phytohormone concentrations, culture filtrates of pathogens, viral infection, wounding and cell culture (Van Loon, 1985; Kombrink et al., 1988).

Other proteins induced by wounding, fungal elicitors or infection are the hydroxyproline-rich glycoproteins, or extensins (Chen and Varner, 1985). Extensins have been postulated to be involved in controlling cell expansion, cell wall strengthening (Wilson and Fry, 1986), and in defense against infection (Corbin et al., 1987). Extensins are coded for by several low copy number genes. Work by Corbin has shown that each transcript exhibits marked differences in accumulation under different stress conditions, suggesting the operation of several distinct intercellular stress signal systems in higher plants.

Altered patterns of gene expression by synthesis of different isoenzymes in response to different developmental stimuli have also been observed with peroxidase. The pattern of peroxidase isoenzymes present is tissue specific (Cassab and Varner, 1988) and is seen to be involved in different metabolic pathways. Peroxidases have been implicated in the generation of free radicals for the polymerisation of lignin precursors, cross linking of proteins, hemicellulose and ferulic acid in cell wall matrices (Fry, 1986), and suberisation during wound healing (Espelie et al., 1986).

Many of the responses mentioned above do not occur only because of wounding but may be triggered by a number of external stimuli. Moreover, the defense reaction of the plant is not always restricted to a defined
region in the near vicinity of the original wound, but may also occur in tissues far from the original wound site, resulting in a systemic reaction. This has been shown to occur in the accumulation of proteinase inhibitors in distal leaves, following wounding (Graham et al., 1986). Members of two small wound-inducible gene families, called Inhibitor I and Inhibitor II have been isolated from tomato and potato (Logemann et al., 1988; Lee et al., 1986; Cleveland et al., 1987). The former exhibits a chymotrypsin inhibiting activity, the latter a trypsin/chymotrypsin inhibiting activity (Sanchez-Serrano et al., 1987). These genes are also expressed in the potato tuber, where they are thought to play a role in the defence against insect or microbial attack by inhibiting their proteinases. In a systemic reaction, the enzymes' synthesis may for example be induced by mechanical wounding in leaves. The diffusible factor postulated to signal the wounding event to more distant tissues in the plant is an oligosaccharide (Sanchez-Serrano et al., 1987).

1.3 Gene Expression in Dedifferentiating Cells

Dedifferentiation is the process by which quiescent cells will enter a new cell cycle and proceed to divide. This occurs in vivo mainly as a result of wounding, and in vitro it is the process by which cell and tissue cultures are initiated. A number of changes occur during dedifferentiation, related to the wounding event itself, and the fact that the cells are re-entering division. They include changes in the expression of several enzymatic activities (Masuda et al., 1988), chromosome number and populations of repetitive DNA (Kikuchi, Takaiwa and Oono, 1987), and ribonucleic acid synthesis (Fleck et al., 1982). These points are amplified in Section 5.1.

The processes involved in the initiation of cell division are not easy to study in plants due to the difficulty of isolating tiny meristems deep within plant tissues, coupled with the requirement for large quantities of physiologically and morphologically similar cells for molecular analysis. Likewise, dedifferentiating cells close to wound sites are very difficult to separate from the surrounding tissues. For these reasons scientists have often turned to alternative in vitro tissue
culture techniques to study the phenomena of division and dedifferentiation. Tissue culture is the process whereby small explants are isolated from an organism and grown aseptically for indefinite periods on a semi-defined or defined nutrient medium. The ideal *in vitro* system should be able to generate large amounts of material, be as near to single cells as possible, to help cytological analysis, and have a high synchrony in cell dedifferentiation, expansion and division. The initiation of proliferating cultures from tissue explants involves profound changes in their developmental state: the basic architecture of the tissue is altered, certain specialised cell types are lost, new cell types arise, and normally quiescent cells begin to divide (Gautheret, 1966)

At the same time we must remember that direct comparison between cultured tissue and meristematic tissue may be erroneous because of a number of basic differences between the two systems. These are mainly caused by the environment of cultured tissues, although some are inherent of dedifferentiated cells. The growth media is rich in sucrose and mineral nutrients, which allows a switch from photoautrophism to heterotrophism. Also the cells now exist as single cells or microaggregates, and are as a result bathed evenly by the nutrients; this is in marked contrast to the nutrient and hormonal gradients present in intact plants. The nutrient gradient probably has no great effect, but the loss of hormone gradient will have drastic effects on the cells; in particular it is interesting to note that a loss in hormone gradient is thought to act as inducer to cell dedifferentiation and proliferation in wound sites (Lipetz, 1970). At the same time, the plasma membrane architecture may have to vary, to change its permeability for obtaining nutrients, and the overall biochemical pathways within the cells will have to adapt to their new environment.

As a result it is possible that a great number of changes occur within cells as they are placed into culture. These are probably best visualised by the work of Ultrich and Key (Ultrich and Key, 1988), showing the complexity of RNA in soyabean suspension cells to be greater then that in hypocotyl tissue from the same plant, and to be affected by hormonal conditions. It is still not known whether this increased complexity in RNA population is retained throughout tissue culture, or
whether it is only present during the dedifferentiation process, and returns to more average levels as the culture reaches its steady state.

Cells can be artificially maintained in a dedifferentiated state in culture. This property of cultures has been used in a number of experiments to try and clarify the processes occurring during dedifferentiation and redifferentiation (see Section 3.1 for details). Moreover, cultures have been used as a tool to separate tissue function from structure, in an attempt to understand the metabolic pathways of plant cells.

1.4 Methodology

Transcription is an early control step in gene expression. In prokaryotes it is also the main control step (Lewin, 1983) and until recently was thought so in eukaryotes as well. The choice of methodology for this thesis relies on the fact that, as discussed in Section 3.2.1, representation of gene expression is easier at the transcriptional level rather than at the protein level. Two approaches have been used in the thesis, the empirical approach and the targeted approach. The empirical approach is theoretically simpler in that it does not require any biological knowledge of the systems under comparison. In this case it involved constructing a cDNA library using mRNA extracted from the cell culture system under study. Radioactive probes were then made by synthesising first strand cDNA using mRNA extracted from the culture, and the intact tissue system used as comparison; replicas of the cDNA library were then probed to detect clones that will light up with one probe and not the other. This system is termed +/- screening and is a relatively unsensitive method, but may be improved by enriching for differentially expressed genes (see Section 4.2). The targeted approach requires a known target molecule to exist. This may be either a polypeptide or mRNA species which is known to appear at definite stages in a process, or in a specific tissue. Here some information of the target is needed, such as protein or DNA sequence, or immunological properties (see Section 5.3).
Both methods have their relative advantages and disadvantages. The empirical approach requires little or no knowledge of the biochemical and physiological differences between the systems being compared. At the same time it is a simplistic approach and the results are often difficult to analyse. The researcher will acquire a family (or families) of transcripts which can be shown to be unique to or upregulated in the system being studied. Because of the scant amount of data available it is unlikely that sequencing of the transcript(s) will show it to code for a known polypeptide; as a result the biological significance of the transcript and its regulatory behaviour will not be apparent. The targeted approach is more focused and will therefore preclude the viewing as a whole of the transcriptional changes occurring; at the same time having a defined protein as the target will allow for data resulting from protein chemistry techniques (such as antibody work) to be accumulated, giving a fuller picture of the biological interactions taking place.

In terms of this study, both approaches have been used in an attempt to understand the underlying factors controlling one aspect of a plastic response in plants. Any information on the nature of the transcripts and proteins responsible for, or occurring as a result of, these responses is of use.

1.5 General Scope of the Thesis

Wounding responses in plants differ depending on the species, organ involved and type of wounding, but all involve isolation of the damaged area to prevent desiccation and infection by opportunist pathogenic fungi and bacteria. A process that occurs at large wound sites is dedifferentiation of neighbouring quiescent cells to give rise to fast dividing cells, so that a callus plug may be formed at the wound site. This process is very similar to that occurring at the onset of plant culture via the placement of plant explants on growth media. Two main questions arise, that will be examined in this project: how controlled is the process of dedifferentiation, and how similar, or dissimilar, are fast dividing, established cultured cells to normal plant cells.
The process of dedifferentiation has been shown to cause specialised mature cells to regress to the state of meristmatic-like cells. It is not known whether this occurs via a breakdown of cell structure and a loss in metabolic functions, or whether it is an ordered process. This question will be considered in Chapters 5 and 6. Secondly, an attempt will be made to examine the similarities and differences in gene expression between an established suspension culture and a normal plant; this will be covered in Chapters 3 and 4.

This thesis is concerned mainly with evaluating whether the process of dedifferentiation and maintenance of the fast-dividing, dedifferentiated state is dependant upon major changes in gene expression. The role of transcription in these changes in gene expression has been explored, as outlined in Section 1.4.

There are also a number of potential applications to this work, including a better understanding of the factors controlling gene expression in cultured callus, which affects the production of secondary metabolites and the tissue's competence to somatic embryogenesis or organogenesis. These will be discussed more fully in Section 3.1. Once differentially expressed cDNAs have been purified, it is hoped their 5' regulatory regions will be isolated and characterised. Such promoter may then be used to drive the expression of useful genes in dedifferentiating cells in homologous and heterologous plant systems.
CHAPTER TWO

METHODS, STRAINS AND MEDIA
2.1 METHODS

All chemicals mentioned in these pages were acquired from Sigma or BDH unless otherwise stated. Nucleotides were acquired from Pharmacia, isotopes from Amersham. Similarly, all restriction enzymes and DNA modifying enzymes, were obtained from Bethesda Research Laboratories (BRL), unless otherwise stated.

All stock solutions mentioned in the text whose recipe is not included, were made according to Maniatis (Maniatis et al., 1982).

2.1.1 Petunia Suspension Cell Cultures

An established suspension cell culture of albino Petunia hybrida cv. Blue Lace (BL) was grown in UM medium (see Section 2.3.2) at 20°C with constant shaking (75rpm) and illumination (2.5-4.0 μmol M⁻² sec⁻²). Every 7 days 15ml of culture were subcultured into 80ml of fresh medium.

2.1.2 Asparagus Culture Initiation

NOTE all manipulations carried out under sterile conditions. Approximately 10g of fronds were sterilised in 10% bleach 20min, and washed thoroughly with sterile tap water. Cladodes were removed from the fronds using sterile gloves. The cells were isolated into 50ml water by scraping the cladodes in a 150mm Petri dish with a polypropylene scraper (3.5cm x 8.5cm). The resultant cell suspension was passed through a 64μm mesh filter. The cells were pelleted by centrifugation at 800rpm in a Sorval RT6000B (H-1000 B rotor) 2.5min, washed in water, in culture medium (see Section 2.3.3), and resuspended in medium. The number of viable cells (determined by the appearance of the cell contents) was counted using a hemocytometer, and the concentration adjusted to 4x10⁵ viable cells per ml. The cells were plated out in 10ml aliquots per 90mm Petri dish, sealed with "Nescofilm", and incubated in the dark at 25°C on a rotating orbital shaker at 35rpm.
2.1.3 Wet and Dry Weight Measurements

Two flasks of cell culture at the appropriate growth stage were vacuum filtered onto Whatman No.1 filter paper using a Buchner flask and funnel. The cellular mass was weighed to ±0.01g, frozen at -80°C and then placed in a freeze drier overnight. The freeze dried material was then weighed to ±0.01g to give the dry weight value.

2.1.4 Protein Extraction for PAGE

A known volume of culture (20ml) was vacuum filtered as above and then ground under liquid nitrogen in a pre-cooled pestle and mortar. Extraction buffer comprising 10% sucrose in glycine buffer (3.0g Tris base + 4.4g glycine in 1000ml H₂O at pH8.3), was added at 3ml/g wet weight of tissue. The mixture was allowed to thaw, transferred to Eppendorf tubes and spun for 2-3 seconds in a microcentrifuge. The aqueous layer was then removed and stored at -20°C until needed.

2.1.5 Protein Quantitation

Protein was quantified according to the method of Bradford (1976). The reagent was made up with 100mg Comassie brilliant blue G-250 in 50ml 95% ethanol + 100ml of 85% w/v phosphoric acid in 1000ml H₂O. This solution was left to stand overnight and then filtered through Whatman No.1 filter paper. A standard protein concentration curve was established with 0 to 100μg BSA in 0.1ml. 0.1ml of protein was added to 5ml of reagent and its absorption read at 595nm.

2.1.6 Protein Extractions in DMF Buffer (Mayer et al., 1987)

The plant material was washed thoroughly, weighed and pulverised in a pestle and mortar under liquid nitrogen. To this 0.5ml/g tissue of 2DMF (2% Ampholines pH 3-10 (BDH) + 300mM NaCl + 1mM EDTA + 1mM EGTA + 2% Triton X 100 + 5mM Ascorbic acid + 100mM DTT + 10μg/ml Leupeptin) and 20μl/g tissue 100mM PMSF (phenylmethylsulphonylfluoride) were added, and the mixture ground further. The powder was transferred to an Eppendorf tube and allowed to thaw.

Once thawed, the tube was spun in a microcentrifuge 10min, and the supernatant collected. To this, 100μl/ml of 3mg/ml DNaseI (Sigma) were added, and the tube incubated at 37°C 15min. The tube was spun (5min)
once more, and solid urea added to the supernatant to 9M.

The samples were kept at -70°C and the protein concentrations quantitated using Bradford's method.

2.1.7 Protein Quantitation in DMF Buffer

The assay uses the Bradford reagent (see Section 2.1.5)

10μl of protein was mixed with 10μl 0.1N HCl and 80μl H2O. The sample was then added to 0.9ml 2xBradford, stood for 5min, and read at 595nm. The standard was ovalbumin in 2DMH and the curve was established with 0-50μg ovalbumin per 10μl.

2.1.8 Plant DNA Extraction Using CTAB

Plant material was freeze dried and ground with alumina (1:0.7g tissue:alumina) in a mortar and pestle. The optimal quantities for a miniprep are 0.1g callus or 0.07g leaf. The powder was then placed in an Eppendorf centrifuge tube and 600μl of extraction buffer (50mM Tris-HCl pH8.0 + 0.7 NaCl + 10mM EDTA + 1% CTAB + 1% 2-mercaptoethanol) added. After mixing the suspension was incubated for 10min at 56°C and then extracted with 600μl of chloroform:isoamyl alcohol (24:1). Denatured protein was collected by centrifugation (4min) and the aqueous layer removed. The denatured protein interface on the organic layer was washed with 100μl of extraction buffer which was pooled with the initial aqueous phase. CTAB was then added to 1% and the extraction repeated.

One volume of precipitation buffer (50mM Tris-HCl pH8.0 + 10mM EDTA + 1% CTAB) was added to the aqueous phase in a clean Eppendorf tube and left at room temperature for 20min. The precipitate was collected by centrifugation (12000g, 1min), the supernatant removed, and the pellet dissolved in 1M NaCl. Two volumes of ethanol at -20°C were added to precipitate the nucleic acids, which were collected by centrifugation and washed 3 times with 65% ethanol, twice with 85% ethanol and finally dried in a vacuum desiccator. The nucleic acids were then redissolved in distilled water and quantitated using the diphenylanine method.
2.1.9 DNA Quantitation Via the Diphenylalanine Assay

NOTE it is important that all work is done in a fume hood.

140μl H₂O, 10μl DNA sample, 150μl 3N perchloric acid (49.2ml perchloric acid per 200ml H₂O) and 180μl diphenylalanine (20μl paraldehyde + 198ml glacial acetic acid + 8g diphenylalanine) were mixed well in an Eppendorf tube and incubated in the dark 16-20 hours at 30°C. Using herring sperm DNA a standard concentration curve was constructed taking into account that the method gives a linear relationship at OD600 with a DNA concentration of 5-50μg/ml. Distilled water replaced the DNA sample in the control and was used each time to zero the spectrophotometer.

2.1.10 RNA Extraction

The method used is a derivation of Covey's (Covey and Hull, 1981), altered so as to minimise contamination with soluble carbohydrates.

An appropriate amount of tissue was ground in liquid nitrogen in a pre cooled pestle and mortar. Grinding medium (6% 4-aminosalicylate + 1% trisopropyl naphthalene sulphonate (Kodak) + 6% phenol + 50mM Tris-HCl pH8.4) was added at 2ml/g of tissue. When ground to a fine powder, the mixture was thawed and poured into 50ml polypropylene tubes (Sorval GSA rotor). One volume of phenol:chloroform (1:1) was added, and the tubes shaken thoroughly and centrifuged at 5K for 10min. The aqueous phase was removed and re extracted once with the phenol:chloroform mixture. Sodium acetate (pH6) was added to 0.2M, together with 2.5 vols. of ethanol at -20°C. The total nucleic acids were collected by centrifugation (10K for 10min). The pellet thus recovered was resuspended in 1ml 100mM Tris-HCl pH 8.3 per gram of tissue, and re-extracted with the phenol:chloroform mixture until the interface between aqueous and organic phase was clear. Sodium acetate pH 6.0 was added to 0.2M and another ethanol precipitation effectuated. The pellet was dissolved in 0.5ml water/g tissue. If the solution appeared cloudy, it was spun at 5K for 5min. The pellet contains some nucleic acid but is mainly carbohydrate. Three volumes of 4M sodium acetate were added to the supernatant to precipitate the RNA. The solution was placed on ice for 3h, and spun at 10K for 30min. The RNA pellet was redissolved in 0.2M sodium acetate and ethanol-precipitated. The RNA was spun down, the pellet rinsed in 70% ethanol once, dried in a
vacuum dessicator, dissolved in water to 5mg/ml and stored frozen (-20°C) in aliquots of 0.5ml.

2.1.11 Spectrophotometry

A dual beam recording spectrophotometer (Perkin-Elmer Lambda 5 UV/VIS) was used routinely for DNA and RNA concentration determination at OD260 and OD280, and for growth determination of bacteria at OD600.

2.1.12 RNA Poly A⁺ Purification

The appropriate amount of oligo-dT cellulose (5mg cellulose/mg RNA) was equilibrated in sterile loading buffer (20mM Tris-HCl pH7.6 + 0.5M LiCl + 1mM EDTA + 0.1% SDS) and poured into a Pasteur pipette previously plugged with sterile polyallomer wool. At the same time 1 volume of 2xloading buffer was added to the RNA sample, the mixture heated to 65°C for 7min and then placed straight on ice for 5-10min. The RNA was now loaded onto the column in a volume of 1000μl and the eluate reloaded onto the column 6 times. The column was washed with 100μl of loading buffer to remove tRNA and rRNA. The elution buffer (10mM Tris-HCl pH7.5 + 1mM EDTA + 0.05% SDS) was heated to 45°C and added in 1ml aliquots to the column. Poly A⁺ RNA was collected in sterile Eppendorf tubes in 300μl aliquots. The RNA poly A⁺ was quantitated and then precipitated with 2.2M sodium acetate pH6.0 and 2.5vols. of ethanol. If required for cDNA synthesis, the RNA was repurified to yield poly A⁺⁺ mRNA.

2.1.13 In vitro Translation of RNA

The method used commercial rabbit reticulocyte lysate (RRL - Amersham No.90) and 35S-Methionine (>800 Ci/mol - Amersham SJ204) both stored in 50μl aliquots under liquid nitrogen. The translation mixture consisting of 7μl RRL + 1μl 35S Methionine + 2μl RNA was mixed gently and incubated at 28°C for 40mins. The reaction was stopped by addition of 10μl "cracking" buffer (2% SDS + 5% mercaptoethanol + 10% sucrose + 0.002% bromophenol blue (Fisons) in distilled water), if the sample was to be run on a one-dimensional PAGE gel; if the sample was to be run on an IEF rod gel, 10μl of 2DMH (see Section 2.1.6) were added instead.

The isotope incorporation into protein was checked by first spotting 1μl of the mixture onto filter paper squares (Whatman No.1) and washing
those with 10% TCA + 0.5% casein hydrolysate (0°C, 10mins.), 5% TCA (100°C, 10mins.) and 10% TCA (0°C, 10mins.). The filters were counted in a scintillation counter.

2.1.14 Scintillation Counting

The sample was placed in 6ml of scintillation fluid (4.0g PPO + 0.8g POPOP in 1000ml toluene) and counted in a 1217 Rackbeta liquid scintillation counter. The channels used were 8-110 for 35S and 110-225 for 32p.

Cherenkov counting for 32p used the 110-225 channel but no scintillation liquid was added to the sample.

2.1.15 IEF Rod Gels (Celis and Bravo, 1984)

The following components were mixed in a Buchner flask and degased 1h on a bench line: Acrylamide:bisacrylamide (BDH, Aristar, deionised, 28.38:1.62%) 823μl, Urea (BDH, Elecran grade) 3.4g, Nonidet (deionised, 10%) 1.237ml, H2O 1.10ml.

At the same time 10 glass rods (bore diameter 2mm) were prepared by sealing one end with two layers of Nescofilm.

After 1h, the remaining components of the gel were added: Ampholines pH 3-10 (BDH) 416.5μl, Ammonium persulphate (10%) 12.6μl, TEMED 18.55μl. The mixture was taken up with a 2ml syringe, a large (1x150mm) needle was placed on the spout, and the mixture carefully injected into the glass rods up to 10cm.

The gels were overlaid with 10μl of 8M Urea with a Hamilton syringe, and left 1h to set. The overlay was then removed and replaced with 20μl of 2DMH+9M Urea followed by 10μl H2O. The gels were now left for 1-2h.

The overlay was removed and replaced with 20μl 2DMH + 9M Urea containing coloured markers (congo red 0.1g/ml + fast green 0.1g/ml). The rods were fitted into the running tank and top (0.02M NaOH degased 4h) and bottom (0.01M phosphoric acid) running buffers added.

The gel tank was attached to a cooling water bath at 10°C. The gels were prefocused at 200V 15min, 300V 30min, and 400V 30min.
The top buffer and overlay were discarded, the samples introduced, and overlaid with 5 \mu l 2DMH. Fresh top buffer was then placed in the running tank, and the gel was run 400V 12h, followed by 1500V 1h.

The gels were now removed from the rods, equilibrated 2h in SDS sample buffer (75mM Tris + 10% glycerol + 2.5% SDS + 5% 2-mercaptoethanol + 0.01% bromophenol blue) and stored at -70°C until further use.

The second dimension was run on a standard denaturing PAGE gel.

2.1.16 Polyacrylamide Gel Electrophoresis - PAGE

The gel was made up of a stacking and a resolving portion. Both portions were made up of the relevant amounts of acrylamide/bisacrylamide (30:0.8%) stock, 250mM Tris-HCl, 0.1% ammonium persulphate and 0.1% SDS. Both gels were polymerised by the addition of TEMED (20\mu l).

The resolving gel was either 12% or 15% acrylamide, or a 10-15% acrylamide gradient gel made up with Tris-HCl pH8.8. The stacking gel contained 4% acrylamide and Tris-HCl pH6.8. The gel was 1.0mm thick and was run at 125V overnight on a vertical electrophoresis apparatus.

The reservoir buffer was a glycine buffer (27.92g glycine + 1g SDS + 3.30g Tris base in 1000ml H2O at pH8.3).

2.1.17 Copy DNA Synthesis

The synthesis was carried out following the Gubler & Hoffman method (1983), with alterations enabling it to be carried out as a one-tube reaction.

The RNA poly A++ was boiled for 2min, and placed on ice (5min). The first strand reaction comprised 2.5\mu l RNA polyA+++ (0.2\mu g/\mu l), 1.0\mu l RT-1 buffer (250mM Tris-HCl pH8.3 + 50mM MgCl2 + 6.25mM dA,dT,dGTP + 2.5mM dCTP), 0.5\mu l 100mM DTT, 0.5\mu l Oligo dT14-18 (1mg/ml) and 0.5\mu l (10U) of Reverse Transcriptase (nbl). This reaction was usually set up as three replicas, with a total of 1.5\mu g initial RNA poly A++. A further tube was also set up containing 0.5\mu l 32P-dCTP (10\mu Ci/\mu l) to monitor yield, and to run on an acrylamide/urea gel. The tubes were incubated at 43°C 1h, then set on ice.

The second strand reaction comprised 20.0\mu l RT-2 buffer (100mM Tris-HCl pH7.5 + 25mM MgCl2 + 50mM (NH4)2SO4 + 500mM KCl + 0.2mM dA,dT,dG,dCTP), 2.0\mu l BSA (BRL, 2.5mg/ml), 5.0\mu l cDNA/RNA hybrid, 10U DNA
Poll (Pharmacia), 0.8U RNase H (Pharmacia), and H₂O to 100µl. 0.5µl 32p-dCTP was also added to one of the tubes. The tubes were incubated at 12°C 1h followed by 22°C 1h and 70°C 10min.

If cloning into lambda vectors was to follow, 0.5µl T4 polymerase (Pharmacia) was added to the tubes and these were incubated at 37°C 10min to blunt end the cDNA.

EDTA pH8.0 (1/10 vol.) was added to the cDNA (whether blunt ended or not) and the reaction loaded onto a Sephadex G50 (Sigma) fine column in a Pasteur pipette, previously equilibrated in TE.1 (10mM Tris-HCl pH7.5 + 0.1mM EDTA). The column was eluted with 100µl aliquots of TE.1 and these run through a scintillation counter to ascertain which aliquots to precipitate. The cDNA should be contained in 3-5 aliquots. Precipitation was by addition of 1/10 vol 2M sodium acetate pH5.5 and 2.5 vol ethanol.

2.1.18 cDNA Yield Estimation and Product Analysis.

The yields for both first and second strand reactions were estimated by TCA precipitation. This involved determining the input radioactivity by spotting a 1µl aliquot of the reaction onto a 2.5cm GFC (Whatman) disc (T). At the same time a 1µl aliquot was added to 0.5ml of 0.5mg/ml carrier DNA; 125µl of 50% TCA were added and the tube incubated on ice for 5min. The precipitated DNA was collected on a GFC filter (I) on a filter tower. The filter was washed with 2x5ml 10%TCA and 5ml ethanol. Both T and I GFC filters were then counted in a scintillation counter. The ratio between total and incorporated counts was used to estimate the DNA synthesis.

The cDNA products were analysed by running them on a 7M urea, 5% acrylamide gel made in 0.5xTBE (5.4g Tris base + 2.75 boric acid + 2ml 0.5m EDTA pH8.0 in 1000ml H₂O). 5x10⁴cpm of sample were mixed with an equal volume of formamide-dye solution (90% formamide + 25mM EDTA + 0.5% bromophenol blue (Fisons) + 0.5% xylene cyanol (Fisons)) and the mixture boiled for 2min. The gel was run in 0.5xTBE at 45mA until the xylene cyanol dye was 3/4 down the gel. The gel was then transferred onto a 3MM paper (Whatman) backing and autoradiographed.
2.1.19 Cloning of cDNA into pUC Vectors.

Tailing of the DNA molecules was done using a 40:1 ratio of dioxy- and dideoxynucleotides which resulted in tails averaging 20 nucleotides on the 3' end of the DNA. The reaction solution contained tailing buffer (100mM K cacodylate pH7.2 + 2mM CoCl₂ + 0.2mM DTT), 15 units of terminal deoxynucleotidyl transferase (BRL), 2000pmols of dCTP, 500pmols of ddCTP and 1pmol of ends of DNA in 30μl. Incubation was for 30min at 37°C and the enzyme was heat inactivated. The reaction could be followed using radioactive dCTP.

The plasmid vector was appropriately tailed with dGTP as above, the tailed vector and cDNA were incubated at different ratios in terms of pmoles of ends, ranging from 10:1 to 1:1, vector:cDNA. The incubation was for 2h at 58°C in the presence of 10mM Tris-HCl pH7.6, 1mM EDTA, and 250mM NaCl. The annealed insert/vector mixture was then used to transform competent E.coli cells.

2.1.20 Cloning of cDNA into Lambda Vectors.

The Amersham 'cDNA cloning into gt10' kit (RP.1257) was used in this process. As it was found that the ligase did not behave satisfactorily, BioLabs T4 ligase was used instead. Also a Sephadex G75-120 (exclusion limit 50kD, or 75bp) column in a Pasteur pipette was used to replace the columns provided with the kit.

The cDNA thus prepared was not cloned into gt10 supplied with the kit, but into Lambda-ZAP (Stratagene).

2.1.21 Ligation

The ligations were performed in 20μl volumes, in a buffer containing 50mM Tris-HCl pH7.8, 10mM MgCl₂, 1mM DTT, 1mM ATP, 50μg/ml BSA (BRL), 10mM spermidine. If a circularisation of the DNA was required, the reaction contained 1pmol DNA ends/ml; if recombination was to be favoured, the reaction contained 5pmol DNA ends/ml (1μg of 1kbp linear DNA = 3.0 pmol ends, etc.). Sticky end ligation utilised 0.1U Ligase, blunt end ligation 1U Ligase.

The DNA molecules were combined, ethanol precipitated, and redissolved in ligation buffer. Ligase was added, and the ligation incubated overnight at 15°C. Ratios of 1:1, 5:1, and 20:1, vector:insert
were used to find the conditions maximising one insert per vector molecule.

2.1.22 Rubidium Chloride Transformation of E. coli

The relevant cell strain was grown in Ψ broth with vigorous constant shaking (250-350rpm) at 37°C to an OD660 = 0.49. The cells were chilled on ice, spun down (5K, 5min) and resuspended in 3/10 volume of Tfb1 (100mM RbCl + 50mM MnCl2 + 30mM K acetate + 10mM CaCl2 + 15% glycerol pH 5.8 with glacial acetic acid, filter sterilised). The cells were stood on ice for 1.5h, then spun down (5K 5min), resuspended in 1/25 volume of Tfb2 (10mM MOPS pH7.0 with NaOH + 10mM RbCl + 75mM CaCl2 + 15% glycerol, filter sterilised) and aliquoted to 0.2ml. The cells could now be used straight away, or stored at -70°C until needed. If previously frozen, the cells were thawed on ice until just liquid. DNA was added at 10ng per 200μl aliquot of cells. These were placed on ice for 20min, then heat shocked for 90s at 42°C, followed by 2min on ice. One ml of Ψ broth was added and the mixture incubated at 37°C for 1hr. The cells were then plated on an L agar plate with the relevant antibiotics and/or IPTG (Pharmacia) and X-gal (Pharmacia) mixes.

2.1.23 Infection of Cells With λ-ZAP

To make plating cells, a BB4 colony was picked from the master plate and grown overnight at 37°C in L-broth/tetracycline (12.5μg/ml) supplemented with 0.2% maltose and 10mM MgSO4. 1ml of overnight culture was then added to 50ml of pre-warmed L broth + 0.4% maltose + 10mM MgSO4 and the cells incubated at 37°C with vigorous shaking until they reached an OD600 = 0.5. The culture was cooled on ice, spun at 3K 10min at 4°C, and the pellet resuspended in 15ml of ice-cold 10mM MgSO4. Aliquots of 150μl were used for transfection.

An appropriate amount of phage mixture in phage dilution buffer (5.8g NaCl + 2g MgSO4·7H2O + 5ml 1M Tris-HCl pH 7.5 + 5ml 2% gelatin per 1000ml H2O) was added to 150μl of competent cells and the mixture incubated at 37°C 15min. Top agar (LB + 7% agarose + 10mM MgSO4) was melted and placed at 42°C in a water bath. To each cell aliquot 4ml of liquid top agar were added, mixed quickly by inversion and poured onto a dry 90mm Petri dish containing L-agar.
2.1.24 Oligo Labelling

This method uses very small amounts of DNA (10 ng). Its advantage is the fact that the fragment to be labelled may be size fractionated by gel electrophoresis; also the method does not require that the DNA fragment to be labelled be recovered from the agarose in which it is fractionated (Feinberg & Volgestein, 1984).

The buffer was made up of three separate components: solution A (625 μl 2 M Tris–HCl pH 8.0, 18 μl 2-mercaptoethanol, 25 μl 5 M MgCl₂, 350 μl H₂O, 5 μl each 0.1 M dATP, dTTP, dGTP), solution B (2 M HEPES pH 6.6) and solution C (hexadeoxyribonucleotides - Pharmacia 27.2166.01 - suspended in 3 mM Tris–HCl pH 7.0, 0.2 mM EDTA at 90 OD units/ml). The solutions were mixed in the ratio 2:5:3, A:B:C.

The DNA fragment was purified by running the restriction digest releasing the cDNA clone in an 0.8% low melting point agarose gel. The fragment was visualised with UV light, cut from the agarose gel and the gel slice stored in an Eppendorf tube at -20°C. Prior to labelling, the Eppendorf was placed in boiling water for 7 min and then transferred to a 37°C water bath for 10 min. An aliquot (10 ng or 3 μl maximum) was now removed for labelling and the rest returned to -20°C for storage.

The labelling mix had a final volume of 15 μl and contained 3 μl buffer, 6 ng BSA, 10 ng DNA, 1.5 μl 32P–dCTP and 0.6 U of DNA Polymerase Klenow fragment (Pharmacia). The reaction was allowed to run at room temperature for 5 hours. It was stopped by the addition of 85 μl stop solution (20 mM NaCl, 20 mM Tris–HCl pH 7.5, 2 mM EDTA, 0.25% SDS) and boiled before addition to the hybridisation solution. The method gives specific activities of about 10⁸ cpm/μg of DNA.

2.1.25 First Strand cDNA as Hybridisation Probe

This is an alteration of the Gubler method, resulting in probes of specific activity of 10⁸ cpm/μg DNA (Gerard, 1988).

The reaction mixture contained 100 ng mRNA, 2 μl FS buffer (250 mM Tris–HCl pH 8.3 + 500 mM KCl + 50 mM MgCl₂ + 2.5 mM each of dATP, dTTP, dGTP + 0.05 mM dCTP), 0.5 μl DTT, 0.5 μl oligo dT (14-18) (Pharmacia), 2.5 μl 32P–dCTP (10 μCi/μl), H₂O to 9 μl, and 1 μl Reverse Transcriptase (nbl). The reaction was incubated at 42°C 1 h and TCA precipitation used to calculate the yield.
2.1.26 RNA Digestion of cDNA:RNA Hybrid

The method involved the use of NaOH as opposed to RNaseA, as further manipulations may involve RNA.

A 1/20 volume of 5M NaOH was added to the cDNA:RNA hybrid solution, and incubated at 80°C 5min. One time the original volume of 5M K acetate (60ml 5M K acetate + 11.5ml glacial acetic acid + 28.5ml H2O) was then added, followed by 3 volumes of ethanol. The sample was incubated at -70°C 30min, and spun to precipitate the DNA. A further ethanol precipitation followed, to remove any residual nucleotides.

2.1.27 Preparation of DDL probe

Cold first strand cDNA was synthesised from 0.5μg 5 day cells poly A⁺ RNA, and the RNA in the hybrid digested using NaOH (see Sections 2.1.17 and 2.1.26). The cDNA was mixed with 50ng of DDL oligomer, placed in a boiling water bath 5min, and cooled on ice. The synthesis reaction was performed in a total volume of 15μl and contained 3μl of AB buffer (see Section 2.1.39), 6ng BSA, 1.5μl 32p-dCTP and 1U of DNA Polymerase Klenow fragment (Pharmacia). The reaction was incubated at the appropriate temperature (see Results) for 3h, and the sample boiled for 7min prior to addition to the hybridisation solution.

2.1.28 Colony Hybridisation

Single E.coli colonies were grown overnight on a nitrocellulose disc (Schleicher and Schuell) placed on L agar. The nitrocellulose was then floated 5min on 1.5M NaCl, 0.5M NaOH to lyse the cells and denature the DNA, followed by 5min on 1.5M NaCl, 0.5M Tris-HCl pH7.5 (neutralising solution) and 5min on 2xSSC. The filters were then baked at 80°C 2h.

The hybridisation was carried out as described below:

Prehybridisation solution consisted of the following: 50% formamide, 6xSSC, 25mM Na phosphate buffer pH6.5, 100μg/ml sheared denatured salmon sperm DNA, 0.1% SDS, 5x Denhart’s solution (0.1% ficoll, 0.1% BSA, 0.1%PVP).

Hybridisation solution: prehybridisation solution + radiolabelled probe (2ng/ml).

The filters were bathed in prehybridisation solution at 42°C for 3h, the solution poured off, and the hybridisation solution added.
Hybridisation was at 42°C for 12-18h in a slow shaking incubator. The filters were then washed at 65°C with 2xSSC, 0.1% SDS twice for 15min, and with 0.2xSSC, 0.2% SDS twice for 30min, before exposure to X-ray film.

2.1.29 Plaque Hybridisation

Phage were plated out, using BB4 plating cells, at an appropriate dilution (400pfu per 90mm plates, 1500pfu per 140mm plates) onto L-plates using top agarose. The plates were incubated at 37°C overnight and then placed at 4°C 1h.

A number of nitrocellulose filters (Schleicher and Schuell) were numbered and placed carefully on the surface of the plates. The filters were allowed to wet, and then carefully peeled with blunt ended forceps. This process is more easily done if the top agarose contains agarose to replace the agar.

The filters were laid plaque side up on 3MM filter paper imbied in denaturing solution (0.5M NaOH, 1.5M NaCl) 5min, followed by neutralising solution (0.5M Tris-HCl pH7.0, 1.5M NaCl) 5min, and 2xSSC 5min. The filters were then air dried, baked at 80°C 1h, and used in filter hybridisations (see Colony Hybridisation, Section 2.1.28).

2.1.30 In situ amplification

This method was used to increase subsequent hybridisation signals, especially when screening with subtracted DNA (Vogeli and Kaytes, 1987).

BB4 cells were grown overnight in L broth. They were then centrifuged and resuspended in an equal volume of L broth + 10mM MgSO4.

Nitrocellulose filters (Schleicher and Schuell) were floated on the bacterial solution and air dried. The filters were subsequently laid, bacterial side down, on the surface of plates containing the plaques to be tested, allowed to wet, and peeled off carefully. The copy filters were laid plaque side up on fresh L plates containing 10mM MgSO4 and incubated overnight at 37°C. During the overnight growth at 37°C the plaques infect the growing E.coli, leading to a substantial amplification of phage DNA.

The next day the filters were removed from the plates, and treated as in the Plaque Hybridisation section (2.1.29).
2.1.31 Autoradiography

Southern, Northern, dot blots and colony hybridisations were set up on Amersham Hyperfilm MP X-ray film with two intensifier screens at -80°C. The length of the exposure depended on the intensity of the signal, but was usually overnight. 2-D gels containing 35S were exposed at -80°C without intensifier screen for 14 days. Sequencing gels were exposed overnight at room temperature without intensifier screen.

2.1.32 Large Scale Plasmid Preparation

Cleared lysate plasmid preparations were performed according to the method of Clewell and Helinski (1969).

Cells containing the plasmid of interest were grown overnight in 500ml of L broth and antibiotic (100µg/ml ampicillin in the case of pUC vectors, no antibiotics with M13).

After the overnight incubation, the cells were cilled to 4°C and spun 7K 0°C 10min. The pellet was resuspended in 3ml TSE (25% sucrose + 50mM Tris-HCL pH8.0 + 1mM EDTA). This was removed to a SS34 rotor tube, and 0.5ml of 10mg/ml lysosyme (Sigma) added. The mixture was stood on ice for 10min, and 5ml lytic mix (2% Triton x100 + 50mM Tris-HCL pH8.0 + 50mM EDTA) added. The tube was spun in an SS34 rotor 19K 60min.

The supernatant was transferred to a Corex tube and 0.92g/ml CsCl added, together with 75µl/ml of 10mg/ml ethidium bromide. The solution was mixed well to ensure all the CsCl had dissolved, and spun 15K 15min to remove any proteins. The supernatant was transferred to another Corex tube and its density measured and adjusted to 1.395, by addition of either water or CsCl as needed.

The solution was transferred to Beckman centrifuge tubes. These were spun overnight at 45K 15°C in a Ti80 rotor.

To extract the plasmid the tube was pierced at the top with a needle. A second 19G needle was then inserted in the tube underneath the band to be extracted and the plasmid band removed (Fig. 2.1). The band thus extracted was placed in a Corex tube. The ethidium bromide was removed with several volumes of isopropanol saturated with 20xSSC until the organic phase was clear. The CsCl was removed by dialysis against TE for 16 h at 4°C. The DNA was quantitated and stored at 4°C.
Figure 2.1 CsCl gradient to separate plasmid DNA from other nucleic acid impurities.
2.1.33 Small Scale Plasmid Preparation

These are based on the method of Birnboim and Doly (1979).

The cells containing the plasmids of interest were grown overnight in 3ml L-broth with the relevant antibiotic. 1.5ml of the cultures were then transferred to Eppendorf tubes and spun 15s. The liquid was poured off and the tubes placed on ice.

100μl of solution A (10mM EDTA + 25mM Tris-HCl pH8.0) were added, the pellets resuspended by vortexing, and 200μl solution B (27ml H2O + 2ml 3M NaOH + 1.2ml 25% SDS) added. The tubes were incubated on ice for 10min, and 150μl 3M Na acetate pH4.8 added.

The tubes were spun 10min, 430μl of supernatant was removed to a second Eppendorf tube, and 1ml ethanol added. The tubes were incubated at -70°C 30min and then spun 10min.

After drying the DNA pellet in a vacuum desiccator, the DNA was resuspended in 80μl of TE. Restriction digests required 5-10μl of DNA in a final reaction volume of 20μl.

2.1.34 Small Scale Phage Preparation

A plaque was cored out, using a sterile Pasteur pipette, into 0.5ml fresh BB4 plating cells (see Section 2.1.23) in a 15ml polypropylene centrifuge tube, and left 15min at room temperature to allow the cells to adsorb the phage. 5ml of L-broth containing 5mM CaCl2 were added, and the cells grown for 4h at 37°C with vigorous shaking. After the incubation period, 50μl chloroform were added, the cells shaken for 5min, and spun in a Sorvall RT6000B refrigerated centrifuge with an H1000B rotor, at 2.5K 10min.

100g of DEAE-cellulose DE52 (Sigma) were placed in a beaker and several volumes of 0.05N HCl added, until the pH read below 4.5. Concentrated NaOH was slowly added with constant stirring until the pH approached 7.5. The resin was now allowed to settle, the supernatant decanted, and the DE52 washed repeatedly with L-broth. A final slurry was made with 75% DE52 resin, 25% L-broth and 0.1% sodium azide to prevent growth of microorganisms, and stored at 4°C until needed.

4ml of phage lysate were mixed with 4ml of 20% PEG, 2M NaCl in phage dilution buffer, and left on ice 1h. The phage were pelleted by spinning at 2.5K 20min, and the supernatant removed by aspiration using a
drawn-out Pasteur pipette. The pellet was resuspended in 750μl L-broth and mixed by inversion with 750μl of DE52 slurry. Centrifugation was used to remove the DE52, and 750μl of the supernatant removed to an Eppendorf tube. 13μl of 0.1mg/ml proteinase K (Sigma) and 32μl 10% SDS were added to the tube, and the mixture left at room temperature 5min. 130μl of 3M K acetate were then added, the tube incubated at 88°C 20min, cooled on ice 10min, and centrifuged in a minifuge 10min. 800μl of the supernatant were transferred to a new Eppendorf tube, an equal volume of cold propan-2-ol added, and the tube placed at -70°C 10min. The solution was allowed to thaw, spun 10min, and the pellet dried and resuspended in 20μl TE. Restriction digests utilised an average of 5μl DNA solution.

2.1.35 Excision of pBluescript from λ-ZAP

200μl of BB4 plating cells (see Section 2.1.23), 200μl of phage stock and 10μl (10⁷ pfu) of R408 helper phage were combined in a 15ml polypropylene centrifuge tube and incubated at 37°C 15min. 5ml of 2xYT media was added, and the cells incubated 4-6h at 37°C with constant shaking.

The tubes were heated at 70°C 20min to neutralise the helper virus, spun 5min at 2.5K, and the supernatant decanted in a sterile tube. This contains the pBluescript plasmid packaged into the f1 (M13) phage particle.

The plasmid was plated by combining 200μl of BB4 plating cells to 100μl of phage stock, incubating at 37°C 15min, and plating on L-agar/ampicillin plates. Recombinant colonies were grown overnight at 37°C.

2.1.36 Restriction Digestion of DNA

Restriction digestions were carried out in the appropriate buffers as suggested by the manufacturers and generally up to 1μg of plasmid DNA in a volume of 20μl, and 1μg of chromosomal DNA in a volume of 15μl. The solutions were mixed and incubated at 37°C for 2h. The restriction enzymes were inactivated by extraction with phenol or heat (65°C, 5min). Samples were stored at -20°C until needed.
2.1.37 Agarose Gel Electrophoresis of DNA Fragments

Electrophoresis was carried out at 80V for 2 hours or 18V overnight in 1xElfo buffer (4.84g Tris base, 2ml 0.5M EDTA per 1000ml H₂O at pH7.7 with glacial acetic acid + 5µg/ml of ethidium bromide). The gels contained 0.6-2% agarose (ICN Biomedicals Ltd) made up in 1xElfo buffer (see above). Restricted DNA samples were mixed with a fifth volume of loading buffer (25% glycerol + 1% SDS + 0.01% bromophenol blue + 0.01% xylene cyanol), and electrophoresed. DNA bands were visualised on a FOTO Dyne UV (300nm) transilluminator and photographed with a Polaroid MP4 land camera through an orange Kodak "Wratten" filter.

2.1.38 Electroelution of DNA from Agarose Slices

The DNA was purified by running the restriction digest releasing the required fragment in a 0.8% agarose gel. The fragment was visualised with UV light, cut from the agarose gel, and the gel slice placed in the basin of the electroeluter (Fig. 2.2). The electroeluter was filled with elution buffer (10mM Tris–HCl pH 8.0 + 1mM EDTA + 5mM NaCl) and a salt cushion of 60µl 3M Na acetate pH 7.9 + Bromophenol blue (for visualisation) placed at the bottom of the V-shaped well via the access tube. The DNA was then electroeluted from the slice by applying a potential of 150V for 45min. The DNA migrates away from the agarose slice, enters the well, and is trapped in the salt cushion. After 45min, the current was switched off, the salt cushion removed via the access tube to an Eppendorf tube (300µl) and the DNA ethanol precipitated.

2.1.39 End Labelling of λ-HindIII Cut Recessed 3' Ends

The reaction was carried out using an AB buffer made up of 2vol solution A + 5vol solution B + 3vol H₂O (see Oligonucleotide labelling, Section 2.1.24). The reaction volume was 15µl and comprised 1µg DNA, 3µl AB buffer, 6ng BSA (BRL), 10µCi ³²P–dCTP and 0.2µl Klenow (Pharmacia). The reaction was allowed to run for 1h at room temperature and the labelled DNA was stored at 4°C. 10⁵ cpm were loaded per track in agarose gels to be used for Southern blotting.
Figure 2.2 Diagram of elecroeluter.
2.1.40 Southern Transfer

Detection of sequences homologous to radioactive probes was
accomplished by the transfer of DNA from agarose gels to Schleicher and
Schuell nitrocellulose filters as described by Southern (1975). Maniatis
et al. (1982) have revised the procedure which was followed with the
additional step of depurinating the agarose gel prior to denaturing to
assist in more efficient transfer of high molecular weight DNA.

The gels were run using the agarose gel electrophoresis system
(Section 2.1.37). They were then depurinated in 250mM HCl 15min,
denatured in 500mM NaCl, 1.5M NaCl 30min, and neutralised in 0.5M NaCl,
0.5M Tris-HCl pH7.5 60min. The gel was placed on a blotting sponge
partially immersed in 20xSSC, overlayed with nitrocellulose pre-soaked in
3xSSC, followed by Whatman 3MM paper and a stack of paper towels weighed
down with a glass plate, ensuring all air bubbles were excluded (see
diagram below). After blotting overnight, the filter was rinsed briefly
in 3xSSC, air dried and baked at 80°C 2h. The hybridisation conditions
were as per Section 2.1.28, with the only alteration of washing the
filters at 42°C.

2.1.41 Northern Blotting

Each sample consisted of one volume of RNA (normally 100ng poly
A+/µl), one volume of LiCl buffer (1M LiCl, 0.05M EDTA, 2% SDS, 0.01M
Tris-HCl pH6.5) and 8 volumes of deionised DMSO. The gel was a 2% agarose
gel made up in MOPS (2.09g Na-MOPS, 0.37g Na acetate, 0.18g Na2-EDTA at
pH7.0 in 500ml of H2O) + 1/6 vol formaldehyde. The samples were run in
MOPS buffer at 150mA for two hours and the gel rinsed in 20xSSC for 30
min followed by blotting onto nitrocellulose as with the Southern
transfer. The nitrocellulose was then baked at 80°C for 2 h prior to
hybridisation.
2.1.42 Dot Blots

The nucleic acid was dissolved in an appropriate buffer (1M NaCl, 0.1M NaOH, 10mM EDTA for DNA; 6xSSC for RNA), heated to denature it (100°C, 2min) and placed on ice. Samples were blotted onto nitrocellulose (Schleicher and Schuell) presoaked in 20xSSC using a BRL 'Hybri-Dot' manifold. The nitrocellulose was baked at 80°C for 2h prior to hybridisation.

2.1.43 Hybrid Release

The DNA clone to be used in the selection was dissolved in water (500μg/ml) heated to 100°C 10min, and chilled on ice. An equal volume of 1M NaOH was added, and the solution incubated at room temperature 20min. An equal volume of neutralising solution (1M NaCl + 0.3M Na citrate + 0.5M Tris-HCl pH 8.0) was added, and the solution left on ice. 5μg of DNA were spotted on a 3x3mm square of Schleicher and Schuell nitrocellulose, the filter air dried, washed in 50ml 6xSSC, and baked at 80°C 2h.

The nitrocellulose filter was placed in 1ml H2O, heated in a boiling water bath 1min, and the water removed. 250μl hybridisation buffer (65% deionised formamide + 20mM PIPES pH 6.4 + 0.2% SDS + 0.4 M NaCl + 100μg/ml tRNA (Sigma) + 10μg/ml poly A (Sigma) + 10μg/ml poly C (Sigma) + 100μg/ml denatured sheared salmon sperm DNA (Sigma)) were added, and the filter incubated at 50°C overnight.

10μg poly A+ RNA from 5 day cells was precipitated, the pellet dissolved in 250μl hybridisation buffer, and heated to 70°C 10min. The filter was placed in this solution, and incubated at 50°C 3h. The mRNA solution was removed and the filter washed at 65°C, 10 times with lml buffer 1 (10mM Tris-HCl pH 7.6 + 0.15M NaCl + 1mM EDTA + 0.5% SDS), and twice with lml buffer 2 (10mM Tris-HCl pH 7.6 + 0.15M NaCl + 1mM EDTA). The filter was now placed in 300μl H2O with 20μg tRNA, incubated in a boiling water bath 10min, and snap freezed in a dry ice/ethanol bath (-70°C). The solution was allowed to thaw, the filter removed, and the eluted mRNA precipitated with ethanol. The pellet was then resuspended in 4μl H2O, and half the volume used in an in vitro translation.
2.1.44 DNA M13 Sequencing-Template Preparation

Overnight cultures of *E. coli* JM103 were diluted 200-fold into 2ml of L-broth, grown for 1h at 37°C, and inoculated with M13 single plaques. The cultures were incubated at 37°C with fast shaking (300rpm) for 7h, decanted into Eppendorf tubes and centrifuged for 5min. To 1ml supernatant, 200μl of 20% PEG 6000, 2.5M NaCl was added, mixed and left at room temperature for 15min. The phage was pelleted for 10min, the supernatant removed by aspiration using a drawn-out Pasteur pipette, and resuspended in 100μl TE (10mM Tris-HCl pH 7.5 + 1mM EDTA). The suspension was extracted by vortexing with 50μl phenol, allowed to stand 5min and vortexed again. After centrifugation for 2min, the phenol extraction was repeated, followed by extraction with chloroform:isooamylalcohol (48:2). DNA was precipitated with 0.1 volume of 2M sodium acetate pH5.5, and 2.5 volumes ethanol at -70°C 1h, centrifuged 10min, washed with 70% ethanol, vacuum dried, and resuspended in 50μl TE.

2.1.45 DNA pBluescript Sequencing-Template Preparation

BB4 cells containing the plasmid were grown overnight in the presence of tetracycline to allow for selection of the F’ episome. 3ml of 2xYT were inoculated with 300μl of the overnight culture, and shaken at 37°C up to OD600=0.3. Helper phage R408 was then added (50μl, or 5x10⁹ pfu), and the culture grown for a further 8 hours.

The cells were spun down and the supernatant transferred to Eppendorf tubes. 1/4 volume of 3.5M NH₄OAc pH 7.5, 20% PEG was added, the solution mixed by inversion, and left to stand at room temperature 15min. The tubes were spun 20min, and the supernatant aspirated using a drawn-out Pasteur pipette.

The pellet was resuspended in 200μl TE, 200μl phenol:chloroform (1:1) added, and the tube vortexed for 1min, left to stand 1min, vortexed 1min, and spun 3min. The aqueous layer was removed to a fresh Eppendorf tube and the phenol:chloroform extraction repeated.

The ssDNA was precipitated by adding 150μl 7.5M NH₄OAc pH 7.5 and 600μl cold ethanol, incubating on ice 15min, and spinning at 4°C 20min. The pellet was dried, and redissolved in 20μl H₂O.
2.1.46 DNA Sequencing

This was carried out by the method of Sanger et al. (1977).

For 10 templates, primer mix was prepared with 5μl primer (17-base single stranded universal primer, 2μg/ml), 33μl SEQ buffer (100mM Tris-HCl pH7.5 + 50mM MgCl2), 42μl H2O. For each template 4 tubes were prepared and 2μl primer mix added, followed by 2μl template. Annealing was carried out at 65°C for 5min; the water bath was then allowed to slowly cool down to room temperature over a 30 minute period.

2μl of the appropriate sequencing mix (see Table 2.1) were added, followed by 2μl Klenow mix (7.8μl TE + 0.22μl Klenow + 1μl 35S-dATP 10mCi/ml). The assays were incubated at room temperature for 35min, and 2μl chase mix (0.5mM of each dCTP, dATP, dGTP, dTTP) added and left a further 15min.

2μl formamide dye (90% formamide + 50mM EDTA + 0.02% bromophenol blue + 0.02% xylene cyanol) were added, and the tubes heated to 80°C with open tops for 7min to reduce the volume of liquid prior to electrophoresis.

Sequencing gels were 39cm long and were prepared as single thickness gels (0.4mm spacers). Acrylamide solution was prepared by mixing 8.55g acrylamide, 0.45g bisacrylamide, 75g urea, 15ml 10xTBE (108g Tris base + 55g boric acid + 40ml 0.5M EDTA pH8.0 in 1000ml H2O), and made up to 150ml with H2O. To this 1ml of 10% ammonium persulphate and 36μl TEMED were added prior to pouring the gel.

The gel was prefocused at 900V 15min, and samples loaded with the use of a saw-tooth comb and electrophoresed at 1500V/60W until the lower tracking dye reached the bottom of the gel. The gel was fixed in 10% acetic acid, 10% ethanol 15min and dried down onto Whatman 3MM filter paper on a heated gel drier. Autoradiography was at room temperature overnight without intensifying screens.

2.1.47 DNA Binding to Epoxy cellulose

The Epoxy-activated cellulose was purchased from BRL, and the DNA bound to it (Moss et al., 1981).

150μg DNA were added to 50mg of activated cellulose in 0.1N NaOH solution to a final volume of 220μl and mixed in an Eppendorf tube. The slurry was pipetted onto a standard siliconised microscope slide and
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Nucleotide mixes used in the DNA sequencing reactions.
spread to half the slide surface area. This was allowed to sit at 21°C in 100% humidity for 6h and then air dried for an additional 2h. The mixture was carefully scraped off the glass surface with a razor blade into an Eppendorf tube and mixed with 1ml H₂O by vortexing. The DNA-cellulose was separated from the aqueous solution by low speed centrifugation and washed until no more DNA could be seen in the supernatant by scanning at 260nm. The resin was then reacted with 2M ethanolamine and 50mM Na borate pH 8.5 to inactivate unreacted oxirane groups.

Binding experiments were carried out with sheared salmon sperm DNA. This was heated to 100°C 5min, and quickly cooled on ice prior to addition to the reaction mixture.

2.1.48 Solution Hybridisation for Probe Enrichment (Solution Method)

Here we refer to the "negative" population as the mRNA population we use as the standard against which we hope to pick differentially expressed genes. In the case of the Blue Lace system, this is mRNA extracted from seedlings.

10μg of mRNA from the "negative" population were precipitated and resuspended in 20μl of hybridisation buffer (80% formamide + 0.4M NaCl + 40mM PIPES pH 6.4 + 1mM EDTA + 50μg/ml oligo dT(14-18) (Pharmacia)). The solution was added to cell culture 32P-labelled cDNA:RNA hybrid previously digested with NaOH to remove the RNA (see Section 2.1.26), heated at 80°C 15min, then placed at 49°C overnight.

The sample was run down a hydroxylapatite column to separate single stranded probe, and the column eluate mixed with 2x standard hybridisation buffer. This probe was used in the plaque hybridisation protocol.

2.1.49 Hydroxylapatite Chromatography

Hydroxylapatite is a form of insoluble calcium phosphate that binds nucleic acids. Double-stranded molecules are bound more tightly than single stranded, and both can be released by treatment with phosphate buffers.

Chromatography was carried out at room temperature in a Pasteur pipette, plugged with polyallomer wool, containing 0.5g hydroxylapatite (BioRad) per 100μg RNA. A terminated solution hybridisation reaction (see
Section 2.1.48) was diluted in 300μl 0.01M NaH₂PO₄ pH6.8, and applied to the column until all radioactivity remained within the column (3-4 times). The column was then washed with 1ml 0.001M Na₂PO₄ pH6.8 buffer.

Single stranded probe was eluted with 1.5ml of 0.13M Na₂PO₄ pH6.8. This value had previously been determined by chromatographing a mixture of single- and double-stranded salmon sperm DNA, and eluting the separate fractions using an increasing concentration gradient of Na₂PO₄ pH6.8 buffer.

2.1.50 Western Blotting

The method involved the use of a semi-dry electoblotter, consisting of two 20x20cm graphite electrodes fitted onto plastic supports. The transfer buffer consisted of 25mM Tris, 192 mM glycine and 20% methanol. All components of the transfer "sandwich" were soaked in the buffer prior to assembly. The proteins to be transferred were run on a PAGE gel. A transfer sandwich was then constructed as in the diagram below, ensuring all air bubbles were excluded.

```
Anode (+)

Capillary mat

3MM filter paper

Nitrocellulose

Gel

3MM filter paper

Capillary mat

Cathode (-)
```

The electrodes were connected to a power supply and run at a constant current of 750mA 1h.

After transfer the blot was transiently stained for protein using 0.2% Ponceau S in 3% TCA, for 5min, followed by washing in water.

2.1.51 Antibody Production to DDI

The production followed the method outlined by Chiles (Chiles et al., 1987). Total protein extract from 8 week cultures was separated by 2D-PAGE and transferred to nitrocellulose by Western blotting. M16 P5.6a
was localised by staining with Ponceau S (0.2% in 3% TCA), the peptide excised, and destained in TBS-Tween (500mM Tris-HCl pH 7.4 + 192mM glycine + 0.1% Tween). The nitrocellulose was then washed thoroughly in TBS (500mM Tris-HCl pH 7.4 + 192mM glycine), rolled into the shape of a cylinder, and inserted into the bore of a 16-gauge hypodermic needle. The nitrocellulose was implanted subcutaneously in the rabbit by expelling it with a stainless-steel rod. The implantation was accompanied by an injection of Freund's adjuvant.

The rabbits were injected with antigen every two weeks and their response monitored by regular bleeding. All manipulations of the animals were carried out by the Animal House staff in the Medical Unit of the University.

2.1.52 Immunoblotting

The method uses alkaline phosphatase-conjugated secondary antibody.

A Western blot was blocked with TBS-Tween (50mM Tris-HCl pH 7.4 + 192mM glycine + 0.1% Tween20) containing 3% Marvel (Cadbury's) at 37°C for 30min. If *E. coli* proteins were present in the total protein extract to be immunoblotted, a 1:30 dilution of λ blocker (see Section 2.1.55) was added to the blocking solution.

It was then incubated with the primary antibody in TBS (50mM Tris-HCl pH 7.4 + 192mM Glycine) containing 3% Marvel (see results for dilutions of antibody used) in a sealed bag 1h at room temperature. Washes were with TBS-Tween for 10min each. Three washes were generally used.

A 1:500 dilution of alkaline phosphatase-conjugated anti-primary antibody (Sigma) diluted in TBS with 3% Marvel was then used, for 30min at room temperature in a sealed bag. Three 10min washes of TBS-Tween followed.

The blot was then equilibrated in substrate buffer (100mM Tris-HCl pH 9.5 + 100mM NaCl + 5mM MgCl2), and developed. The developing solution contained BCIP (5-bromo-4-chloro-3-indoyl-phosphate, 50mg/ml in DMF (dimethyl sulphonate, Sigma)), at 50μl/5ml substrate buffer.
2.1.53 Protein Transfer to PVDF membrane

PVDF is polyvinylidene difluoride (Millipore). The method used a semi-dry elecroblotter.

The transfer buffer consisted of 48mM Tris, 39mM glycine and 10% methanol. The filter paper and gel components of the transfer "sandwich" were soaked in the buffer prior to assembly, while the PVDF membrane was soaked in methanol and washed twice in water.

The protein(s) to be transferred were run on a PAGE gel. A transfer sandwich was then constructed as in the diagram below, ensuring all air bubbles were excluded.

\[
\begin{align*}
\text{Anode (+)} & \quad \text{Cathode (-)} \\
\text{3M filter paper, 3 sheets} & \quad \text{3M filter paper, 3 sheets} \\
\text{PVDF membrane} & \quad \text{Gel} \\
\end{align*}
\]

The electrodes were connected to a power supply and run at a constant current of 0.8mA/cm² of gel 3h at 4°C.

After transfer, the blot was stained for protein using 0.2% Comassie blue in 50% methanol for 5min, followed by destaining in 50% methanol and 10% acetic acid. The membrane was then placed in a sealed bag and stored at -20°C.

2.1.54 Expression of Clone 34

3ml of L-broth/ampicillin were inoculated with 300μl of an overnight culture of BB4 and shaken at 37°C up to OD600 = 0.3. 10mM IPTG was added, and the cells incubated at 37°C 3h, with occasional shaking.

The cells were pelleted by centrifugation (2min), resuspended in 100μl cracking buffer, boiled for 3min, and the debris pelleted. Approximately 50μl were loaded onto PAGE for immunoblotting.
2.1.55 Production of λ Blocker

BB4 plating cells were infected with wild type λ-ZAP in the presence of 10mM MgCl₂ at 37°C for 15min. The cells were added to 11 of NZM medium (5g NaCl + 10g NZ amide + 2.03g MgCl₂) and shaken at 37°C overnight.

5ml chloroform were added to the culture, and this returned to the shaker for 15min. The cells were removed by spinning at 6000g 30min, and the proteins in the supernatant precipitated by addition of 80% NH₄SO₄ (516g/l) and incubating at 5°C 1h with stirring. The proteins were collected by spinning at 6000g 30min, the pellet resuspended in 20ml TE (10mM Tris-HCl pH 7.5 + 1mM EDTA) and dialysed overnight against TE. The suspension was stored at 4°C.

The blocker was added in a 1:30 dilution to the blocker in immunoblotting reactions (see Section 2.1.52) whenever λ lambda or E. coli proteins were known to be present in the protein samples being immunoblotted.

2.1.56 Immunoprecipitation

Three *in vitro* translation reactions were pooled and added to 200µl of solution A (10mM Tris-HCl pH 7.5 + 20mM EDTA + 15mM NaCl + 0.5mg/ml RNase A (Sigma)), incubated at room temperature 30min and spun 1min in a minifuge. The supernatant was added to 200µl solution B (25mM Tris-HCl pH 7.4 + 10mM EDTA + 350mM NaCl + 0.1% SDS + 1% Na deoxycholate + 1% Triton X-100) and 100µl of antibody in a fresh Eppendorf tube. The tube was placed on a rotor at room temperature 3h.

Anti-rabbit-agarose (Sigma) was washed several times with solution B. 200µl of the agarose slurry were added to the Eppendorf containing the antibody/*in vitro* mixture, and placed back on the rotor at room temperature 3h. The agarose was sedimented by spinning at low speed in a microfuge, and washed several times with solution B. The agarose was finally resuspended in 40µl cracking buffer, boiled 3min, and the supernatant run on PAGE.
Growth and collection of *Petunia* seedlings material

*Petunia hybrids* cv. Blue Lace seeds were soaked in water overnight and spread on Vermiculite trays. The seeds were then allowed to germinate in growth conditions of 25°C and 200-280 μmol m⁻² sec⁻² illumination (day length 14h). 5 weeks after sowing, the plantlets were picked, the Vermiculite washed off the root system, and the plant material stored under liquid nitrogen.

Protein staining in PAGE

Gels were soaked in Comassie solution (25% methanol + 10% glacial acetic acid + 100mg/l Comassie Blue) at 37°C 30min with gentle shaking. The gels were then removed to destaining solution (25% methanol + 10% glacial acetic acid) and placed on an orbital shaker at room temperature. The destaining solution was replaced every hour, until the required staining intensity was achieved.
2.2 VECTORS AND HOST STRAINS

**PUC9**
A 2297 \text{Pvu II} \text{EcoR I} fragment of pBR322, with inserted a 433bp HaeII \text{lacZ} fragment containing the polylinker sequence from M13mp9 (Vieira and Messing, 1982).

**λ-ZAP**
An engineered lambda vector, it contains the phagemid vector pBluescript SK(−) replacing the red\(^+\), gam\(^+\) internal fragment. A number of unique cloning sites can accept foreign inserts of 0-10Kb within the pBluescript sequence. The phagemid can be automatically excised by a helper phage and recircularised to generate plasmid-carried subclones.

**pBluescript SK(−)**
An engineered ColEl derivative that carries a gene for ampicillin resistance, an inducible lac promoter upstream of the N-terminal coding region of β-galactosidase, and an f1-phage origin. The plasmid contains a synthetic polylinker containing 21 unique restriction sites, flanked by T7 and T3 RNA polymerase promoters located within the N-terminal coding portion of β-galactosidase. The insertion of the T7 promoter/multiple cloning site/T3 promoter sequence, which does not interfere with α-complementation, is located in the proper reading from within the N-terminal coding region of the β-galactosidase gene.

**JM 103**
An \textit{E. coli} strain
Genotype Δlac, pro, supE, thi, F\(^'\), tra, D36, proAB, lacI\(^a\), 2ΔM15, RecA\(^−\)
Grown on minimal media, plus 2mg/l thiamine, and 10μM FeCl\(_3\).
BB4
An *E. coli* strain:
Genotype supF58, supE44, hsdR514 (rk−, mk−), galK2, galT22, trpR55, metB1, tonA, λ−, Δ(arg-lac)U169 [F', proAB, lacI^Q^ZΔM15, Tn10(tet^R)]

XL1-Blue
An *E. coli* strain
Genotype endA1, hsdR17 (rk−, mk^+^), supE44, thi-l, λ−, recA1, gyrA96, relA1, (lac−) [F', proAB, lacI^Q^ZΔM15, Tn10(tet^R)]
2.3 MEDIA

2.3.1 MS Basic Salts

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl(\cdot)2H(2)O</td>
<td>440.000</td>
</tr>
<tr>
<td>NH(4)NO(3)</td>
<td>1650.000</td>
</tr>
<tr>
<td>KNO(3)</td>
<td>1900.000</td>
</tr>
<tr>
<td>KI</td>
<td>0.830</td>
</tr>
<tr>
<td>CoCl(2)(\cdot)6H(2)O</td>
<td>0.025</td>
</tr>
<tr>
<td>KH(2)PO(4)</td>
<td>170.000</td>
</tr>
<tr>
<td>H(3)BO(4)</td>
<td>6.200</td>
</tr>
<tr>
<td>Na(2)MoO(4)(\cdot)2H(2)O</td>
<td>0.025</td>
</tr>
<tr>
<td>MgSO(4)(\cdot)7H(2)O</td>
<td>370.000</td>
</tr>
<tr>
<td>MnSO(4)(\cdot)4H(2)O</td>
<td>22.000</td>
</tr>
<tr>
<td>CuSO(4)(\cdot)5H(2)O</td>
<td>0.250</td>
</tr>
<tr>
<td>ZnSO(4)(\cdot)7H(2)O</td>
<td>8.600</td>
</tr>
<tr>
<td>Fe Na EDTA</td>
<td>36.700</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.000</td>
</tr>
<tr>
<td>Inositol</td>
<td>100.000</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.500</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.500</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.900</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30000.000</td>
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2.3.2 UM Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D(+)</td>
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</tr>
<tr>
<td>Kinetin</td>
<td>0.25</td>
</tr>
<tr>
<td>Thiamine</td>
<td>9.90</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>4.50</td>
</tr>
<tr>
<td>Casein Hydrolysate</td>
<td>2000.00</td>
</tr>
</tbody>
</table>

pH 5.8 with NaOH
2.3.3 Asparagus Medium

Modified from Nagata and Takebe (1971)

NH₄NO₃ " 825.000mg/l
KNO₃ " 925.000 "
CaCl₂·2H₂O " 220.000 "
MgSO₄·H₂O " 1233.000 "
KH₂PO₄ " 680.000 "
Na₂EDTA " 37.300 "
FeSO₄·7H₂O " 27.800 "
H₃BO₃ " 6.200 "
MnSO₄·4H₂O " 22.300 "
ZnSO₄·7H₂O " 10.580 "
KI " 0.830 "
NaMoO₄·2H₂O " 0.250 "
CuSO₄·5H₂O " 0.030 "
Mannitol " 30000.000 "
Sucrose " 10000.000 "
Myo Inositol " 100.000 "
Thiamine HCl " 1.000 "
NAA " 1.000 "
6-BAP " 0.300 "

pH5.8 with KOH

Just prior to use, add 3.4ml of filter sterilised glutamine (2.35g/100ml) per 80ml aliquot of medium.

2.3.4 Luria Broth

Bacto tryptone at 10.0g/l
Bacto Yeast extract " 5.0 "
NaCl " 10.0 "

pH7.5 with NaOH

L agar plates contain 1.5% agar
L soft top contains 0.7% agarose
2.3.5 2% Broth
2% Difco Bacto Tryptone
0.5% Difco Bacto Yeast extract
0.4% MgSO4
10mM KCl

2.3.6 Minimal Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na4PO4</td>
<td>at 6.0 g/l</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>&quot; 3.0 &quot;</td>
</tr>
<tr>
<td>A) NaCl</td>
<td>&quot; 0.5 &quot;</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>&quot; 1.0 &quot;</td>
</tr>
</tbody>
</table>

pH 7.4

B) 1M MgSO4       | " 2.0 ml/l    |
C) 20% glucose    | " 10.0 "      |
D) 1M CaCl2       | " 0.1 "       |

B, C, and D autoclaved separately and added prior to use.

When making plates 2x salts and 2x(3%) agar solutions are made and autoclaved separately; they are mixed just prior to pouring.

2.3.7 2xYT Broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto tryptone</td>
<td>at 16g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>&quot; 10 &quot;</td>
</tr>
<tr>
<td>NaCl</td>
<td>&quot; 10 &quot;</td>
</tr>
</tbody>
</table>

pH 7.2-7.4 with NaOH
CHAPTER THREE

CELLULAR AND MOLECULAR CHARACTERISATION OF PETUNIA BLUE LACE CELL CULTURE
3.1.2 General Features of Callus and Suspension Cell Cultures

3.1.2.1 Callus Culture

Callus cultures consist of an amorphous mass of cells growing on solid medium. During incubation the biomass of this tissue increases; this increase occurs only for a limited time and the culture reaches a point of maximum yield of cell material. If at this point portions of these calli are subcultured, growth will be resumed. Work on nucleic acid synthesis in calli has shown that growth occurs concomitantly with periods of increased synthesis producing transient periods of accumulation of RNA at discrete stages (Aitchinson, 1977). At the same time, the growth pattern occurring after each subculturing seems to vary when compared to the previous growth cycle, and no reproducible growth curve, as seen in suspension cultures (Fig. 3.1) has been produced.

Most of the work carried out using plant calli has been useful as the system does allow some separation of tissue structure from function, and the possibility of distinguishing primary responses to hormone changes or stress, from the multitude of secondary responses caused by such stimuli in the intact plant. However for the above conditions to be fulfilled, a complete physical separation between cells if required, and this is not the case in callus. Indeed, calli grown on solid media show the presence of nutritional and hormonal gradients within the tissue, which make the definition of experimental conditions difficult. The different biological responses to known culture conditions can be seen in the example of Ruta graveolens (Reinhard, Corduan and Volk, 1968) where the pattern of coumarins present in the suspension cultures differ from those present in callus cultures of the same plant. At the same time, callus and suspension systems have sometimes been shown to display similar responses, such as has been shown in some vascular differentiation experiments using beans (Bolwell and Northcote, 1983b). Here, a relation of arabinan and xylan synthase and PAL activity to primary and secondary wall formation and lignification during cell division, growth and differentiation similar to that found in callus, has been demonstrated in cell cultures.

On the whole, while callus culture may be more useful in practical studies on totipotency, suspension cultures have been used in an attempt
Figure 3.1 Increase in cell number per unit volume of culture to time in a typical batch cell suspension culture.
to define the biochemical pathways of plant metabolism and their capacity to respond to environmental/external stimuli.

3.1.2.2 Suspension Cultures

Established batch suspension cultures consist of cells and cell aggregates dispersed and growing in moving liquid medium. During incubation the amount of cell material increases; this increase occurs only for a limited time and the culture reaches a point of maximum yield of biomass. If at this point the culture is diluted back by subculture to a cellular content similar to the one at the beginning of the antecedent culture, it will, in a subsequent and similar incubation period, go through a similar pattern of growth and yield a similar amount of cell material. In this way the culture can be propagated *ad infinitum* by successive subculturing into fresh media.

The earliest suspensions of cultured plant cells capable of repeated subculture (Muir, Hilderbrant and Riker, 1954) were developed as a source of single viable cells from which single-cell clones could be established; these were derived from *Nicotiana tabacum* and *Tagetes erecta* plants. Similar suspensions of free cells and aggregates were also obtained from cultured carrot root explants (Steward and Shantz, 1956).

There are two main types of cell suspension cultures in use today: batch cultures and continuous cultures. Most of the published physiological and chemical biochemical investigations of growth and cell division in higher plant cell cultures have been carried out on batch cultures. This technique involves the isolation of an inoculum of cells in a finite volume of nutrient medium in a system which is closed except for exchange of gases and volatile metabolites with the outside air. The change with time in the number of cells present in a typical batch culture follows the course shown in Fig. 3.1. This pattern of growth over the span from initiation to the next subculture of stationary phase cells is termed the growth cycle. The rate of increase in cell number during this growth cycle changes almost continuously from the initiation to the cessation of cell division. The occurrence and duration of each phase shown in the model curve depends very much upon the cell type, the frequency of subculture, the initial density and the culture medium used (King and Street, 1973).
In batch cultures, cell division and the synthesis of metabolites are initiated or cease at different points in time. The cells are therefore said to be in a state of unbalanced growth. This may limit the value of batch cultures in the study of metabolic regulation in actively dividing cell cultures (King and Street, 1973), as this phase is so transient within the batch culture's growth cycle. A technique which realises balanced growth is continuous culture. Here the culture is continuously supplied with nutrients by the inflow of fresh medium, while keeping the culture volume constant. Under these conditions the rates of biosynthesis and cell division in a random population of cells are constant and equal, and therefore the main composition of cells does not vary with time.

In typical batch suspension cultures, the growth profile follows the course shown in Fig. 3.1, while in continuous cultures cells are always in their exponential phase of growth. But in neither of the two culture types can cells be said to be behaving in a synchronous fashion. In a synchronous system, changes in the culture as a whole are an amplification of events of the cell cycle of an individual cell. This would enable a detailed description of the events of the cell cycle and allow identification of the factors operating to control the orderly sequence of biochemical events which separate one generation of cells from its daughter generation. Synchrony has been reported to occur *in planta* during female gametophyte and pro-embryo development in a number of conifers (King and Street, 1973). Cells in culture may be artificially synchronised by external means such as nutrient deficiency (Gould et al., 1981), hormone starvation (Bayliss, 1985), hormone addition (Roberts and Northcote, 1970) and ethylene (Constabel et al., 1971).

Cell suspension cultures have been used to study metabolism and growth of dedifferentiated plant cells. In a batch culture, inoculation with starved cells initiates a new growth cycle which includes the transport of available nutrients, the formation of cellular metabolic pools and the activation of the cellular anabolic capacity. This growth cycle comprises four main stages, the lag phase, cell division, cell elongation and the stationary phase (Thom et al., 1981). The lag phase is characterised by uptake of medium phosphate to establish a high cellular
phosphate concentration which stimulates nucleotide synthesis (Meyer and Wagner, 1985a). The cell division phase is characterised by the complete uptake of medium sucrose; also the contents of nucleotides, RNA and protein attain their maximum levels and the rate of DNA synthesis is at its peak (Wylegall, Meyer and Wagner, 1985). The cell elongation phase starts when the cellular phosphate pool decreases below a low threshold value, provided the carbon-source is not exhausted; it is characterised by a halt in nucleotide, protein and RNA synthesis, coupled with an increase in cell mass (Wylegall, Meyer and Wagner, 1985; Meyer and Wagner, 1985b). The starvation, or stationary, phase occurs when both the phosphate and carbon sources within the media have been exhausted. It is interesting to note that, even during starvation, important internal cellular pools such as nucleotide pools are never completely exhausted (Meyer and Wagner, 1985a).

3.1.2.3 Primary Metabolism of Cell Suspension Cultures

During development and differentiation a cell undergoes a complex sequence of metabolic alterations involving synthesis of novel enzymes and the loss of others. Changes in the protein content of the cell during its development may imply differences in the rate of synthesis of the individual protein components which are correlated with the changes in several enzymes closely related to each other (Verma and Van Huystee, 1970). Accordingly morphological and physiological changes during plant development are associated with changes in protein constitution which are not only characteristic of different organs but also of different stages of development within the same organ. Metabolism studies within the whole plant are complicated by the array of different tissues performing varied functions. For example, a plant will have photoautotrophic (mesophyll) and heterotrophic (meristem) cells, as well as anabolic (storage organs) and catabolic (root hairs) cells, and cells in various differentiated states. In contrast, cultured cells are structurally simpler, with fewer variations in the overall metabolic patterns.

Associated with growth and structural changes in culture, are changes in physiological activity (Street, 1977). Measurement, on a per cell basis, of individual enzymes show that peaks of activity occur. These may be quite sharp and are not coincidental. Even when maintained
under apparent stable culture conditions, cultured cells exhibit variation in morphology and metabolism which is the product of the physiological and genetic constitution of the original cells and the "developmental stimuli" supplied in the form of the culture conditions (Lindsey and Yeoman, 1985). Primary metabolism in the cells will affect cell division, cell expansion, synthesis of macromolecules, and gene expression. Cell metabolism in tissue culture will be affected by the culture conditions such as nutrient availability, presence of growth regulators, and stress. Thus when examining gene expression in such systems, it is important to be aware of any media or environmental factors that may affect gene expression.

3.1.3 Factors Affecting Gene Expression in Cultured Cells

3.1.3.1 Nutrient Availability and Utilisation

Selective permeability of living cells to concentrate important nutrients against a concentration gradient has become a well-established phenomenon in plant and animal tissues, as well as in microorganisms. The facilitation of movement of organic substances across membrane barriers by specific and active transport systems appears to be an essential feature common to all biological systems.

One of the key factors in the well being of a culture is its ability to transport nutrients from the media into the cells. Sucrose, glucose, fructose or any combination of these sugars may be used to support growth of cell cultures. While some cell cultures grow equally well in any of these carbon sources (Ferguson, Street and Davis, 1958), other show a clear preference for a specific sugar. This may be because of the inability of the cells to take up these sugars (Chin et al., 1981) or because of the presence of a sucrose (Daie et al., 1987; Lemoine et al., 1988) or glucose (Marezki and Thom, 1972) carrier in the cells' plasmolemma. Changes in the carbohydrate status of a cell have also been demonstrated to affect protein profiles and gene expression (Baysdorf and Van der Wounder, 1988). For example, in *Nicotiana plumbaginifolia*, a massive induction of manganese superoxide dismutase occurs in the presence of sucrose (Bowler et al., 1989).
The calcium content of the culture medium may also affect cell division. There is evidence that Ca++ ions inhibit cytoplasmic streaming in higher plants (Minorsky, 1985). For example, raising Ca++ levels in the external medium of *Elodea* cultures results in a decline in the cytoplasmic streaming rate, and increases in cytosolic calcium calcium have been shown to promote microfilament disassembly (Minorsky, 1985).

Another nutrient found to have a major effect on culture growth is nitrogen, especially in terms of whether it is supplied in its organic or inorganic form. If supplied in its inorganic form (NO₃ + NO₄), preferential uptake of one of the two forms (eg. NO₄ in *Ipomea*, Martin *et al.*, 1977; NO₄ in asparagus, Jullien and Guern, 1979) may affect the pH of the culture, with subsequent cell mortality. In this case the pH of the culture must not be allowed to fall past physiologically harmful levels by external control such as ammonium chloride of sodium hydroxide addition throughout the culture period.

Nitrogen may also be introduced in the media as an organic source such as glutamine or other amino acids. Here, organic nitrogen uptake is probably controlled by a transport system. For example, DNP and azide inhibitor studies have shown that alanine uptake in soyabean suspension culture is closely linked to an energy source (King and Oleniuk, 1973). Sugarcane cells, on the other hand, are thought to possess multiple transport systems for basic amino acids (Maretzki and Thom, 1970); these are interconvertible in their amino acid specificity depending on the exogenous concentrations of amino acids available within the culture media.

### 3.1.3.2 Growth regulators

The effect of plant growth regulators on cultured cells has been studied extensively. Many of the published data are apparently contradictory, and their analysis is complicated by the fact that hormones of the same type will affect tissues in different ways. An example of this can be seen with cultures of carrots and soyabean, both of which need 2,4-D for growth. If 2,4-D is removed from the media, the carrot culture will produce somatic embryos, while the soyabean culture will not (Jacobsen, 1983). Experiments have shown that carrots are capable of excreting 2,4-D but in soyabean 2,4-D is conjugated to amino
Acids and is therefore immobilised.

Auxins have been shown to have a direct effect on protein and nucleic acid synthesis in cultured cells. Auxin addition results in enhancement of DNA and histone precursor production in cultures of *Allium cepa* roots (Jacobsen, 1983), the incrementation of RNA transcription by increased RNA polymerase I and II activity in Jerusalem artichoke, and increased RNA polymerase activity in soyabean nucleoli (Bevan and Northcote, 1981b) resulting in accumulation of rRNA.

Cytokinins are also thought to be involved in the stimulation of cell division. This is shown by the fact that they are sometimes used to synchronise the mitotic cycle in cultured cells (Jounneau and De Marsac, 1973). Moreover, cytokinins have also been found to influence polysaccharide synthesis by cultured cells. For example, high kinetin levels produce a denser and more granular extracellular hemicellulose in sycamore cultures (Stepan-Sarkissian and Fowler, 1986). These changes have been ascribed to kinetin suppression of phosphoglucose isomerase and other enzymes participating in monosaccharide conversion.

It is known that subculturing results in a sharp increase in nucleic acid and protein synthesis (Jounneau, 1970). As most maintenance media contain hormones, the question arises of whether this increase in synthesis is caused by subculturing *per se* or as a direct result of application of new supplies of exogenous hormones. Work with soyabean and bean cell cultures has shown that the primary stimulus for *de novo* transcription or the recruitment of previously untranslated mRNA into polysomes is brought about by subculture. Both auxins (Bevan and Northcote, 1981b) and cytokinins (Bevan and Northcote, 1981a) are shown to have a modulatory effect on the appearance of active mRNA molecules, but the response is still dependant upon subculture taking place, as addition of either hormone to hormone-starved cultures will have no effect on bulk protein synthesis or the synthesis of a particular polypeptide.

Ethylene production is a property of meristematic tissues and it has been postulated that its synthesis is directly related to cell division, but the data presented so far is often contradictory. It also is a regular metabolite of plant cell culture and its production, although increased in the early part of the stationary phase, is not associated
with a particular growth phase (La Rue and Gamborg, 1971; Mackenzie and Street, 1970); the amount of production seems to be species dependant. Studies of the effect of exogenous ethylene addition to cultures have yielded contradictory results. Work with Ruta, rose (La Rue and Gamborg 1971), sycamore (Mackenzie and Street, 1970), and tomato (Huxter et al., 1981) has shown that exogenous ethylene has no effect on cell growth and will not replace hormone requirement for cell division. At the same time experiments on sealed-vial cultures of artichoke explants indicate that differentiation is strongly suppressed without significant effect on cell number as compared with other open vial controls (Phillips, 1980); whether this is caused as a result of general gas-exchange restriction or specifically by ethylene buildup is still unknown. Other work on the effect of ethylene on differentiation and cell division (rice and tobacco callus, Huxter et al., 1981; soyabean, Constabel et al., 1977), show the responses of the cells to be mediated by the differentiation and growth state of the culture at the time of ethylene addition. Ethylene has also been shown to be produced in whole plants as a result of plant-pathogen interaction (Ecker and Davis, 1987) or wounding (Smith et al., 1986), and to induce transcription of specific genes (eg. PAL and chalcone synthase). The compound may therefore be a stress signal for plants, to activate defense mechanisms against invading organisms. This is not necessarily the only physiological function of ethylene, but it remains the only identified role.

3.1.3.3 Responses to Stress

Cultured cells are thought to be constantly in a stressful environment per se (Logemann et al., 1989). Any displacement from the status quo in terms of culture conditions would also bring additional stress to the cells. This includes chilling, heating, wounding, deficiency of existing nutrients or addition of chemicals such as metabolic inhibitors. The influence of any of these stressful stimuli might bring about changes in gene expression. For example, work with tomato suspension cultures has shown that doubling times change from 2-3 days at 28°C, to 3-8 days at 12°C, and cell division is inhibited at 8°C (Du Pont et al., 1985). Wounding has been shown to lead to drastic changes in the physiology of plant cells partly as a consequence of
changes in the expression pattern of several genes (Logemann et al., 1989). These include phenylalanine ammonium lyase (PAL) (Edwards et al., 1985), pathogenesis related proteins (Van Loon, 1985), and stilbene synthase (Vornam et al., 1988).

In a typical batch culture, the cell expansion growth phase is followed by a stationary phase. This is usually concomitant with the exhaustion of a particular nutrient supply, such as phosphate and carbon sources (Meyer and Wagner, 1985a), and may bring about the initiation of some facets of secondary metabolism (Hahlbrock et al., 1971a). For example, in Citrus peel tissue and sycamore cell suspension cultures a three- to four-fold increase in PAL activity can be correlated with the exhaustion of nitrogen from the medium in the latter half of the growth cycle, prior to an increase in the synthesis of tannins (Lindsay and Yeoman, 1985).

Addition of particular chemicals will also affect gene expression and cell metabolism. For example, actinomycin D will inhibit transcription and can therefore be used in mRNA stability studies (Jones and Northcote, 1981). Addition of certain herbicides will result in overproduction of the natural amino acid corresponding to the herbicide analog by resistant culture lines (Gonzales and Widholm, 1985). Addition of hormones may affect gene expression by varying the availability of mRNA to translation (eg. PAL, Jones and Northcote, 1981).

In vitro cultures of plant cells are an invaluable tool for the researcher as they separate tissue structure from function. Experimental conditions can be closely monitored and faithfully reproduced, and large amounts of tissue may be made available for sampling. All the factors discussed above, which may affect primary metabolism, may represent ways of potentially inducing specific genes and mimicking processes occurring in the whole plant. At the same time, it must be remembered that any alterations in the culture conditions will affect the cells, and must be avoided if we do not wish to disrupt the "normal" metabolic activities of the system under study.
3.1.4 Uses of Suspension Cultures

Suspension cultures offer a population of cells growing in a defined environment. This environment may be changed, within limits, and manipulated by addition of radioactive tracers, hormones etc, in known amounts for a known period of time. Conditions may be monitored quickly and accurately, enabling experiments to yield realistic figures for the rate of incorporation or metabolism of compounds. These properties of suspension cultures have been used to clarify a number of metabolic processes occurring in cells, and to use cultures in the production of specific compounds. In addition, the dedifferentiated cells may be stimulated to undergo cellular redifferentiation, and develop into organs or whole plants. Suspension cultures have also been used for gene transfer experiments.

3.1.4.1 Membrane Transport

Cell cultures offer a number of advantages over the use of fresh tissue preparations for the study of membrane transport in plants. Bacterial contamination is not a relevant factor in these experiments because the cells have been cultured under sterile conditions. Also, since the sterile cultures do not require the inclusion of an antibiotic, there is no fear of an antibiotic effect on the cells. Differences attributable to individual plants or tissues are eliminated since the history of cultivation of the cells is constant, and uniform pretreatment possible. The relative homogeneity of cells in suspension culture ensure uniform sampling and facilitate experimental design (eg. rapid, repeated sampling necessary to establish uptake rates). Finally, plant cell cultures can provide information about transport-linked reactions which were previously not amenable to experimental manipulation in higher plants.

3.1.4.2 Primary Metabolism and the Cell Cycle

Suspension cultures, with the increased advantage of a homogeneous population, and smaller organisational entities afforded by a quasi-single cell system, have been used in detailed biochemical studies. Examples of this are the study of acetate metabolism in rose cells.
(Fletcher and Beevers, 1970), and the analysis of enzymological activity and carbon flux in *Acer pseudoplatanus* cells (Stepan-Sarkissian and Fowler, 1986).

Cellular pathways thought to be directly involved with cell proliferation, namely phosphorylation (Draetta and Beach, 1988) and changes in cytosolic calcium concentration (Berridge, 1984), have also been investigated to try and correlate results with those collected from animal systems. It is already known that in plant cell cultures biomass increase is achieved in two distinct phases, cell division and cell elongation, and that the cell elongation phase starts when the cellular phosphate pool decreases below a low threshold value, provided the carbon source is not exhausted (Wylegall *et al.*, 1985). Work with *Catharanthus roseus* suspension culture showed that diphosphorylated phospholipids were seen to vary in concentration with the growth cycle of the culture (Hein and Wagner, 1987). It was also shown that an operational P1 cycle is essential for cell division; the finding that addition of inositol 1,4,5 triphosphates to zucchini hypocotyls results in the release of divalent calcium ions (Hein and Wagner, 1986), suggests even more firmly a close relationship between phosphorylation and cell proliferation. More recently, immunological techniques have shown the presence of a non-histone protein associated with the nuclei of rapidly dividing cells (Smith *et al.*, 1988). The protein was detected in a number of unrelated plant species, and its correlation with cell proliferation is similar to the cyclin proteins found in the nuclei of dividing animal cells.

3.1.4.3 Differentiation in Culture: Cytodifferentiation

The ability of cells to cytodifferentiate in culture has been used in the study of the synthesis of the cell wall. For example, suspension cultures have been used to elucidate the biosynthesis of extensin (Wilson and Fry, 1986), mainly because cultures seem to form larger amounts of hydroxyproline-glycoproteins than ordered plant tissues. Sugar cane cultures were used in the study of post translational modification in extensin, and sycamore and tomato cultures to clarify the mechanism of secretion and insolubilisation in the cell wall.

The alteration of wall components during cytodifferentiation has been shown to be controlled by the induction or repression of the
activities of various enzymes that occur at or after the onset of secondary wall thickening but not before it (Phillips, 1980). The direction of polysaccharide synthesis is a major feature of the state of differentiation of plant tissue. A marker often used for studies in lignin synthesis is PAL, as this represents the first step in the process. Peroxidases studies (peroxidase is the final step in lignin synthesis) have also taken place. They have shown the presence of different isoenzymes of wall-bound peroxidases, with different substrate-specificity that seem to be induced at different stages in cytodifferentiation.

During secondary wall thickening there is an increase in the production of cellulose and hemicellulose, and a cessation of protein synthesis. In bean suspension cultures two synthases are involved, concerned with the formation of neutral pectin (arabinan synthase) and hemicellulose (xylan synthase) in the cell wall (Bolwell and Northcote, 1983b). Inhibitor studies show that both transcription and translation are required for the rise in synthase-specific activity. Specific gene products are therefore required either as direct products of the mRNA molecules for the synthases, or to process the mRNA, or to activate pre-enzymes postranslationally. During this period the incorporation of arabinose or xylose into the type of polysaccharide that is accumulated is a function of the state of differentiation of the cells, which in turn can be regulated by the application of different growth factors (Bolwell and Northcote, 1983a).

3.1.4.4 Differentiation in Culture: Embryogenesis

Embryogenesis is a major pathway for plant regeneration from cultures of somatic cells and male generative cells (immature pollen grains). Embryo formation is achieved experimentally by successive changes in the nutrient media, an important aspect of which includes the nitrogen to auxin ratio (Wareing and Phillips, 1978). This process is associated with substantial changes in cytology, enzyme activity and biochemical pathways.

Studies have taken place on the protein and mRNA profiles during embryogenesis in carrot, pea, rice, and Dactylis. In carrot, embryo formation in culture is suppressed by the use of 2,4-D and high cell
density (Borkird et al., 1988). The tissue may form pre-embryonic masses (PEM) under these conditions. If cell density is lowered, and 2,4-D removed, the PEMs will differentiate into heart- and torpedo-stage embryos. Work comparing callus (Sung and Okimoto, 1981), PEMs (Wilde et al., 1988) and heart-stage embryos (Borkird et al., 1988) to torpedo-stage or somatic embryos, has shown that a very small number of changes occur in the overall protein patterns between the various developmental stages. The same has been found in pea and rice (Hahne et al., 1988). On the other hand, if cultured cells and PEMs are compared in carrot (Sacco de Vries et al., 1988) in an attempt to explore the acquisition of embryogenic potential, a number of in vitro translation products are seen to appear transiently, during the acquisition period, and to disappear in the somatic embryos. Before any conclusions may be drawn from these results, it must be stated that 2D gels of in vitro translated mRNA products may not be sensitive enough to detect genes preferentially expressed in either of the states under scrutiny. An example of this can be seen in Smith's publication (Smith et al., 1988), where immunodetection is used to purify a cDNA clone, whose corresponding in vitro product cannot be visualised on a 2D autoradiograph.

3.1.4.5 The Production of Secondary Metabolites

Secondary metabolites are a broad class of products, termed as such to distinguish them from so called primary metabolites, like amino acids or nucleic acids, that perform vital physiological functions. Their physiological function include warding off potential predators, attracting pollinators, or fighting infectious diseases.

There are a number of economic and practical advantages to producing secondary metabolites from culture. Economically, plants are a major source of pharmaceuticals, flavours and fragrances (Whitaker and Hashimoto, 1986). Technically, cultures afford strictly controlled nutritional and environmental conditions, giving independence from seasonal factors. The cultures are aseptic therefore eliminating the problems associated with microorganism contamination. Also, liquid culture presents us with a very effective way of incorporating precursors which may often be difficult to administer to the whole plant. Finally, the technology available today allows large scale production of batch
cultures in chemostats or turbidostats.

Large scale production of certain metabolites by microbes (e.g. insulin and interferon by genetically engineered *E. coli*) is already a reality; these are single gene products of simple sequence and simple three-dimensional structure. A group of dicotyledonous secondary metabolites, typically synthesised from amino acids, are the alkaloids. These are the products of multi-gene sequences coding for complicated and highly integrated biochemical pathways. The cloning of the genes coding for enzymes responsible for the synthesis of a particular plant-specific indole alkaloid would therefore involve the isolation and expression of roughly 10-15 as yet unknown genes, in the proper sequence and orientation, probably coupled to a major modification in the genetic regulation of the biochemical pathway for the primary precursor in the host organism. This renders the system unsuitable for the construction of genetically engineered microbes to perform these syntheses. It therefore seems that with the technology available to date, the only option open to us for *in vitro* production of secondary metabolites is the utilisation of cultures resulting from the plants themselves.

Extensive studies on the production of useful compounds such as alkaloids, steroids, terpenoids, quinones and other pigments (Mantell & Smith, 1984; Fujita *et al.*, 1983) by cultured plant cells or organ cultures, have been undertaken, for possible industrial application. The main problem arises from the fact that cell cultures often have altered secondary metabolism that may result in either overproduction or underproduction of metabolites, or production of novel metabolites. For example, aposcopalamine in *Datura* is present at very high levels in culture extracts, but only in trace amounts *in vivo*, and cell cultures from the *Aspenula, Galium, Rubia* and *Sheradia* genera have been reported to produce anthraquinones at higher levels than those found in intact plants (Schulte, El-Shagi and Zenk, 1984).

Optimisation of secondary metabolite production in cultured plant cells has relied on extensive manipulation of cultural conditions to influence the degree to which these substances accumulate. For example, *in vitro* cultures of *Chinchona ledgeriana* have been manipulated by either feeding L-tryptophan to the culture (Hay *et al.*, 1986), or changing other parameters in the medium such as mineral salts, auxins, cytokinins and
organic nutrients concentration (Harkes et al., 1985), or altering the physical environment. Nutrient optimisation is also often combined with the induction and recovery of genetic variation by somaclonal variation technology (Whitaker and Hashimoto, 1986). In some instances, cell cultures have been selected that produce significantly higher concentrations of a particular secondary metabolite that is found in the intact plant, for example anthocyanins production by *Euphorbia millii*, (Yamamoto, Mizuguchi and Yamada, 1982), and reserpine production in *Rouwolfia serpentina* (Yamamoto & Yamada, 1986).

A simple need for nutrient optimisation is not always the answer to problems of metabolite yield. Caffeine, for example is a secondary product which arises from a modification of purine. Work with tea leaves has shown that maximum production occurs in callus cells that have reached the end of the growth stage and are autolysing (Ogutuga and Northcote, 1970). Production is further increased by fragmenting the callus, suggesting that caffeine is formed during the catabolic breakdown of nucleic acids. *In vivo*, the withering process in the fermentation of tea leaves results in an increased caffeine content and in loss of RNA (Bhattachayya and Gosh, 1968).

What can be generalised from these examples is: a) an inverse relation exists between the growth rate and alkaloid production in cell cultures. For optimum secondary metabolite production a two-step system should be used, the first medium for growth, followed by a production medium in which the alkaloid level is increased in the cells; and b) most importantly, even for related species no generalisation is possible for the factors inducing secondary product formation. This leads to the necessity for extensive tissue culture studies for each individual system of interest; it is very unsatisfactory as it involves a great deal of work with no certainty of success.

It is at this point that molecular biology might come in useful in producing a promoter which is independent of the repression/feed-back mechanisms controlling secondary metabolite production. It is very likely that the pathways controlling such production will be affected at one, or at mostly two, key steps. If the sequences coding such enzymes can be cloned and constructd into a chimeric gene with a promoter active in cell cultures, then secondary metabolic pathways might be built up in
cultured cells. If this proves to be possible, the applications of such a system will be endless.

3.1.4.6 Biotransformation

Biotransformation is a technique which utilises enzymes located in a plant cell to alter the functional group chemistry of externally supplied chemical compounds. It is used in many cases to increase the biological activity of a chemical structure and usually involves the action of one of several enzymes coupled in sequence to perform a series of specialised chemical reactions. In such systems the plant cells' primary metabolism is harnessed in such a way as to provide a mechanism for regeneration of the appropriate co-enzymes/cofactors necessary to sustain catalysis.

Biotransformation by plant cell components can take two forms: the use of whole cells where the cells can be either in the form of completely free suspensions or immobilised by anchoring to an external support, or the use of immobilised cell component preparations, where the specific enzymes concerned are isolated, purified and immobilised on an external support. The enormous biochemical potential of plant cells to perform specific biotransformations on particular natural or synthetic substrates to produce more useful substances leads to a number of possible applications. In the case of synthetic compounds, analogues of intermediates or products from other species which are not normally available in the plant may be used as substrates to produce new compounds of unique biological activity. Natural intermediates of the plant may also be used to produce compounds of similar activity to those obtained from plants grown in the field.

3.1.4.7 Genetic Transformation

Plant cell transformation takes place via two main techniques: Agrobacterium infection and direct DNA transfer. The former relies on the natural ability of the soil bacterium Agrobacterium tumefaciens to transform plant cells at wound sites. This occurs in two main steps: attraction of bacteria to wound sites by a gradient of phenolic compounds (eg. acetosyringone, Stachel et al., 1985), and attachment of Agrobacteria to the plant cell surface (Gurlitz et al., 1987). Wounding of the plant tissue is a prerequisite for successful infection, and it is
generally accepted that dedifferentiating cells undergoing DNA synthesis are competent for transformation. DNA-mediated gene transfer involves direct uptake of vector DNA by protoplasts using liposome-mediated uptake, chemically-activated endocytosis (PEG), fusion with bacterial spheroplasts, electroporation, or microinjection (Draper et al., 1986). Successful transformation seems to rely on cells undergoing dedifferentiation and DNA synthesis as a response to wounding or stripping of the cell wall (protoplasts). Such transformed cells will be dedifferentiated and will have to be capable of redifferentiating into embryos or shoots for the transformation process to be successful.

A large number of vector plasmids have been designed (e.g. Bevan, 1984; Herrera-Estrella et al., 1983; Fromm et al., 1986). They have a number of common features including: an E. coli origin if replication to facilitate recombinant DNA work, an Agrobacterium origin of replication (if Agrobacteria-directed transformation is required), and the border signals of the portion of Ti plasmid transferred to the plant's genome containing a selectable marker and the gene to be inserted into the host plant. In addition there are so called cassette plasmids; here a CAT, NPT II or GUS coding region is preceded by unique restriction enzyme sites (e.g. Herrera-Estrella et al., 1984). If a putative plant promoter sequence is cloned into the cassette plasmid, and the vector introduced into a host plant, expression of the CAT, NPT II or GUS gene products, as driven by the plant's promoter, can be quantitated by biochemical or immunochemical methods. A lot of work has been published, where whole plant genes were transformed into heterologous systems to look for integration sites, inheritance patterns, and retention of tissue specific expression, or where putative promoter and/or enhancer element sequences were tested using cassette plasmids (for review see Weising et al., 1988).
3.1.5 Conclusions and Aims of the Project

*In vitro* cultured cells can be artificially retained in a dedifferentiated state. Work has been undertaken to define many tissue cultures *per se*, in terms of their biochemistry and cytology. The factors affecting primary metabolism such as nutrient availability, the presence of growth regulators, and the effect of external stress on the biochemistry of the cells have been investigated. Biochemical and molecular changes have been shown to occur the establishment of cultures, and whenever conditions within the culture are altered. The results attained have shown the importance of stringent definitions of culture conditions for experimental reproducibility. At the same time, alterations in experimental conditions have been used as a tool to induce expression of specific genes.

Plant cultures have been used as tools to separate tissue structure from function, in an attempt to simplify the study of plant metabolism. For example, cultures have been used to separate the primary causes of chilling injury (prevention of cell division or cell expansion) from secondary effects such as phloem transport malfunctions resulting in nutrient imbalance or dessiccation, and eventual cell death in the whole plant (Du Pont *et al.*, 1985). In this way it has been possible to dissect cellular metabolism from the metabolism of the plant as a whole. This has simplified studies on membrane transport, primary metabolism, the cell cycle, and cellular differentiation.

Tissue culture has also surfaced as useful for practical applications such as the production of secondary metabolites and biotransformation. Experiments have been carried out to try and understand the underlying biochemistry governing natural product synthesis, with the aim of altering culture conditions for optimum metabolic activity. Cultured tissue has also been used as a transitional state during genetic transformation.

Many of the studies on cellular metabolism in culture have been carried out with the assumption that the metabolic models resulting from the data will be applicable to the more complicated system of a whole plant. The question arises on how closely related the *in vivo* and *in vitro* systems are, and how safely can we apply results achieved in one
system to responses to similar conditions in the other. We already know that even when maintained under apparently stable culture conditions, cultured cells exhibit variations in morphology and metabolism which are the product of the physiological and genetic constitution of the original cells and the "external" stimuli supplied in the form of the culture conditions (Lindsey and Yeoman, 1985). It is in fact the culture conditions themselves that cause the most salient differences between cultured cells and whole plants. There are differences in organelle structure, development of the cell walls and reliance by the cells on an exogenous supply of nutrients and hormones, and in culture the molecular conversation between cells (e.g. hormone gradients) is disrupted because of physical separation. Hahne (Hahne et al., 1988) has indeed stated that: "callus is a tissue type without direct equivalent in whole plants and thus contains unique proteins".

This part of the project is aimed at investigating the gene expression changes that occur in culture to bring about the biochemical and cytological differences apparent between the two systems. In particular it is proposed to investigate the role of transcription in controlling changes in gene expression. This choice arises for two main reasons: representation of gene expression is easier at the transcriptional level rather than the protein level (see Section 3.2.1), and transcription is expected to be one of the major regulatory steps in determining the synthesis of a particular protein in cultured cells (Bevan and Northcote, 1981a). Once such a transcript has been identified, the 5' sequence will be investigated to pinpoint the promoter and possible enhancer elements. This may then be used in the future to drive expression of useful genes when reintroduced into host plants.

Finally, possible applications of this work will be considered before defining the systems to be compared. Tissue culture is being actively used in genetic transformation and secondary metabolite production. The vectors for genetic transformation contain a selectable marker. Selection of transformed tissue is undertaken mainly via resistance to kanamycin afforded by the Neomycin phosphotransferase (NPT II) gene product. The expression of the gene is usually driven by the 35S promoter from Cauliflower Mosaic Virus (Fromm et al., 1986), which is constitutive. The energy drain caused by the constitutive expression of
such a "superfluous" gene may result in a drain on the resources of the host plant, causing physiological abnormalities (such as stunted growth), or a reduction in yield of storage products (reducing a plant's commercial value). The occurrence of bacterial enzymes and their metabolic products in crop plants may also be undesirable. An inducible promoter, such as one which is preferentially expressed in actively dividing tissue culture, would therefore be very useful as it would only be expressed in cultured tissue, and not in regenerated plants.

Production of secondary metabolites in plants relies on the presence and activity of a very complex set of enzymatically controlled metabolic pathways. It is likely that these will be controlled, for example, by determining the amounts of precursor substances, or by a key step within the metabolic pathway itself. These controls will probably act during the growth or stationary phases of the culture's growth cycle, respectively. If the sequences coding for such rate controlling enzyme(s) can be cloned and constructed into a chimeric gene with a promoter preferentially active in a specific growth phase, these metabolic pathways might be built up in cultured cells.
3.2 Characterisation of *Petunia* Cell Suspension Culture

3.2.1 Introduction

As already discussed in the preceding section, this part of the project is aimed at investigating steady state transcriptional differences between cultures and intact plants, in the belief that they will mirror the biochemical and cytological differences apparent between the two systems.

An empirical approach was taken which relies on differences between the two systems under study reaching levels appropriate to the sensitivity of the screening system, but required no previous knowledge of the nature of such transcripts, nor their biological significance. The choice of methodology for this project relies on the fact that representation of gene expression is easier at the transcriptional level rather than at the protein level. This is because comparison of steady-state protein levels is very difficult, whilst recombinant DNA technology allows amplification of the low abundance transcriptional signals present in the cells. If mRNA is extracted from an active cultured cell, its DNA template can be reconstructed via the use of viral reverse transcriptase; this can then be cloned in a suitable vector, and introduced into *E. coli* hosts where the sequences will be amplified *ad infinitum*. This process is referred to as cDNA (copy DNA) cloning. Gene expression in the two different plant systems can now be compared at the mRNA level by cross hybridisation techniques. This involves making a $^{32}$P-labelled DNA species ("probe") from one of the two systems, and using this to screen the cDNA from the second system (+/- method). The probe will hybridise to all homologous sequences, and after washing off non-bound species these can be visualised by autoradiographic techniques. Such methodology has been used for example to show up differences in gene expression in the search for fruit ripening-specific expression in tomatoes (Mansson *et al.*, 1985). Once differentially transcribed sequences have been purified, they can themselves be used as probes to locate the related genomic sequences. These may then be used to study the promoter
structure, gene products and other features as discussed in later sections. The same system can also be used to detect highly expressed sequences as the strength of signal (mRNA abundance) attached to a DNA clone is often proportional to the transcriptional level of that particular gene.

As suspension cultures exist as single cells and microaggregates, it is felt they represent a more homogenous population than calli, and have therefore been chosen for this study. The albino *Petunia hybrida* cv. Blue Lace (BL) cell culture used in this project was established some years ago. Continuous subculture in fresh medium have brought about a number of changes which render the BL line extremely useful. In the first place the cells are completely heterotrophic; this means that they have to rely on the medium for all their nutrients. As a result the cells will enter log phase and stationary phase depending on the availability of nutrients and can therefore be synchronised at will if starved of nutrients. Secondly, the culture itself is under constant selection for the most actively dividing cells; this is of advantage because it is hoped that the differences between dividing cultured cell and normal, quiescent, differentiated plant cells will be magnified as a result.

In an attempt to define the system under study, it is felt that a stage within the growth cycle of the culture has to be chosen, as the gene expression profile of the cells may change within the cycle. As discussed in Section 3.1.5, from a practical point of view two stages may be of interest. Analysis of gene expression in the log phase (actively dividing cells) may allow the isolation of promoters to be used in the construction of selective markers for use in transformation systems (as selection is usually done on actively growing callus), or in boosting enzymatic pathways leading to the production of secondary metabolite precursors. Promoters active only within the stationary phase are also of interest as it is when the cells have stopped growing that the production of secondary metabolites in culture is most active.
3.2.2 Definition of Growth Stages

*Petunia* is particularly suitable to be grown in suspension culture as its callus is very friable, and gives rise to small cellular microaggregates of about 6-8 cells each (Fig. 3.2). As the cell culture cDNA library had to be constructed from poly A⁺ RNA specific to both the logarithmic and stationary phases, the first goal was to determine the conditions within the Blue Lace cell culture, that would produce a line whose growth profile was easily reproducible. For practical reasons it was also hoped that conditions could be found that produced a growth profile with a full cycle (lag, log, and stationary phase) of 7 days; this would facilitate experimental design in terms of subculture and sample collection. For this purpose separate lines were started, differing in the amount of inoculum used for subculture; the lines were left to stabilise and at the fourth subculture (7 day intervals) a number of parameters were measured. These included wet and dry weight and DNA concentration measurements. The DNA concentration measurements were considered to be the most reliable indication of cell number.

Table 3.1 and Fig. 3.3 show the results from one of the experiments. Fig. 3.3 clearly shows that growth rate is initially slow at all inoculation levels, but increases after about day 3 (except for the 5ml inoculum) to reach a peak at day 5 or 6 and then decreases, until at days 8 and 9 no more growth occurs. The sudden dip in DNA extracted from the cells after day 5 is thought to be as a result of carbohydrate production within the culture; this physically binds to the nucleic acids and prevents their efficient recovery during DNA extraction.

A 15ml inoculum was found to produce the best growth conditions for two reasons: the rate of DNA concentration (and therefore growth) increase is smoother with this inoculum, and the culture reaches late log/beginning of the stationary phase in 7 days.

Once the culture conditions were determined, the Blue Lace line was further characterised to produce a more accurate growth profile. The parameters considered included dry and wet weight, together with DNA and protein content. The results are shown in Table 3.2. The experiments were
Figure 3.2 Blue lace suspension cultured cells.

X 300 magnification

Scale bar = 10µm
TABLE 3.1

Growth of *Petunia* Blue lace suspension cultures with different inoculi. See Fig. 3.3 for graphical representation.

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>5ml inoculum</th>
<th>10ml inoculum</th>
<th>15ml inoculum</th>
<th>20ml inoculum</th>
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<tr>
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<td>6.5</td>
<td>11.5</td>
<td>31.0</td>
<td>73.0</td>
</tr>
<tr>
<td>1</td>
<td>9.0</td>
<td>45.0</td>
<td>80.0</td>
<td>145.0</td>
</tr>
<tr>
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<td>65.0</td>
<td>45.0</td>
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</tr>
<tr>
<td>3</td>
<td>23.0</td>
<td>35.0</td>
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</table>
Figure 3.3 Growth of *Petunia* cell suspension culture with different inoculi. For tabular representation see Table 3.1.
repeated four times, to give the general growth trend as mirrored by each parameter. This profile can be better understood by looking at the four parameters together, as shown in Fig. 3.4. From the values reached, days 2, 5, 7, and 9 were assigned approximately as representative of the lag, mid log, late log and stationary phases respectively.

3.2.3 Extraction and Purification of RNA

Recent advances in our knowledge of eukaryotic messenger RNA have permitted a rational approach to its identification and isolation. In particular, the development of mammalian cell-free systems that will synthesise defined proteins in response to exogenous RNA (Pelham & Jackson, 1976) has provided a precise means for identifying unique mRNA species.

The work presented in this section is aimed at developing methods for the isolation of high purity mRNA in sufficient quantities to be used in \textit{in vitro} translation experiments, and cDNA cloning.

The RNA extraction method used in this project has been modified from Covey and Hull (1981) to counteract some of the problems associated with plant RNA extraction. The method is based on the use of phenol as a protein denaturant, and in particular to dissolve compounds such as phenolics. A detergent (triisopropylarnaphthalene sulphonate, TNS) is also added to the tissue homogenate to dissolve nucleoprotein complexes and facilitate protein denaturation, improve RNA yields and inhibit ribonuclease activity. The extraction buffer is at pH 8.4. This is because it has been observed that at neutral pH, the mRNA is mainly found in the denatured protein interface during phenol extractions, the interaction between the mRNA and proteins being promoted by monovalent cations such as Na$^+$ and K$^+$; it is therefore necessary to employ an alkaline buffer during phenol extraction, to allow most of the mRNA to partition into the aqueous phase. The phenol/chloroform extraction also serves the purpose of removing all phenolic compounds from the RNA-containing phase, thus reducing complexes between RNA and hydrophobic impurities that will alter the fractionation profiles of higher plant poly(A)-containing RNA (Takahashi & Nitta, 1986).
TABLE 3.2

Cell culture growth parameters for an inoculum of 15ml of Blue Lace culture. See Fig. 3.4 for graphical representation.

<table>
<thead>
<tr>
<th>TIME (days)</th>
<th>WET WEIGHT (g/100ml)</th>
<th>DRY WEIGHT (g/100ml)</th>
<th>DNA (μg/100ml)</th>
<th>PROTEIN (mg/100ml)</th>
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<tr>
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<tr>
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<td>0.13 0.20</td>
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<tr>
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<td>1.35 1.43</td>
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<td>150 180</td>
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<td>0.14 0.15</td>
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<td>12.2 12.8</td>
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<td>0.15 0.18</td>
<td>150 180</td>
<td>12.4 13.0</td>
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</tbody>
</table>
Figure 3.4 Growth of *Petunia* cell suspension culture with 15ml inoculum, showing range bars. For tabular representation see Table 3.2.
DNA is removed from the nucleic acid precipitate by washing the salt-ethanol nucleic acid precipitate with 3M sodium acetate by resuspending and resedimenting. This solubilises the DNA as well as tRNA and 5S rRNA. An additional benefit of the sodium acetate washing is that the remaining mRNA becomes a more efficient template in protein synthesis assays.

The RNA extracted by this method contains no protein impurities and is very low in carbohydrate contamination. The poly A+ RNA fraction may be extracted via the use of oligo-dT cellulose columns (see Methods). After a single column step, the poly A containing RNA preparation (polyA+) usually contains contaminating rRNA, which can account for up to 50% of the eluate. However, repassing the RNA sample through a second column step is usually sufficient to yield essentially pure poly A containing RNA. This is termed poly A+ RNA and is the substrate used for cDNA cloning. The poly A+ fraction is pure enough to be used in \textit{in vitro} translations.

On average \textit{Petunia} seedlings yielded an average of 1mg total RNA/g fresh weight tissue. The poly A+ fraction represented around 1% of the total RNA. Cultured BL cells yielded 10mg RNA/g fresh weight tissue, and poly A+ was 1.2-1.4% of the total RNA. The poly A++ fraction represented 30% of poly A+, mainly because of loss through non-specific binding.

### 3.2.4 Steady State Proteins and Transcripts at Different Growth Phases in Suspension Cell Culture

As already discussed in Section 3.2.1, the best method available to date to look at differentially-expressed genes, when no target molecules in the form of transcripts or proteins are available, is via the study of the mRNA population within the tissues under study. A useful method to visualise this population is via \textit{in vitro} translation of the mRNA, carried out in the presence of $^{35}$S methionine with the products fractionated on a PAGE gel and then visualised by autoradiography. The steady state protein population was also looked at.

Total protein extracted from different stages within the culture's growth cycle were run on an SDS-PAGE gel and stained with Comassie blue
to visualise any major changes in pattern. As can be seen from Fig. 3.5, the protein bands do not show any marked up-regulation or down-regulation in terms of their relative abundance. The loss of higher molecular weight bands at the later stages within the cycle seem to suggest some degradation taking place, possibly because of cell death occurring within the culture; this may be as a direct result of culture starvation at these stages. Again, this showed the advantage of subculturering the cells at day 7, thus reducing mortality within the culture, and the adverse effects resulting from phenolic and other substances produced by dying cells, on the general well-being of the culture.

Poly A+ RNA extracted from cells on days 2, 5, 7, and 9 was translated *in vitro* and the products run on an SDS-PAGE gel and visualised by autoradiography (Fig. 3.6). No major differences were apparent between the different stages within the culture. The protein bands did not show any marked up- or down regulation in their intensity, suggesting that no quantitative changes occurred in polypeptide populations within a specific molecular weight range as resolved by one-dimensional PAGE.

As changes within the population of polypeptides at a specific molecular weight will not be detected by one-dimensional PAGE, a two-dimensional system was set up (O'Farrell, 1975). This involves separating the *in vitro* translation products with different pKa values (iso-electric focusing) prior to the molecular weight separation afforded by PAGE under denaturing conditions. Iso-electric focusing (IEF) is a method specifically intended for the fractionation of molecular species differing only in net charge. Since separation is not due to any molecular size effect during electrophoretic transport through the medium, it is performed in essentially non-sieving media, such as polyacrylamide gels of high porosity. A pH gradient is established within the polyacrylamide gel with the aid of a mixture of low-molecular weight amphoteric substances, commonly referred to as ampholines. Most carrier ampholines in general use are polyaminopolycarboxylic acids, possessing a number of ionizable groups; these have a higher conductivity and better buffering capacity at the iso-ionic point than a single bivalent one such as phosphate or Tris buffers (Andrews, 1987). During IEF macromolecules migrate through the pH gradient as long as they retain a net positive or
**Figure 3.5** PAGE of total protein extracts from cultured *Petunia* Blue lace cells.
Lanes 1 and 13. Protein markers.
Lanes 2–12. Extracts from cell cultures 0–10 days respectively after subculturing.

**Figure 3.6** PAGE of *in vitro* translated products from Blue Lace cell culture mRNA.
Lanes 1–4. Days 2, 5, 7 and 9 respectively.
negative charge until they reach the point in the pH gradient which corresponds to their iso-electric point, where the net molecular charge will be zero, and migration ceases.

The proteins are dissolved in a buffer containing a non-ionic detergent, 9M urea and 100mM DTT. The non-ionic detergent will ensure that the pKa value of the protein is not affected by the solvent, while the 9M urea denatures the proteins so that their charge is sequence and not conformation dependant; this is further ensured by the presence of 100mM DTT which destroys disulphide bonds thus aiding the unraveling of the globular proteins. The IEF is run in rod gels, which facilitates sample application to the second dimension. Prior to running the second dimension, the rods are equilibrated in a buffer containing 2.5% SDS. This will ensure differences in charge within the native proteins do not affect separation in PAGE.

The results are a very good example of the increased resolution afforded by two-dimensional systems (Fig. 3.7). mRNA from day 5 appeared to translate with a much higher efficiency. As the same amount of incorporated counts was loaded in all gels, translations requiring a lower loading volume will have a lower background, and polypeptides with low incorporated radioactivity values will be easier to visualise over the background activity (Fig. 3.7B). A large number of polypeptides oscillated in terms of their relative abundance within the translation products population throughout the growth cycle of the culture. In particular a polypeptide (arrowed by the closed triangle) seemed at its most abundant 2 days after subculture. The two polypeptides marked with an open triangle showed some upregulation at the exponential phase, and the group of polypeptides within the open circle showed a marked up-regulation at day 5 (Fig. 3.7). Only minor quantitative differences were observed in the profiles of the in vitro translation products of the different stages within the culture's growth cycle. No "novel" or grossly upregulated polypeptides could be seen.
Figure 3.7 2D PAGE of *in vitro* translated products from Blue lace cell culture mRNA, 2 (A), 5 (B), 7 (C) and 9 (D) days after subculture. Small letters denote enlarged detail of the respective gels; polypeptides upregulated at the exponential phase (△, ○), or after subculturing (▲) are shown.
3.2.5 Comparison of Steady State mRNA Profiles Between Cultured Cells and Seedlings

The project is aimed at investigating transcriptional differences between cultured cells and plants. The plant system used for comparison was the seedling. This is because it was not considered adequate to compare fast dividing, heterotrophic cells (culture) to quiescent, photoautotrophic cells (leaf), as the whole plant does not contain only mesophyll cells but an array of tissues performing different functions. In particular it was decided to include meristematic (fast dividing) and root (anabolic and heterotrophic) cells. Seedlings were chosen as they contain functional leaves, meristematic tissue, and a root system. Five week-old seedlings were chosen as this is the stage that yields the largest amount of RNA/g tissue, when compared to 4, 5, 7 and 8 week seedlings. These plantlets have 2-3 pairs of true leaves and a root system about 3cm long.

In vitro translation products of seedling and cultured cells mRNA were run on denaturing PAGE and autoradiographed. Some bands were seen to decrease in intensity in the culture samples, when compared to seedling in vitro translation products (not shown), but the gel did not show the appearance of new major cell culture-related translation products. Running the translation products on a 2D gel (Fig. 3.8) improved the resolution and allowed the qualification of the changes in band intensity seen with the 1D system. In particular, two basic polypeptides of molecular weight 27kD (open square) present at high levels in translation products of seedlings, disappear in culture. Two more peptides of the same molecular weight, but more acidic pI (open rhombus) are strongly down-regulated in culture. The relative amounts of some polypeptides, with respect to the total translation population, are higher in cultured cells than seedlings (open circles). No polypeptide spots were reproducibly present in the culture translation products that could not be detected in the seedling samples. The exact activity incorporated into the various polypeptides within the two samples varied when the translations were repeated, so that the up- and down-regulation trends
Figure 3.8 2D PAGE of *in vitro* translated products from Blue lace cell culture (A) and seedling (B) mRNA. Small letters denote enlarged detail of the respective gels; polypeptides present in seedlings only (□), downregulated in culture (◇), and upregulated in culture (○) are shown.
mentioned above could not be quantitated accurately. This was partly due to the different efficiencies of translation within the mRNA samples; such discrepancies have indeed been shown even with separate \textit{in vitro} translations of a single batch of mRNA (Harikrishna \textit{et al.}, 1989a).

3.2.6 Conclusion

The gene expression profiles of the cell culture system may be accounted for by the cells behaving in a completely desynchronised fashion. There is evidence of structural and biochemical heterogeneity at every stage of development of cell cultures (Lindsey and Yeoman, 1985). For example, work on carrot suspension cultures (Gould, Bayliss and Street, 1974) shows the presence of cells of differing cell cycle times, depending on the length of the G1 phase. As a result, even though the culture as a whole displays a reproducible growth profile, individual cells within the population are at differing stages of their own growth cycle, resulting in overall similar (if not identical) mRNA steady state populations.

Moreover, the method used for RNA extraction does not differentiate between cytosolic and polysome-bound mRNA. As a result, these translational profiles mirror both the translating and non-translating pools of mRNA present in the culture at the time of sampling. It has been shown that certain responses, such as that to subculturing, are partially mediated by untranslated transcripts becoming available for translation (Bevan and Northcote, 1981a and 1981b). If this feature of mRNA is being used by the cells to control their polypeptide synthesis and other facets of metabolism, it will not become apparent with the methodology used in these experiments.

The one-dimensional gel electrophoresis system is a very unsensitive technique. It is only expected to detect changes in protein quantities of 1% or above of the total incorporated activity. The two-dimensional gel electrophoresis system, on the other hand, allows a 100-1000 fold improved resolution (O'Farrell, 1975). In this laboratory, the resolution of 2D-PAGE was quantitated at 0.018% of incorporated activity (see Section 5.2.4).
The definition of growth stages was nonetheless maintained unaltered. It was felt that it was important to continue this study on the culture as a whole system, rather that focus on the division cycle of an individual cell within the culture population. This general-view system was considered important as it could be related to that of actively-growing callus and to that of culture conditions in systems such as fermenters; this was bearing in mind that individual cells under such growth conditions are behaving in a highly asynchronous manner in relation to individual cell cycles, even though the system as a whole may appear to be following a steady growth profile. At the same time an attempt to synchronise the cells by external means (such as nutrient deficiency, Gould et al., 1981; hormone deficiency, Bayliss, 1985; hormone addition, Roberts and Northcote, 1970; and ethylene treatment, Constabel et al., 1977) would bring stress to the system, and disrupt the steady state, thus complicating the analysis of results attained. A study on the cells' cycle was not considered appropriate to the Petunia culture, and a different system would have to be set up. This appears in the form of the Asparagus single cell system (see Chapters 5 and 6).

At this point it is useful to place the project in a temporal context. The Petunia cDNA library in pUC9 (see Section 4.1) was constructed and screened for differential clones soon after 1D PAGE was run. The simple +/- screening technique used in Section 4.1 has a reported sensitivity of 0.1% (Sargent, 1987) and was therefore considered appropriate from the results in our possession at the time. The 2D gel system was set up by K. Harikrishna, and it was not reported to be working to a satisfactory standard until 12 months later. The 2D gels therefore confirmed the fact that the +/- screening system was not sensitive enough for this system. It was at this point that a second library was constructed in λ-ZAP and enrichment techniques used to prepare the probes for screening (see Section 4.2). Such enriching has been reported to increase the screening sensitivity to allow identification of RNAs representing 0.01% of the total mRNA population (Sargent, 1987). This is a level of sensitivity very similar to that achieved with the two-dimensional gel system, and was therefore used to verify the results attained with the 2D PAGE.
CHAPTER FOUR

SCREENING FOR GENES PREFERENTIALLY EXPRESSED IN SUSPENSION CULTURES
4.1 Construction of a cDNA Library into a pUC9 Vector, and Screening for Clones Preferentially Expressed in Cultured Cells

4.1.1 Introduction

4.1.1.1 Aims

The goal of this section of the project is to detect both highly expressed sequences, and possibly to detect cDNA clones representing genes that are expressed preferentially in the culture system. In the latter situation, ideally some colonies would light up strongly with cell culture-specific probes but not with seedling-specific probes. Such colony screening procedures can detect clones homologous to mRNAs of an abundance of 0.1% or above (Sargent, 1987), and would therefore have to be employed with the assumption that there are significant changes in relatively abundant mRNAs within the two systems. A summary of the method is shown in Fig. 4.1. As stated in section 3.2.6, at the time the cDNA library was being screened, the two-dimensional gel system was still being perfected; the 1D PAGE gels of the in vitro translated products did not show the appearance of major cell culture-related transcripts, but as changes within the population of peptides at a specific molecular weight will not be detected by a one-dimensional PAGE, it was hoped that the screening would prove more sensitive and still show the presence of novel cell culture-specific transcripts.

4.1.1.2 cDNA Synthesis

Copy DNA (cDNA) molecules can be made from an mRNA template using a viral reverse transcriptase. The molecules can then be attached to a vector plasmid such as pUC9 (Fig. 4.2) which replicates in E. coli and carries a selective marker (usually g-lactamase production). The plasmid is then introduced into its E. coli host, where each individual cDNA molecule (clone) can be amplified ad infinitum and reach levels high enough for experimental purposes. The advantage of this system is the ease of selection of E. coli colonies containing recombinant plasmids as they will result in white colonies (as opposed to blue) when the E. coli
Figure 4.1 Differential hybridisation to detect a tissue specific transcript.
pUC 9 Polylinker

CCAAGCTTGGCTGCAGGACGCTCCGGGAATTCA

Sali SmaI
HindIII EcoRI
PsI AccI BamHI XmaI

Figure 4.2 Restriction map of pUC 9, and sequence of the polylinker.
cells are grown on L-Amp plates containing X-gal and IPTG. This is a result of the cloning site of pUC9 being within an active lacZ gene which is inducible by IPTG and able to convert a colourless substrate (X-gal) to a blue product. Recombinants in which the insertion of DNA has not caused a frame shift mutation to the lacZ gene fragment, can also be recognised as they give rise to pale blue colonies as opposed to the very dark blue obtained with the intact pUC9 plasmid.

Once the library from a designated mRNA sample has been constructed, it can be used to compare gene expression at the steady state mRNA level with a different system. In this case the cDNA library has been constructed from steady state mRNA from suspension BL cells, at mid log phase, 5 days after subculture. The plant system used for the comparison is 5 week BL seedlings (see Section 3.2.5).

Comparison of the two systems involves making $^{32}$P-labelled first strand cDNA (probe) from the seedling mRNA, and using this to screen the newly constructed cDNA library. The probe will hybridise to all homologous sequences, and after washing to remove non-bound species these can be visualised by autoradiographic techniques. Such methodology has been used for example to show up differences in gene expression in the search for fruit ripening-specific expression in tomatoes (Mansson et al., 1985), in the isolation of tissue-specific cDNAs from tomato pistils (Gasser et al., 1989), and in the identification of genes expressed during floral initiation in tobacco (Meeks-Wagner et al., 1989). Plasmid screening at low (Grunstein and Hogness, 1975) and high (Hanahan and Meselson, 1980) colony density are now standard procedures. The procedure is usually repeated in replica with culture mRNA-directed DNA probes as positive controls.

4.1.2 Construction of the Library

Double stranded cDNA molecules were made from poly A$^{+}$ RNA extracted from 5 day cultured cells, via a reverse transcriptase-catalysed reaction followed by a DNA Pol I-catalysed reaction. The molecules were then tailed with terminal dioxxygentransferase and dCTP, and annealed to a previously dG-tailed pUC 9 vector (see Methods for details), and the
recombinant plasmids used to transform JM103 cells. The library contained approximately 5000 recombinants. Twelve plaques were picked at random, the plasmid extracted, cut with Pst I, and run on a 0.8% agarose gel to size the inserts. An average insert size of 700bp was found.

4.1.3 Screening the Library

2000 independent recombinant colonies were picked onto nitrocellulose filters, replica-plated, and probed with 32P-labelled first strand cDNA from both cell culture day 5 and seedling mRNA (Fig. 4.3). The screening identified several clones that were putatively differentially expressed in seedlings and suspension cultured cells. These were purified, and small scale plasmid DNA isolation was used to extract the recombinant plasmids. These were cut with the appropriate restriction enzyme and run on an agarose gel, which was then Southern blotted; replica blots were then probed with 32P-labelled first-strand cDNA from both cell culture day 5 and seedlings mRNA (Fig. 4.4). This was both to prove that the probe was indeed binding to the cDNA and not the pUC portion of the plasmid, and to confirm the previous results of Fig. 4.3 and discard any positives which may have arisen out of non-specific binding. The results, as seen in Fig. 4.4, suggest some of the clones represent cell culture related transcripts, and one, 4B (lane 5) to be present in both leaf and cell culture mRNAs.

The differential hybridisation test was repeated using DNA dot blots, which have a higher sensitivity (Fig. 4.5). This time, known quantities of insert DNA from the recombinant plasmids were blotted onto nitrocellulose in replica and again probed with 32P-labelled first-strand cDNA from cell culture day 5 and seedling mRNA. In this instance the dot blots show that none of the clones were stringently cell culture specific, although some seemed to be present as a large fraction of the mRNA populations, and may be more highly expressed in the cell culture system. The clones seen to represent an mRNA species present at high levels within the mRNA population (as shown by the arrows) were further characterised.
Figure 4.3 Colony hybridisation of cDNA library replica filters with $^{32}\text{P}$-labelled first strand cDNA from day 5 culture (A, B, C) and seedling (a, b, c) mRNA. The clones chosen for further characterisation are arrowed.
Figure 4.4 Southern blot of positive clones from the cDNA library probed with 32p-labelled first strand cDNA from cell culture (A) and seedling (B) mRNA.

Lanes 1 and 15. λ-Hind III markers.

Lane 2. pUC 9 cut with Pst I. Its position on the agarose gel is arrowed.

Lanes 3 to 14. Clones 3B, 4A, 4B, 4D, 4E, 6A, 7D, 7E, 7P, 8A, and 8B respectively, cut with BamH I/Hind III.
Figure 4.5  Dot blots of cDNA clones containing 1μg DNA per sample. The location of the clones is shown in the box below. The two replicas are probed with cell culture (A) and seedling (B) $^{32}$P-labelled first strand cDNA.

1A 1B 3A 3B 4A 4B 4D
4E 4F 4G 4H 5A 6A 7B
7D 7E 7G 7L 7I 7Q 7P
8A 8B 8C 8D 10A 10C pUC9
7S

The arrows represent clones picked for further characterisation.
4.1.4 Characterisation of the 6A Clone Family

Large scale plasmid preparations were made of the clones arrowed in Fig. 4.5. Table 4.1 shows the size of the clones as estimated from running \textit{Pst} I-digested recombinant plasmids on a 2\% agarose gel. These clones were then used in a series of cross-hybridising dot blots to ascertain whether any of them were copies of a common transcript (Fig. 4.6). All clones except 8B were seen to cross react, while 8B seemed only to hybridise to 6A, 4B and 7S. The suggestion that all clones may be copies of a common transcript was confirmed by three further experiments. The recombinant plasmids were digested with \textit{Pst} I and the reaction loaded on a 0.8\% agarose gel. Slices of agarose containing the inserts were then cut away from the gel, and the DNA extracted by electroelution. These inserts were then cut with a number of restriction enzymes capable of recognising 4-nucleotide sites. The probability of an enzyme finding a specific 6-nucleotide site on a stretch of DNA is 1/4096, while the probability of a 4-nucleotide site being found is 1/256. As a result, the use of 4-cutter enzymes (ie. restriction enzymes recognising specific 4-nucleotide sites) is more likely to yield a significant number of internal fragments. Cutting the larger inserts with the enzymes \textit{Sau} 3A, \textit{Msp} I and \textit{Alu} I, and running the samples on a 2\% agarose gel, showed a number of common bands appearing. Unfortunately the digestion patterns resulting from digests using one or two enzymes were too complicated for a restriction map to be drawn.

DNA extracted from cultured cells was also digested with either \textit{EcoR} I or \textit{Hind} III, run on a 0.8\% agarose gel and Southern blotted. Oligolabelled probes from the various clones were then used to hybridise to replicas of the blot (Fig. 4.7). As can be seen from the figure, the Southern blot patterns are the same for all clones, the number of \textit{EcoR} I restriction fragments lighting up was directly proportional to the length of the clone used as probe.

Finally, poly A\(^+\) RNA from cell culture was run on a denaturing 2\% agarose gel and Northern blotted. Oligolabelled probes from the various clones were then used to hybridise replicas of the blot (Fig. 4.8). All clones corresponded to the same RNA species; the transcripts comigrated with the 28S rRNA subunit as seen in both 2\% and 1.5\% agarose gels, with
TABLE 4.1

Sizes of clones showing putative differential expression, as identified from differential hybridisation screening.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Size (bp)</th>
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<td>6A</td>
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<tr>
<td>3B</td>
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</tr>
<tr>
<td>4B</td>
<td>1450</td>
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<tr>
<td>4D</td>
<td>295</td>
</tr>
<tr>
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<td>420</td>
</tr>
<tr>
<td>7P</td>
<td>610</td>
</tr>
<tr>
<td>7S</td>
<td>1485</td>
</tr>
<tr>
<td>8B</td>
<td>1320</td>
</tr>
</tbody>
</table>
Figure 4.6 Cross hybridisation dot blots for the 6A clone family. The location of the clones is shown in the box below. The clone used as probe for each hybridisation is shown on the figure.

<table>
<thead>
<tr>
<th>3B</th>
<th>4B</th>
<th>4D</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>7I</td>
<td>7P</td>
</tr>
<tr>
<td>7S</td>
<td>8B</td>
<td>pUC9</td>
</tr>
</tbody>
</table>
Figure 4.7  A. Southern blot of *Petunia hybrida* DNA probed with selected cDNA clones. The tracks contain 3µg DNA cut with EcoR I (R) or Hind III (H). Lane λ denotes the radioactive λ-Hind III markers. The clone used as probe for each hybridisation is shown on the figure.

B. Northern blots of cell culture (2% gel and C track on 1.5% gel) and seedling (SL) mRNA. The clone used as probe for each hybridisation is shown on the figure.
Figure 4.8 Enzyme restriction maps of the 6A clone family
an approximate size of 4.7Kb (Carmichael and McCmoster, 1980).

Once satisfied that the clones indeed represented copies of a common transcript, clone 6A was chosen as the representative of this group, because it is the longest (1960bp) and binds to the largest number of restriction fragments in the EcoR I-cut DNA (three bands, at 2.3, 4.5 and 20Kb). Restriction mapping with EcoR I, BamH I and Hind III as single or double digests were also carried out. The resulting maps and relationship between clones can be seen in Figure 4.8.

4.1.5 Sequencing the 6A Clone

The 6A plasmid was cut with BamH I and Pst I and subcloned into the M13mpl9 vector, previously digested with BamH I and Pst I. The ligation mixture was used to infect competent TG2 cells and the resulting plaques picked and grown in L broth for 5h prior to DNA extraction via the miniprep method. The DNA was then cut with BamH I and Pst I to detect any inserts and their respective sizes. A clone containing the 570bp fragment of 6A was picked for sequencing using Sanger's dideoxynucleotide method (Sanger et al., 1977). The 233bp sequence resulting from this (Fig. 4.9) was used in a data search of the EMBL nucleic acid sequence database. This showed the fragment to bear a high sequence homology with mouse 28S ribosomal rRNA (88%), yeast 25S rRNA (80%) and rat 28S rRNA (67%).

The previous transcript-sizing result using Northern blotting, confirms the sequence result. Furthermore, Southern blotting analysis with DNA from wheat, pea, tobacco and flax (fig. 4.10) shows the pattern of 6A-homologous fragments to resemble that for ribosomal probes already published (Gerlach and Bedbrook, 1979; Appels et al., 1980; Ellis et al., 1984; Uchimiya et al., 1982; Goldsbrough and Cullis, 1980). Also, samples containing BL DNA equivalent to one million haploid genome copies (1.6μg) were run on an agarose gel with samples containing plasmid reconstructions equivalent to 1, 2, 10 and 50 thousand million copies of the 6A clone and Southern blotted. The Southern blot was probed with oligolabelled 32P-6A to attempt a quantitation of the copy number of the gene. Fig. 4.10 shows this to be estimated at 1-2 thousand.
Figure 4.9 Sequence of 233bp internal fragment of 6A.
Figure 4.9B Sequence of rat 28S ribosomal RNA showing region of homology to 6A.
Figure 4.9C  Sequence of mouse 28S ribosomal RNA showing the region of homology to 6A.
Figure 4.9D Sequence of yeast 25S ribosomal RNA showing the region of homology to 6A.
Figure 4.10 Southern blot of DNA extracted from a number of different plant species, cut with EcoR I (odd numbers) or Hind III (even numbers), and probed with 6A. The amount of DNA in each lane has been adjusted to give one million copies of each haploid genome.

Lanes λ. λ-Hind III radioactive markers.
" 1, 2. Wheat
" 3, 4. Tobacco
" 5, 6. Flax
" 7, 8. Oat
" 9, 10. Pea
" 11, 12. Petunia Blue lace
" 13, 14. P. mitchell
" 15, 16. P. inflata
" 17-20. 6A cut with Pet I, loaded in quantities equivalent to 1, 2, 10, and 50 thousand copies of cDNA

\( \lambda \text{ million} \)
4.1.6 Self Priming of Poly A+ RNA

The question now posed was: why were copies of ribosomal RNA present in the cDNA library? RNA copying into cDNA involves the denaturation of the RNA template by boiling, and the use of oligo dT(14-18) as primer to the reaction. This should ensure that only RNA molecules which possess a poly A tail will be copied. But mRNA preparations from cellulose-oligo dT columns are known to contain rRNA molecules (Spradling, Pardue and Penman, 1977), and these are often represented in cDNA libraries as well (Meeks-Wagner et al., 1989); indeed, the presence of such clones at 5-10% of the total cDNA recombinants is probably a normal event.

A pilot first strand cDNA reaction was run using 200ng poly A+ RNA, with or without oligo dT(14-18) primer. The reaction was carried out in a total volume of 10ml, thus containing 5nmol dCTP (see Methods for reaction and incorporation calculation details). The results are shown in Table 4.2. It is therefore apparent that the poly RNA has a self-priming capacity. It is not known whether this is caused by a snap-back effect within an RNA molecule (Meeks-Wagner et al., 1989) thus initiating self-priming, or if two separate RNA molecules hybridise under the reaction conditions (Maxwell and Martin, 1986).

Within the 6A family of clones, the majority have a common EcoR I end fragment of 440bp. This is therefore the site at which self-priming is probably occurring. The fragment was subcloned into M13mp19 and sequenced. Klenow is known to be unable to copy DNA successfully when encountering sequences with a high GC content, especially in the case of templates containing inserts with dG·dC homopolymer regions (Gomer, Datta and Firtel, 1985). Klenow was in fact unable to copy the DNA template distal to the poly dC run created by the tailing reaction, and the sequencing reaction resulted in a high number of artificial terminations. A method was reported in Focus (Gomer, Datta and Firtel, 1985), using Hinc II buffer (20mM Tris-HCl pH 7.4, 5mM MgCl2, 50mM KCl) and a temperature of 50°C, which apparently relaxes the secondary structure of the template that may cause the Klenow fragment to stop. It did not improve the results.

The sequencing was therefore attempted using Sequenase (United States Biochemical Corporation). This is a new sequencing enzyme which is
Table showing the extent of self-priming in a first-strand synthesis reaction under the conditions outlined in the cDNA cloning method.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Total Counts</th>
<th>Incorporated Counts</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ oligo dT</td>
<td>409106</td>
<td>12926</td>
<td>97%</td>
</tr>
<tr>
<td>- oligo dT</td>
<td>458886</td>
<td>1794</td>
<td>12%</td>
</tr>
</tbody>
</table>
reported to eliminate compressions caused by sequences with high GC contents (Tabor and Richardson, 1987). While Sequenase passed successfully through the dG·dC homopolymer region, the synthesis of nascent DNA seemed to experience a number of artificial terminations further down the template. These terminations were thought to be caused by Sequenase detaching from the template as a result of response to a specific DNA sequence. Sequenase is known to sequence successfully inverted repeat sequences (REF) and therefore the inability of the enzyme to cope with the sequence is not thought to be because of snap-back resulting in double-stranded DNA. At the same time, the 3' end of the sequence did not contain any poly (A) sequences longer than 5bp, making the hypothesis that the oligo dT\(^{(14-18)}\) molecules may have served as primers unlikely.

4.1.7 Conclusion

The main conclusion to be drawn from the results shown above is that, whatever the nature of any differential transcripts between the culture and seedling systems, they are not present at a frequency of 0.1% or more of the total mRNA population. This is in contrast to other systems where direct differential screening has been successful, such as studies in ripening in tomato (Mansson et al., 1985), flowering in tobacco (Meeks-Wagner et al., 1989) and tissue specific expression in tomato pistils (Gasser et al., 1989); screenings with these systems have shown the presence of both elevated or exclusive gene expression in the tissues under study. Unfortunately, there have been no reports on such methodology being used to study differences in steady state transcripts between dividing cultured cells and whole plants, and it is therefore not possible to compare these results with other systems.

Consideration of the abundance level of mRNA sequences is of critical importance in the understanding of these results. A typical eukaryotic cell contains approximately 1pg of mRNA, equivalent to about \(10^6\) molecules, transcribed from about 15000 different genes (Sargent, 1987). Most genes are members of the low abundance class, and are represented by about 20 mRNA copies per cell or 0.002% of the total
population. A small number are expressed at higher mRNA levels (medium abundance), and a few mRNAs accumulate to a few percent of cellular mRNA, or even more in some cases (Sargent, 1987). Probe design in this type of screening involves synthesis of first strand cDNA from poly A⁺ RNA by a method that yields DNA with a maximum activity of about $10^6$ cpm/μg. If 100 ng of poly A⁺ RNA are used, the activity incorporated into an RNA species representing 0.1% of the total mRNA, will be 100 cpm. Assuming the background activity on the filter because of non-specific binding to be 5-10 cpm, this method would not detect any signal given by a probe representing less than about 0.05% of the total mRNA population. Therefore, a differential screen will only detect medium to high abundance mRNA species and will fail to detect low abundance transcripts.

To investigate further the transcriptional profiles of the culture and seedling systems, two-dimensional protein gel electrophoresis methods were developed. This was thought to be necessary in an attempt to define the cloning strategy needed to continue the study.

There is also an apparent discrepancy in the results attained with the Northern blots shown in Fig. 4.7B. The samples added to the gel were poly A⁺ RNA extracted from cultured cells and seedlings respectively; the apparent modulation in the relative amounts of rRNA in the samples was probably a result of the extraction and purification procedures. As the same chemicals were used in the production of both samples, this must have occurred as a result of some impurities inherent to the samples themselves.

The presence of rRNA copies (crDNA) in the cDNA library is still unexplained. From the sequence analysis of the putative 3' end of the molecule (Section 4.1.6) we know that priming was probably not caused by binding of the oligo dT(14–18) to the rRNA molecules. Also, first-strand reaction does show DNA synthesis to occur in the absence of the oligo dT(14–18) primer (Section 4.1.5). To understand this we have to look at the function of the reverse transcriptase in the first-strand reaction. The enzyme embodies two functions \textit{in vitro}, a polymerase activity and an associated progressive 5' and 3' (RNase H) ribonuclease activity that is specific for RNA:DNA hybrids (Maniatis \textit{et al.}, 1982). The polymerase, the
desired activity, requires a template RNA molecule hybridised to a DNA primer with a 3'-hydroxyl group, and all four deoxyribonucleoside triphosphates, in order to synthesize a DNA molecule which is a faithful complement of the RNA. During this process, the exonuclease is fully active. The products of the exonucleolytic cleavage reaction are oligomers 2–30 bases in size. RNA fragments produced by RNase H after the polymerase has synthesized complementary DNA can act, inefficiently, as primers (Krug and Berger, 1987); owing to their small size, oligomers of RNA dissociate from the newly synthesized cDNA at moderate temperatures. When such fragments are reverse transcribed, the resultant DNA oligomers are excellent primers. The presence of such "artificial" primers in the reaction mixture could explain the synthesis of crDNA. The other method of priming may also involve secondary structure formation within the rRNA molecules, or the presence of smaller RNA molecules in the mRNA preparation which already show homology to the rRNA. For example, mouse 5 and 5.8S rRNA has been shown to hybridise to 18 and 28S rRNA (Maxwell and Martin, 1986). Unfortunately none of the data collected so far can discount either process (or indeed a combination of the three) being the cause of rRNA self-priming.
4.2 Construction of a cDNA library into a Lambda Vector, and Screening for Genes Preferentially Expressed in Culture

4.2.1 Introduction

Minor changes within a population of polypeptides of common molecular weight are difficult to detect by one-dimensional PAGE. In one dimensional gels of the \textit{in vitro} translated proteins from \textit{Petunia} mRNA, up-regulation or down-regulation of one transcript, resulting in a change in the relative amount of its translation product within the total polypeptide population, could not be resolved satisfactorily. As discussed in Section 3.2.4, a two-dimensional gel system was set up and used to resolve the population of polypeptides produced in the \textit{in vitro} reactions. As seen in Section 3.2.6, the \textit{in vitro} translation product profiles from 5 day old cultured cells and seedling mRNA were remarkably similar, with a number of peptides being down-regulated or undetectable in culture. When radioactive polypeptides appearing on the two-dimensional gel were scintillation counted, and the values compared to the total number of counts applied to the gel, it was calculated that a low abundance spot represented 0.018\% of total radioactivity applied (see Section 3.2.6). This showed why a method which can only pick up differences in an mRNA population greater than 0.1\% failed to show any differential transcripts.

Therefore the failure of direct colony hybridisation screening to pick out differentially expressed clones was presumably the result of the very strong similarity in the transcription profiles of the two systems, coupled with the relatively insensitive methods used.

The improvement of cDNA differential probing could be achieved via the use of two parallel strategies. Firstly a new library would be constructed into a lambda vector, \lambda-ZAP (Fig. 4.11A). The use of a phage as vector has a number of advantages, the main one being its transformation efficiency via transfection. Competent cells can be made to accept plasmids such as pUC with an efficiency of $10^7$ cpn/$\mu$g plasmid DNA; higher transformation efficiencies have been quoted by commercial
Figure 4.11  A. Restriction map of $\lambda$-ZAP.

B. Restriction map of pBluescript.
suppliers such as Amersham and Bethesda Research Laboratories (BRL), but
the above is a value routinely reached in our laboratory. Lambda
packaging extracts will produce particles capable of infecting competent
E. coli cells with an efficiency of \(10^8-10^9\) pfu/\(\mu\)g lambda DNA.
Furthermore, in a vector such as pUC 9 (2.7Kb) a cDNA insert of 1Kb will
represent 30% of the recombinant plasmid. Using the transformation
efficiency of \(10^7\) cpu/\(\mu\)g plasmid DNA quoted above, we can deduce that
30ng of double-stranded DNA will be required to produce a library of \(10^6\)
recombinants. With \(\lambda\)-ZAP (50Kb) the same cDNA insert will now represent
2% of the recombinant phage, and therefore 0.2ng of double-stranded cDNA
will now be sufficient to produce a library of \(10^6\) recombinants assuming
the transfection efficiency of \(\lambda\) to be \(10^8\) pfu/\(\mu\)g lambda DNA. Although
the above calculations are an overt simplification, as they do not take
into account experimental error, they give a reasonable idea of the way
in which use of the \(\lambda\) vector reduces the amount of cDNA needed to reach
the amount of recombinant molecules that are needed to produce a library
containing representatives of low abundance transcripts.

Secondly, the phage naturally produces lysis plaques within an E.
coli lawn. These plaques will contain a large amount of DNA which can be
absorbed onto nitrocellulose via plaque lifting methods. As already seen
in Section 4.1.2, colony hybridisation suffers from a problem of high
non-specific background activity. This is because of the large amount of
cellular debris (proteinaceous and carbohydrate) formed as a result of
artificial cell lysis. This both entraps radioactive probe, increasing
the background, and to some extent may shield the plasmid DNA from
hybridisation. Cellular debris is far less abundant in plaques, and tends
to remain within the soft agarose top layer when plaque lifts are
effectuated, thus decreasing the background problem.

Libraries constructed in lambda vectors are also easier to store;
mature phage particles can be stored indefinitely at 4°C in SM buffer
(50mM Tris-HCl pH7.5, 100mM NaCl, 10mM MgSO\(_4\), 0.01% gelatin) and may be
plated as and when needed (Vogeli and Kaytes, 1987). Amplification is
also very efficient; if done in situ on L-Agar (rather than in solution)
competition between different phage particles is minimised thus ensuring
that the amplified phage population is very similar to the original one.
In the case of plasmids on the other hand, the biological host has a
limited lifespan, and storage can only be implemented by either glycerol freezing of the bacteria or purification of the recombinant plasmid population, followed by subsequent introduction into host cells, before the library can be reused; these procedures are time consuming and may lead to loss of individual clones within the library because of their low efficiency of transformation.

The λ-ZAP system also has a number of advantages in respect to its phage vector counterparts. In λ-ZAP (Fig. 4.11A), the internal $\text{red}^+ \text{gam}^+$ fragment has been replaced by a Bluescript plasmid sequence (Fernandez et al., 1986). This contains a polylinker within the lac promoter and lacZ gene, similar to that in pUC, and a defective M13 origin of replication. The former allows the phage to be used as an expression vector so that specific clones may be isolated by antibody screening. The latter enables the vector to be excised as a functional pBluescript plasmid (3.0Kb, Fig. 4.11B) when complemented in trans by a helper virus; this removes the need for any subcloning of the chosen clones into handier sized plasmids. The pBluescript plasmid can also be used directly in dideoxy-terminal sequencing.

The system used for the differential hybridisation can also be improved. A probe which has been enriched for cell culture specific sequences may be produced. One such method involves binding seedling cDNA covalently to cellulose (Noyes and Stark, 1975) and hybridising this to $^{32}\text{P}$-labelled first strand cDNA from cell culture specific RNA. The probe can be enriched with successive cycles of hybridisation, and then used to probe the cDNA library under study. The method has already been shown to be very effective for systems in which the traditional direct colony screening procedures were not strong enough (eg. Scott et al., 1983).

Another method involves solution hybridisation of $^{32}\text{P}$-labelled first strand cDNA from cell culture specific RNA, with a 100-fold excess of seedling specific RNA poly (A$^+$). The mixture is then passed through a hydroxylapatite column to remove all cDNA:RNA hybrids, and the resulting single stranded cDNA used to probe the cDNA library under study (Sargent, 1987).

Both these methods should minimise the appearance of false positives from direct differential screening that may occur as a result of increased probe from a specific transcript because of transcriptional
upregulation, while enhancing the presence of \textit{de novo} transcripts of very low abundance. Work with \textit{in vitro} translation of mRNA samples (see Section 3.2.5, Fig. 3.8) shows that the only differences apparent from the \textit{in vitro} translation products profiles are those caused by transcript up-regulation or down-regulation in culture. The 2D method has been shown to resolve polypeptides of 0.02% abundance. Thus, if any genes present are preferentially expressed in the culture stage, they must be present at a level below 0.02% of the mRNA population, as they are not detected with the 2D method. They will only be detected by differential cDNA library screening, if the summation of the methods discussed above is able to increase the screening sensitivity to below 0.02%.

4.2.2 Construction of the Library

RNA poly A\(^{++}\) extracted from day 5 suspension cultured BL cells was used to synthesise 130ng of double stranded cDNA. This was ligated into \(\lambda\)-ZAP which had been previously cut with \textit{Eco}RI and dephosphorylated. The recombinants were packaged and titrated. The library was seen to contain 1.75\times10^5 pfu at a concentration of 3.5\times10^5 pfu/ml. This gave a cloning efficiency of 7.5\times10^5 pfu/\mu g cDNA.

Twelve plaques were picked at random, grown for 4h and the DNA extracted. This was cut with \textit{Eco}RI to excise the cDNA insert and run on a 2% agarose gel for sizing. The average size of insert was seen to be 500bp.

The library was also probed with the 6A ribosomal probe to ascertain the frequency of ribosomal clones. A plate containing 350 plaques was used. As can be seen in Fig. 4.12, 26 plaques lit up with the probe. This is well below the 10% maximum limit that may be considered acceptable in a library. The library was then stored in SM buffer over chloroform.
Figure 4.12 Plaque lift of 350 plaques from the cDNA library probed with 6A.
4.2.3 Probe Enrichment

When a gene is differentially expressed but is not represented by an mRNA of abundance much greater than 0.1%, it becomes difficult to isolate it by simple differential screening. In such cases it is possible to increase the effective concentration of the desired sequence or sequences by subtraction hybridisation. This procedure is made possible by the fact that mRNA populations can be readily hybridised to complete kinetic termination with homologous cDNA. In this case, mRNA from cultured cells is used as a template to produce a radiolabelled tracer cDNA. The cDNA is hybridised to an excess of mRNA isolated from seedlings. The unhybridised first strand cDNA represents an enriched population of sequence expressed in culture but not seedlings. Two such methods have been used in this project. In the semi-solid method, the seedling sequences (as cDNA) are bound to cellulose, in the solution method the nucleic acids are unbound. Separation of double stranded seedling:culture nucleic acid hybrids from the enriched population of single stranded culture "specific" first strand cDNA occurs by centrifugation or hydroxylapatite chromatography respectively.

Probes derived from differentially expressed genes enriched by the methods discussed above have been used to identify RNAs representing only 0.01% of the total mRNA population (Sargent, 1987). This is still a level of sensitivity that may not be good enough to identify very low abundance RNAs (0.002% of total mRNA). At the same time it is a level of sensitivity very similar to that achieved with the two-dimensional gel system, and may be used to verify its results.

4.2.3.1 The Semi-Solid Method

The first step in this method was the binding of DNA to activated cellulose. For this purpose epoxy-cellulose (BRL) was used; this is expected to bind DNA at concentrations 4µg/mg cellulose with 90% efficiency. Sonicated herring sperm DNA was used to test the system. The reaction conditions were as outlined in Moss et al. (1981). The aqueous waste from the reaction and aliquots of water used in the washes were placed in a spectrophotometer to quantitate the amount of DNA which bound successfully to the cellulose; the results are in Table 4.3a.
TABLE 4.3

(a) Table showing the amount of DNA lost in water washes after binding to epoxy-cellulose.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DNA loss in aqueous waste</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% DNA loss in 1st H₂O wash</td>
<td>14.6</td>
<td>15.0</td>
<td>18.7</td>
<td>19.8</td>
<td>16.5</td>
</tr>
<tr>
<td>% DNA loss in 2nd H₂O wash</td>
<td>2.5</td>
<td>3.0</td>
<td>2.8</td>
<td>2.2</td>
<td>3.9</td>
</tr>
<tr>
<td>% DNA loss in 3rd H₂O wash</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(b) Table showing DNA binding values after specific times of incubation under the solution hybridisation conditions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sol. A</th>
<th>Sol. B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding after 30min</td>
<td>2.06%</td>
<td>1.15%</td>
</tr>
<tr>
<td>1h</td>
<td>2.78%</td>
<td>1.96%</td>
</tr>
<tr>
<td>4h</td>
<td>2.57%</td>
<td>2.58%</td>
</tr>
<tr>
<td>24h</td>
<td>14.20%</td>
<td>8.60%</td>
</tr>
<tr>
<td>72h</td>
<td>16.10%</td>
<td>9.60%</td>
</tr>
</tbody>
</table>
The solution hybridisation was carried out by two separate methods. The first, outlined in Moss et al. (1981) used 200mg cellulose/ml in a solution containing 0.6M NaCl, 10mM PIPES pH7.0, 2mM EDTA and 0.2% SDS (sol. A) and was carried out at 65°C. The second, outlined in Scott et al. (1975), used 180mg cellulose/ml in a solution containing 40mM PIPES pH6.5, 1mM EDTA, 0.1% SDS, 50% formamide, 0.25 mg/ml poly C + poly U and 0.5mg/ml yeast tRNA (Sol. B) and was carried out at 37°C.

The hybridisations were carried out using time courses, with 0.3μg nick-translated single stranded herring sperm DNA as substrate. The reactions were carried out in 4 replicas. Table 4.3b showing the average binding results. Changing the container in which the hybridisation took place from 1.5ml Eppendorfs to 1.5ml screw-capped tubes or 2.0ml Eppendorfs, did not seem to affect the reaction. It is not understood why our results did not mirror the ones published. The system was dropped as unworkable.

4.2.3.2 The Solution Method

This method consists of two main steps, the solution hybridisation, and the separation of single and double-stranded nucleic acids using a hydroxylapatite column.

The hydroxylapatite (BioLabs) was swollen in 0.01M sodium phosphate buffer pH6.8 and poured into a Pasteur pipette previously plugged with polyallomer wool to a height of 1cm. A mixture of single and double-stranded sonicated herring sperm DNA was loaded onto the column in 0.01M phosphate buffer and the column washed with the same buffer. A series of buffer aliquots at concentrations from 0.05M to 0.30M, in 0.05M steps, were then passed down the column. Each aliquot consisted of 10ml, of which the first ml was ethanol precipitated to concentrate any nucleic acid. Samples from each concentration were then run on a 0.8% agarose gel to view the separation on single and double stranded DNA. It was found that single stranded DNA eluted with 0.15M phosphate and double-stranded DNA with 0.20M phosphate. The experiment was therefore repeated using phosphate concentrations of 0.10M to 0.20M at 0.01M intervals. From this the values of 0.13M phosphate buffer for elution of single-stranded DNA and 0.18M phosphate buffer for elution of double-stranded DNA were derived.
The solution hybridisation was standardised as follows. The hybridisations were carried out in replicas (see Methods), one tube containing 1μg globin mRNA, 9μg plant RNA and 10ng 32p-labelled globin first strand cDNA; the other tube did not contain any globin mRNA to show the extent of non specific hybridisation. The reactions were placed at 39°C, 49°C, 54°C, and 59°C overnight, and a hydroxylapatite column used to separate single and double-stranded nucleic acid. These were placed in scintillation vials and counted using Cherenkov counting. The results are shown in Table 4.4. It can be seen that 49°C is the temperature at which the highest specific binding of globin mRNA to the radioactive probe is coupled to the lowest non-specific binding by plant RNA. At the same time it is important to point out that these conditions may only be used to enrich the culture-specific probe in the BL system, not to remove all transcripts also present in the seedling mRNA population.

4.2.4 Screening of the Library

It was expected that cell culture specific transcripts, if present, would be found at very low abundance (less than 0.01%). To increase the probability of such low abundance transcripts binding to DNA from plaque lifts, *in situ* amplifications were carried out (see Methods). This method is used routinely to increase hybridisation signals when screening with subtracted DNA (Vogel and Kaytes, 1987), and circumvents the need for replica screening to confirm true positives.

Ten 150mm Petri dishes, each containing 1500 pfus were grown overnight, and the plaques amplified *in situ*. They were then probed with an enriched probe made with the solution hybridisation method. Replica filters were also probed with 32p-labelled first strand cDNA from seedling mRNA poly A+. This is because the probe is simply enriched for culture-specific transcripts and contains seedling-related transcripts as well; as a result, the replica filters can be used to discount all common transcripts. One of the replica filters is shown in Fig. 4.13 and it can be seen that the enrichment did not remove all seedling related transcripts. At the same time some spots (open arrow) are present only on the filter probed with seedling cDNA (suggesting that although the
Table showing relative amounts of single- and double-stranded nucleic acid species after solution hybridisation, as separated by hydroxylapatite.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Globin mRNA + CPM</th>
<th>%</th>
<th>Globin mRNA - CPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ss</td>
<td>ds</td>
<td>ss</td>
<td>ds</td>
</tr>
<tr>
<td>39</td>
<td>633</td>
<td>1389</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>49</td>
<td>284</td>
<td>1069</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>54</td>
<td>363</td>
<td>570</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>59</td>
<td>336</td>
<td>321</td>
<td>51</td>
<td>49</td>
</tr>
</tbody>
</table>
Figure 4.13 Replica plaque lifts from the cDNA library probed with seedling (SL) and enriched probe (ENR).
transcripts are present in the library, their cDNA have been removed from
the probe mixture by the enrichment process), and some are present only
on filters probed with the enriched probe (closed arrows). This is in
marked contrast with filters where the more direct differential method of
probing replica filters with culture cDNA and seedling cDNA does not give
any differences, suggesting the absence of differentially expressed genes
at levels of 0.1% or higher.

In all 32 clones that appeared culture specific were picked,
together with 2 clones that appeared to be seedling specific. The 32
culture specific clones were grown and reprobed with first strand cDNA
from culture and seedling RNA poly A+; 15 were seen to respond to the
seedling probe and were therefore discarded. The remaining 16 were used
as probes in a series of dot blots containing 50ng, 5ng, and 500pg RNA
poly A+ from the two populations. Of these 3 showed similar binding to
the two RNA populations, 12 showed upregulation of the transcript in
cultured cells, and one showed no response at all, suggesting very low
transcript abundance. The two "seedling specific" clones showed a strong
down regulation in culture. Some examples of the blots are shown in Fig.
4.14, and the results are tabulated in Table 4.5.

4.2.5 Conclusion

The sensitivity of the two-dimensional gel system used in our
laboratory (0.018%) and screening of cDNA libraries with enriched probes
(0.01%) is very similar. In view of this, it was not surprising that the
screening simply confirmed the results achieved with the gel system.
These results emphasise that two-dimensional gels should be a
prerequisite to starting a project looking for differentially expressed
transcription products. If the technique had been available two years
earlier, this work would probably never have been attempted.

The results seen in Section 4.2.4 with one of the two "seedling
specific" probes are also of interest. As the cDNA clones arise from a
culture library, we must presume that the respective transcripts are
indeed present in the culture mRNA population. But one of the clones (BL
101, Fig. 4.14) was not capable of lighting up culture RNA when used as a
Figure 4.14 mRNA dot blots probed with various clones to show the differences in steady state mRNA levels within the culture and seedling populations. The amounts of mRNA on the blots is shown below.

<table>
<thead>
<tr>
<th>Culture mRNA</th>
<th>50ng</th>
<th>5ng</th>
<th>0.5ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seedling mRNA</td>
<td>50ng</td>
<td>5ng</td>
<td>0.5ng</td>
</tr>
</tbody>
</table>
TABLE 4.5

RNA Dot Blots Results for Putative Culture Specific Clones in Blue Lace

<table>
<thead>
<tr>
<th>Clone</th>
<th>Signal with culture mRNA</th>
<th>Signal with seedling mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50ng</td>
<td>5ng</td>
</tr>
<tr>
<td>BL 1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BL 2</td>
<td>+</td>
<td>+</td>
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<td>BL 4</td>
<td>+</td>
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<td>BL 9</td>
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<td>BL 10</td>
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<td>BL 14</td>
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<td>BL 16</td>
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<td>BL 21</td>
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<td>BL 30</td>
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<td>+</td>
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<tr>
<td>BL 101</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BL 102</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
probe in dot blots. We must therefore infer that this transcript is present at an extremely low abundance level in the mRNA population. This shows us that the technology used in the construction of cDNA libraries is adequate to clone transcripts of low abundance. It therefore seems that the screening technique themselves are at fault and need improving if we are to purify such low abundance clones. Finally, it must be emphasised that the inability of a probe to produce a positive signal in an RNA or DNA dot blot is not conclusive evidence of the absence of homologous sequences in the nucleic acid populations being tested. It is always extremely dangerous to define a transcript as tissue or stage specific on such evidence alone.

This part of the project was now considered terminated, as it was not expected to find de novo transcripts in this particular culture system with the technology available. This by no means rules out the use of empirical approaches to the problem of finding genes preferentially expressed in a particular system, but highlights the need for more thorough basic studies before two systems are considered adequate for such a study.

The question remains on how suspension cultures appear to retain a transcriptional profile so intrinsically similar to that of normal growing plants containing a whole range of differentiated tissues. This is probably because of a number of factors, some of which result from the methodology used in this project. As already discussed, the sensitivity of the screening processes and resolution of the in vitro translation products are by no means optimal and do not allow a complete view of the transcriptional profiles in the two types of tissue. The work has also looked at steady state mRNA levels; therefore transcriptional control, mRNA stability and availability to translation by the mRNA molecules, and their role in controlling gene expression, are not dissected. Finally, it is possible that transcriptional control does not play such a prime role in directing gene expression, and that the major control points are further downstream. These points, and other possible control levels in gene expression are discussed in detail in Section 4.3.
4.3 General Conclusions to Chapters 3 and 4

It has been stated that in rapidly growing tissue such as dedifferentiated cultured cells, the major regulatory event determining the synthesis of a particular protein is the transcription and possible subsequent processing of an active mRNA (Bevan and Northcote, 1981a). The striking structural, physiological and biochemical differences found between cultured cells and differentiated plant cells may therefore be expected to be mirrored in transcriptional changes. From our results, it seems that this may be a gross oversimplification. At the same time, there are a number of inherent limitations with the methodology used:

a) the limits of detection are not low enough to guarantee a total overview of the transcriptional profiles;
b) the methodology does not provide differentiation between translating and non-translating pools of mRNA;
c) the further regulatory event of post-translational modification has not been investigated.

As discussed in Section 4.1.7, most gene transcripts are members of the low abundance class, and are represented by about 0.002% of the total mRNA population. Neither two-dimensional gel electrophoresis, nor screening with enriched probes can positively detect differences of less than 0.01%, and this is probably not enough to detect very rare mRNA species. At the same time, autoradiography of two dimensional gels of \textit{in vitro} translated $^{35}$S-labelled proteins showed spots which were deemed to be about 5-fold less dense than the low abundance spots representing 0.018% of the total radioactivity incorporated into the translated polypeptides; unfortunately it was not possible to quantitate the amount of radioactivity by scintillation counting, as this was very close to background activity. O'Farrell (1975) states that two-dimensional gel systems are sensitive enough to detect minor protein species that contain between 0.0001-0.00001% of total radioactivity applied. If we consider that in an average \textit{in vitro} translation 1% of the total radioactivity applied is incorporated into polypeptides, the 2D gels should be able to detect 0.01-0.001% of the total radioactivity incorporated. As an average
mRNA constitutes 0.001-0.005% of the total RNA in eukaryotic cells, it should be possible to detect the proteins encoded by these mRNAs if they are translated in proportion to their abundance. But the results achieved routinely in our laboratory are not within those limits, and we accept the possibility that transcripts of extremely low abundance may still escape detection. These limitations have also been reported by other workers. For example, immunological techniques were used to detect gene products preferentially expressed during the early phases of somatic embryogenesis in carrots (Smith et al., 1988). A 45kD polypeptide was detected, thought to represent less than 0.01% of the total cellular protein. Translational profiles of carrot embryonic proteins separated by two-dimensional gel electrophoresis were either autoradiographed or transferred to nitrocellulose for immunodetection. The polypeptide corresponding to the 45kD immunoreactive spot could not be detected on the autoradiograph.

At the same time, developmental processes in culture, resulting in gross biochemical and physiological changes, such as cytodifferentiation and somatic embryogenesis, have also failed to yield large changes in the transcriptional and translational profiles of the systems under study. During cytodifferentiation in mechanically isolated Zinnia cells (Fukuda and Komamine, 1985), an investigation of proteins extracted from 35S-labelled cells using 2D electrophoresis revealed that only a few proteins were associated with tracheary element differentiation. Two differentiation-specific proteins were newly synthesised and the synthesis of two other proteins was shut off only in differentiation-induced cultures, at a time when protein synthesis is a prerequisite for cytodifferentiation. Unfortunately no data was presented on in vitro translated proteins, and a direct comparison of transcriptional profiles in induced and non-induced cells is not available. When the translational profile of cultured carrot cells is compared with that of the somatic embryos derived from them (Sung and Okimoto, 1981), the profiles from the two tissues are very similar, except for two peptides found in somatic embryos but undetectable in callus. If such processes, normally associated with substantial changes in cytology, enzyme activity, and biochemical pathways, are not reflected
in changes in transcriptional or translational profiles, other regulatory steps must be present.

A known regulation step in gene expression is the availability of mRNA to translation. Subculture has been shown to stimulate protein synthesis by stimulating the recruitment of previously untranslated mRNA into polysomes (Bevan and Northcote, 1981a). Also, the readily reversible differentiation states observed in plant cell cultures raise the possibility that cytodifferentiation in culture does not depend on the transcriptional activity of different sets of genes for each kind of tissue cell but that the 'specialised' physiology of such cells may represent the influence of cytoplasmic factors (such as plant hormones) on the stability and transport of RNA species and other aspects of translational steps in gene expression (Street, 1977). Differentiating between translatable and non translatable mRNA in cells involves purification of mRNA from polysomal preparations, which are then compared to cytological mRNA preparations. The purification method used in our laboratory is such that it does not differentiate between translated and non-translated pools of mRNA. As a result, any regulatory events at the translational stage would have been overlooked.

A number of regulatory events in gene expression also occur post translationally. A typical such regulatory event is phosphorylation. A major role for phosphorylation in eukaryotic cells is in the control of their mitotic cycle (Russell and Nurse, 1987a). This is suggested by the observation that a number of proteins undergo changes in their state of phosphorylation during mitosis; evidence shows that phosphorylation may control a number of events such as nuclear membrane breakdown and reformation, chromosome condensation and spindle formation (Russell and Nurse, 1987b). Studies on the cell cycle in eukaryotic cells (mainly yeast, frog oocytes and human HeLa cells) have individuated a maturation promoting factor (MPF) which promotes the transition between the G2 and M phases in the cell cycle (Cyert and Kirschner, 1988). MPF is a phosphoprotein (from immunoprecipitation studies) present throughout the cell cycle in Xenopus and HeLa cells; it is activated by phosphorylation prior to mitosis and rapidly deactivated thereafter (from 2D protein gels; Cyert and Kirschner, 1988; Draetta and Beach, 1988). The protein kinase responsible for phosphorylation and dephosphorylation of MPF has a
molecular weight of 34KD, and is termed p34. P34-like proteins have been isolated from yeast (Gautier et al., 1988), frog eggs (Cyert and Kischer, 1988) and human HeLa cells (Draetta and Beach, 1988); they all have homologous sequences, similar immunogenetic properties, and the human HeLa protein was isolated by virtue of its ability to rescue temperature sensitive mutants of fission yeast (Draetta and Beach, 1988). The expression of p34 itself is controlled in yeast by positive and negative regulators (Gautier et al., 1988), which also seem to encode proteins with consensus sequences for protein kinases (Russell and Nurse, 1987a, 1987b). On the whole the basic mechanism of mitotic initiation is thought to be highly conserved in all eukaryotic cells, p34 being a key component of the control exerted by MPF (Gautier et al., 1988).

Phosphorylation as well as protease activation (e.g. the opioid peptides precursor, pro-opiolanacortin, or pre-insulin, Creighton, 1984), or modification of glutamic acid to γ-carboxyglutamic acid (Gla) in the activation of blood-clotting enzymes (Creighton, 1984) are only some of the examples of post translational control in eukaryotic cells.

Street (1977) states that: "although from a number of plant species, cell cultures can be readily initiated from different organs (root, stem, leaf petiole or lamina, cotyledons, etc.) or from different living tissues within an organ (parenchymatous cells of pith, cortex or mesophyll, etc.) there is no very convincing evidence that they retain, in culture, characteristics of their in vivo origin". We have shown that the transcription profiles of cultured cells and seedlings in Petunia hybrida mirror the loss of some biochemical synthetic pathways (such as those related to photoautrophy) but show no production of novel transcripts within our detection limits. This seems to disprove the idea that, at least for this system, the major regulatory event determining gene expression is transcription. It does not, in our minds, prove an inherent similarity between the two systems as many more regulatory steps (some of which have been discussed above) have not been investigated. At the same time, it shows up the risk of oversimplification in looking at changes in transcription levels to explain differences between developmental stages in whole plants. Plant cells are highly plastic, developed systems, and we should never forget that transcription is but one level in a miriad of regulatory events.
CHAPTER FIVE

CELLULAR AND MOLECULAR CHARACTERISATION OF ASPARAGUS CELL CULTURE
5.1 Dedifferentiation and Cell Culture Initiation

5.1.1 Introduction

Dedifferentiation is the process by which specialised, quiescent cells give rise to heterotrophic, dividing cells. It is the initial process in the establishment of cultures from tissue explants, cells or protoplasts. Dedifferentiation is a developmental pathway, but differs from all other such processes in that it often represents a transition between two differentiated cell types. Cellular development during this transition must be controlled by changes in the amounts and activity of various enzymes and structural proteins. There is little known about differences in the sets of proteins synthesised during dedifferentiation. Previous studies have concentrated on looking at end products of the dedifferentiation process (see Chapter 3), i.e. cultured cells, but have not attempted to study this process of dedifferentiation at a molecular level.

A number of changes are expected to occur during dedifferentiation: gene expression related to stress and medium constituents, a change in the mode of primary metabolism from photoautotrophic to heterotrophic, the redistribution of plastids, cell expansion, cell division, changes in nuclear morphology, synthesis of cell walls and changes in composition to contain less cellulose and an altered arabinose/xylose ratio (Blaschek et al., 1981), increase in vesicular fusion (Fransz and Schel, 1987), an increase in orithine decarboxylase activity (Chiriqui et al., 1986), changes in the type of lipid synthesised and isoperoxidase profiles (Goldberg et al., 1986). The above changes are expected to contribute to an altered protein profile. At the same time it is quite possible that there are no novel proteins synthesised during dedifferentiation, and that all the observed structural and physiological changes observed are due to differential processing of proteins, mRNA stability and/or translatability, or post translational modification such as phosphorylation, acetylation or glycosylation.
5.1.2 Culture Initiation

The wounding of plant tissues during culture initiation activates a number of metabolic changes which can be sustained, and to some extent regulated, by transfer of the cells to the appropriate culture conditions. As the tissue proliferates in a more or less disorganised manner, the expression of primary and secondary metabolism is disturbed (Aitchison et al., 1977). Two responses are known to occur during culture induction, the wound response and the growth response. The former is characterised by a rapid increase in metabolic activity and does not usually result in callus formation; it may be identified when, for example, explants are cultured on auxin-free medium. The growth response, resulting in cell division, is dependant upon an exogenous supply of auxin, and results in changes in the cell structure which may be viewed under the light and electron microscope (Yeoman and Street, 1977). These changes reflect an altered metabolic activity and include the disappearance of storage products such as starch, and an increase in mitochondria and polyribosomes.

Aitchison (Aitchison et al., 1977) divides the course of callus development into three main stages:

a) induction, during which metabolism is activated and the cells prepare to divide, their number remaining constant;
b) division when active synthesis of protein and nucleic acids occurs, accompanied by a regressive change (dedifferentiation) leading to the formation of a particular growth pattern. During this period the mean cell size and all cell constituents decrease, except for the amount of RNA (mostly rRNA);
c) differentiation, during which a new course of development is initiated.

A number of morphological and metabolic changes occur during the induction period. A common change is the increase in respiration rate that accompanies the excision and culture of quiescent tissue, such as that of Helianthus tuberosum (Yeoman and Street, 1977); this is usually concomitant to structural rearrangements (especially in the mitochondria) suggesting a general increase in metabolic activity, and, in the case of explants, to the establishment of a wound cambium of decreased cell
size. Wounding is not always the trigger for dedifferentiation. For example, if light-grown cultures with mature chloroplasts are transferred to the dark, a dedifferentiation to proplastids is observed, paralleled to a dedifferentiation of the vacuolated cells leading to a less differentiated meristematic state (Yeoman and Street, 1977).

The earliest detectable change in the dedifferentiation of parenchyma cells of carrot and artichoke (Yeoman and Street, 1977) is an increase in the number of polyribosomes, suggesting an increase in protein synthesis. This activation of protein synthesis may result in the production of polypeptides undetected in the original tissue. For example, immunoelectrophoresis shows a change in the antigen population during the culture of tobacco, from excision to regeneration of the whole plant (Butenko and Volodarsky, 1968). Some proteins disappear during dedifferentiation while others appear. The latter set seem characteristic of the undifferentiated phase as they are also detected in normal meristematic tissue. Some of these proteins, such as non-histone proteins (NHP) have been characterised further (Guerri et al., 1982). A comparison of embryogenic and single cells in Dactylis glomerata (Hahne, Mayer and Lorz, 1988) has also taken place by silver staining of total protein extracts run on two-dimensional PAGE, and in vivo and in vitro labelling. 21 polypeptides were detected in the embryogenic cells only, and 6 in the single cell suspension only. Of the former, 10 were found in various tissues of the mature plant; all the others were undetectable. Unfortunately the methods used suffer from two resolution deficiencies: a) silver staining of 2-D total protein gels may only detect a polypeptide spot representing 0.002%, or more, of the total protein; this value may be too high to reveal rare peptides especially as the technique is blighted by problems of protein accumulation; b) there are no allowances for proteins which have been modified to different extents in the systems under comparison.

Other systems, such as carrot (Choi and Sung, 1984), sorghum (Wozniak and Partridge, 1988), pea and rice (Hahne, Mayer and Lorz, 1988) only show one or two peptides which are differentially detectable in differentiated or dedifferentiated tissue.

Dedifferentiation of intact tissues also causes significant changes in the expression of several enzymatic activities (Masuda et al., 1988).
This is sometimes accompanied by the appearance of different isomeric forms of proteins such as peroxidases and esterases. For example, alterations in the activity of peroxidases in cultivated *Sinapsis alba* (Reinert, Bajaj and Zbell, 1977) and tobacco stem sections have been shown to be connected with dedifferentiation, the original state being restored at the beginning of redifferentiation. Another example is rice, where the esterase isoenzyme pattern of various somatic organs show characteristic differences in the number and density of bands upon gel separation, whereas callus cultures, irrespective of source, display a different but common pattern (Wu and Li, 1970).

Nucleic acids are also affected by the dedifferentiation process. In rice, for example, pollen derived callus shows an increase in chromosome number in 65.5% of the cell population (Kikuchi, Takaiwa and Oono, 1987). After root decapitation in *Vicia faba*, cell division is preceded by DNA synthesis (Cionini, Zolfino and Cavallini, 1985), even though the root cells are cell cycle arrested at the postsynthetic phase. Repetitive DNA is also underrepresented in differentiated *Vicia* cells (Bassi *et al.*, 1984) and there is thought to be a relationship between the DNA synthesis observed in root cells which dedifferentiate and DNA sequences which have become underrepresented during the process of cell differentiation. This quantitative change in the population of repetitive DNA has also been shown in rice (Kikuchi, Takaiwa and Oono, 1987). During callus formation a cloned repeat sequence (pBR 301) was seen to increase 50 fold; this change is reversed during redifferentiation. The wound and growth responses during dedifferentiation are also characterised by distinct periods of RNA synthesis (Lindsey and Yeoman, 1985). Ribonucleic acid synthesis results mainly in the production of rRNA; in some instances culture induction will result in the breakdown of existing polyribosomes and the synthesis of novel ones (Fleck *et al.*, 1982). In all the systems viewed so far (eg. Sacco de Vriers *et al.*, 1988) the transition between the differentiated and dedifferentiated states is accompanied by only minor changes in gene expression as visualised by 2D PAGE analysis of *in vitro* translated mRNA populations. This suggests that transcription is by no means the sole control in the switch of gene expression in this developmental pathway, and may actually be shown to be only a minor component.
5.1.3 Choosing a Model System

Three main systems may be used to study the changes occurring during dedifferentiation and cell culture initiation: large organ explants, protoplasts and mechanically isolated cells. Large organ explants have been used for most of the work mentioned above. Their main advantage is the possibility of utilising them for any plant system, both in the study of the wound response and, if the right culture conditions are attainable, the growth response. Two serious disadvantages occur. Firstly, because only the cells neighbouring the wound site respond, there is a problem of dilution of signal which can only be solved by careful dissection prior to tissue collection. This is labour intensive and time consuming. Secondly, only limited amounts of tissue can be collected, making molecular biology studies problematic.

Protoplasts may be prepared in large amounts using simple efficient techniques. Additionally, all protoplasts within the culture will be exposed to the same conditions, thus facilitating experimental design and reproducibility. At the same time, because the cells are being stripped of their cell walls, a highly artificial system ensues, which complicates any results. Firstly, cell wall degradation and placing the protoplasts in culture results in the synthesis of a number of stress proteins (Fleck et al., 1982) similar to those seen after sugar deprivation, anaerobis or heat shock. Secondly, it has been reported that in some instances isolation of protoplasts triggers the rapid hydrolysis of several membrane lipids (Goldberg et al., 1986), and in protoplasts from maize roots the new wall has a different polysaccharide composition from that found in the original cells (Goldberg et al., 1986). Alterations in the new cell wall are also found with respect to protein content; in Vigna radiata, for example, all newly synthesised cell walls contain isoperoxidase patterns different from the ones present in the original hypocotyl cells (Goldberg et al., 1986). All these changes will affect the overall results, and as the protoplast system is so artificial, it may respond to the culture conditions in a completely different manner to cells that have not been stripped of their cell walls. Finally, this project is concerned with investigating the role of transcriptional regulation in dedifferentiation. Fleck (Fleck et al., 1980) has shown
that the difference in gene expression between protoplasts and mesophyll cells in tobacco is entirely caused by transcriptional differences (from \textit{in vitro} translations of mRNA populations), and therefore protoplasts may not be the right system to use.

Mechanically isolated cells may be prepared from only a restricted number of plants, but they can be produced in large amounts using simple efficient techniques. They have all the advantages of protoplasts without the major disadvantages connected to the loss of the cell wall, and are discussed in the next section. Mechanically isolated cells of \textit{Zinnia} have been successfully used to study cytodifferentiation.

5.1.5 Mechanically Isolated Cells in the Study of Cytodifferentiation

Cells at a relatively early stage of development, such as quiescent (non-dividing) types of parenchyma and the cells in meristems, vascular cambial tissues and embryonic tissues, are in a condition referred to as "undetermined". Undetermined cells are by definition capable of switching to different pathways of development depending on the environment imposed on them. Cytodifferentiation is the process or processes by which a previously undetermined plant or animal cell may be induced to differentiate into a different specific cell type \textit{de novo}.

The study of cytodifferentiation has often used as a model system the differentiation of mature parenchyma cells into tracheary elements. Tracheary element cytodifferentiation \textit{in vitro} is composed of four ontogenetic phases: acquisition of competence of the target cell to commence cytodifferentiation (lag phase), cell enlargement, secondary wall formation, and secondary wall lignification and autolysis (Fukuda and Komamine, 1983). It is at phase III that differentiation-specific proteins are synthesised, and sensitivity to DNA and RNA synthesis inhibitors is at its highest level.

The use of \textit{in vitro} culture systems have brought about an improvement of the study of tracheary elements by rendering possible the use of homogeneous tissue (or cells) as the initial material. At present, isolated single-cell systems are the nearest to an ideal system for the investigation of cytodifferentiation. A synchrony of tracheary element
formation of 30-40% mesophyll cells differentiating within a 20h culture period has been reported (Fukuda and Komamine, 1985) for *Zinnia*. The system developed by these workers has a number of inherent advantages: as the initial cell population is composed of only single cells, each cell can simultaneously receive equal stimulation through a given liquid medium to divide and differentiate; the process can be followed visually in individual cells. Single cell suspension also means that the effects of cell-to-cell interaction can be excluded in analysing the process of cytodifferentiation. Fukuda and Komamine (1985) report a high frequency and high synchrony in the differentiation process, with circa 60% of the tracheary elements formed within 3 days of culture without intervening mitosis. Using the *Zinnia* mesophyll cell system, the relationship between cytodifferentiation, the cell cycle, and biochemical mechanisms of cytodifferentiation has been investigated.

A number of physiological conditions are thought to influence cytodifferentiation, including hormones, carbohydrates, inorganic nitrogen sources, amino acids, light, temperature, and osmotic pressure. Auxins and cytokinins are the most important factors in the initiation and sequence of differentiation in higher plants; an exogenous supply of both hormones is essential for cytodifferentiation, although the optimum concentrations may vary (Phillips, 1980; Mizuno and Komamine, 1978). On the whole, the data collected on the effects of auxins and cytokinins on cytodifferentiation seem to suggest that the primary role of the hormones lies in inducing the expression of some specific genes, rather than simply cell division and elongation (Fukuda and Komamine, 1985). This may be likened to the role of different cytokinin/auxin ratios in promoting shoot and root formation in tobacco tissue culture, or xylem and phloem differentiation in sycamore callus (King and Street, 1973). At the same time, *in vivo* labelling studies using $^{35}$S-Methionine (Fukuda and Komamine, 1985) reveal that only a few proteins are associated with tracheary element differentiation in *Zinnia*.

A close relationship between tracheary element differentiation and the cell cycle has also been suggested. Many reports suggest that there is the need of an intervening round of cell division prior to cytodifferentiation in culture; other reports show that this is not the case, but the possibility that the cells were committed to
differentiation before plating is not ruled out. It is with *Zinnia* (Fukuda and Komamine, 1980) that the first direct evidence for cytodifferentiation without prior cell division has been presented.

5.1.5 Mechanically Isolated Asparagus Cells as a Model System

Mesophyll cells of asparagus will undergo dedifferentiation when placed in culture medium. The cells may be mechanically isolated from asparagus cladodes rapidly and in sufficient quantities to enable a study of this switch in development at the molecular level. Work in this laboratory (Paul et al., 1989; Harikrishna, 1989a) has shown that during dedifferentiation a reproducible, ordered sequence of events occurs that includes reactivation of the cell cycle followed by cell expansion. This is a highly active process as shown by a large increase in RNA levels and a change in the relative protein composition, as determined by denaturing PAGE.

Dedifferentiation in asparagus is extremely complex, and includes wound responses, hormonal effects, dedifferentiation and cell division. The wound response is expected as mechanical isolation disrupts the plasmadesmatal connection between the mesophyll cells. It is also well established that the presence of pectic fragments can illicit defense- (Templeton and Lamb, 1988) and wound- (Ryan, 1988) regulated gene expression; the asparagus cells are exposed to cell wall fragments for a period of time up to 40min during isolation. Reactivation of the cell cycle is a fundamental event occurring during dedifferentiation. Little information has been obtained on the events controlling the cell cycle, as an examination of the process within the whole plant meristem at the molecular level is complicated. Results from cell culture systems, which can be more easily studied and manipulated, may be useful in understanding the control of the cell cycle. However, it should be pointed out that division during dedifferentiation differs from that in the meristem as the cells are vacuolated and are not contained within an organised tissue; comparisons between the *in vivo* and *in vitro* systems should therefore proceed with great caution.
Asparagus is also very useful as a molecular system. The organism is a monocotyledon, while still retaining a low ploidy level and small genome size. It is a diploid of relatively small genome size, with a $1C$ value of 1.2pg (Paul et al., 1989), as compared to other monocots such as wheat (17.3pg) or oat (13.2pg). The main advantage of a small genome size is the relative ease of isolation of plant nuclear genes from genomic libraries; furthermore asparagus, being a diploid, presents a homologous nuclear background, where studies on exogenous genes or re-introduced plant gene fusions can be carried out with relative ease. The amenability of asparagus to tissue culture and transformation by $A. \text{tumefaciens}$ (Bytebier et al., 1987) is a further advantage of the system. The organism has been shown to transform with reasonable efficiency, and both the upkeep of transformed cell culture lines and the regeneration of transformed plants is feasible. This enables the re-introduction of plant gene fusions in its homologous host, in order to study plant gene regulation.

5.1.6 Investigation of Differential Gene Expression

Any information gained in analysing protein profiles would greatly contribute to our understanding of the processes of dedifferentiation. The aim of this project is to examine protein profiles, and if any novel or highly upregulated polypeptides appear, to study their accumulation and finally clone the genes coding for them. Resolution is an important consideration for the choice of methodology to employ, as it has been demonstrated (Kamalay and Goldberg, 1980) that rare mRNAs make up over 95% of nucleotide sequence diversity, and it is this fraction that contains differences in mRNA species between organ types.

The choice of methodology for this project relies on the fact that, as discussed in Section 3.2.1, representation is easier at the transcriptional level rather than at the protein level. The approach involves defining specific switches in gene expression that result in changes in steady state mRNA levels (as mirrored by $\text{in vitro}$ translation products), or total protein levels. $\text{In vitro}$ translation products, or total protein extracts, are run on polyacrylamide gels to pinpoint
polypeptides that are being produced to a higher level in one of the systems being compared. "Novel" peptides may also be individuated this way, but we must be wary of calling them novel, as they might be present at levels below detection in the putatively negative system. These polypeptides may then be isolated and used for antibody production, in the case of total protein extracts, and/or microsequencing. As an \textit{in vitro} translation does not yield enough product for microsequencing, this is only possible if the mature protein resulting from the same mRNA transcript in the native plant cell can be identified; this is relatively easy if the mature protein is not post-translationally modified to any great extent (see Section 5.3.3). Microsequencing can then be used to predict the genetic code of the transcript and produce synthetic DNA oligomers to be used as gene probes. The probes thus defined may now be used to extract specific sequences from a cDNA library. This targeted approach is more restricted than the empirical approach used with the \textit{Petunia} project, and will therefore preclude the viewing as a whole of the changes occurring; at the same time having a defined protein as the target will allow for data resulting from protein chemistry techniques (such as antibody work) to be accumulated, giving a fuller picture of the biological interactions taking place with any particular polypeptide.

As already mentioned, resolution is the most important factor in the choice of methodology. The approach chosen for this project is \textit{in vitro} translation followed by two-dimensional gel electrophoresis. This is because changes within the population of polypeptides at a specific molecular weight will not be detected by one-dimensional PAGE. Two-dimensional gel electrophoresis involves separating the \textit{in vitro} translation products with different pKa values (iso-electric focusing) prior to the molecular weight separation afforded by PAGE (O'Farrell, 1975, see Section 3.2.4). IEF gels are broad pH range gels and were chosen in preference to non-equilibrium isoelectring focusing gels (NEPHAGE) because better acidic resolution can be obtained. Since the majority of plant proteins have pIs that fall within the pH 4–6 range, an increased resolution of the acidic range is preferable to increased resolution of the basic range obtainable by NEPHAGE.

This method has been reported to be able to resolve down to 0.1–0.001% of total incorporated activity. Since an average rare mRNA
constitutes between 0.001% and 0.005% of total eukaryotic RNA, if these mRNAs are translated in proportion to their abundance their gene product should be resolved, as it is well within the detection limits of the two dimensional gel system (see Section 4.3).

This approach has a number of potential shortcomings in that in vitro translated mRNA profiles may represent a distorted view of gene expression during dedifferentiation. Firstly, eukaryotic mRNAs are translated with unequal efficiencies in vivo and in vitro (Joblin and Gehrke, 1987). This effect has been attributed to the 5' untranslated leader sequence of the mRNA; for example, a chimeric mRNA composed of the barley α-amylase coding region and the alfalfa mosaic virus leader sequence, shows a 35-fold increase in translation efficiency compared to the native α-amylase mRNA. Secondly, each mRNA is assumed to be the product of a single gene, but this is not a valid assumption in all cases, as can be shown by the extensin gene (Chen and Warner, 1985), which produces two different sized transcripts from a single gene, as a result of its two first exons, whose presence in the mature mRNA molecule is determined by splicing.

In vitro translation also has the disadvantage that it simply mirrors changes in steady state mRNA levels, and is unaffected by other gene regulation mechanisms. Evidence from yeast and animal research is strongly in favour of post translational control being involved in regulating the cell cycle and mitosis (Dunphy and Newport, 1988), so cell cycle mediated changes may not be observed on the translated asparagus mRNA profiles. There are also examples, eg. seeds and pollen grains, where mRNA is stored stably, and only translated at particular stages of development (Gordon and Payne, 1976), in which case any mRNA extracted from these tissues may not represent active protein synthesis; such stable messages would give the wrong impression about gene expression at that point in time. These factors, combined with the differences in plant and animal initiation codons usage when more than one initiation codon is present in the mRNA molecule (Lutcke et al., 1987), may lead to artifacts in the interpretation of gene expression during dedifferentiation. However, if the translated mRNA profiles are compared against each other, and only quite clear differences are highlighted, artifactual interpretations can be minimised.
The ultimate aim of the study is to clone any differentially expressed messages, if they exist, so the use of *in vitro* translation is the most logical approach to use, in order to study mRNA profiles to decide at which stage a cDNA library should be constructed, to maximise the probability of finding differentially expressed proteins.

5.1.7 Summary

Dedifferentiation is a complex response to environmental factors, involving a number of closely related processes: stress-related responses, the loss of specialisation, and cell division. Once again, cell culture is the best system available to date to study dedifferentiation. The main advantages of an *in vitro* system are ease of manipulation, strict control and reproducibility of experimental conditions, and the large amount of homologous tissue rendered available to the researcher by the technique. Against this we always have to bear in mind that we are looking at a highly artificial system, and care must be taken when extrapolating any results to the *in vivo* system.
5.2 Characterisation of Asparagus Cell Culture System

5.2.1 Introduction

Mesophyll cells of asparagus may be mechanically isolated from cladodes and, when placed in the appropriate culture conditions, will undergo dedifferentiation. During dedifferentiation a reproducible, ordered sequence of events occurs, including stress response, reactivation of the cell cycle, cell expansion, and cell division. The culture method allows rapid cell isolation in sufficient quantities to enable a study of this switch in development at the molecular level. The first goal in this project is the separation of the various stages within dedifferentiation to qualify switches in gene expression.

5.2.2 Initiation and Maintenance of Culture

The isolation of mesophyll cells method was based on the procedure of Jullien and Guern (1979) which utilises small glass homogenisers to macerate asparagus cladodes. This method only produces a relatively small number of cells at each grinding, and a number of changes were therefore made to the procedure (see Methods), resulting in a method that enables the rapid isolation of a large number of cells for each experiment (>100 million). The growth of cells in Petri dishes with very gentle agitation (35rpm) is also thought to be less stressful (Paul et al., 1989), while easing microscopic observations. Preliminary experiments indicated that a cell concentration of 4x10^5 ml^-1 gives the most reproducible cultures, while still allowing microscopic examination at this cell density. A high division frequency was achieved (>90% of viable cells, Fig. 5.1) after only 6 days with cells grown in the dark on a slow orbital shaker.

The morphology of asparagus cell cultures after the initial homogenisation up to the end of the first division is shown in Fig. 5.2. The onset of the various phases of the reactivation of cell division was also determined by experiments estimating the onset of DNA synthesis by microdensiometry, and using staining techniques to quantify mitotic
Figure 5.1 Percentage of cells that have divided (Δ) and the area of undivided (○) and divided (●) cells in culture from day 0 to 6.
Figure 5.2 Development of cell cultures. (A) Freshly isolated cells on day 0. (B) Slightly expanded cells on day 3. (C) Divided, expanded cells on day 5. (D) Finely dispersed cells in Petri dish following isolation. (E) Aggregated cell culture on day 2.
events, nuclear division and cell plate formation (Paul et al., 1989). A summary of the events during the various dedifferentiation phases can be seen in Fig. 5.3. For a more detailed account the reader is referred to Paul et al., 1989.

After 14 days cells from two Petri dishes were added to 40ml of media in a 250ml conical flask and placed on an orbital shaker (75rpm) at constant illumination (2.5-4 µmol M⁻² sec⁻²). The cells were further subcultured every 14 days. By day 14 cells had formed microaggregates; these did not disperse, but continued to grow until, by 4-5 months in culture, they formed spherical entities 2-6mm in diameter (Fig. 5.4). This has been found to occur in other systems (e.g. Antirrhinum) and is thought to be hormone dependant (King and Street, 1973). No further growth of the spheres was noticed after 6 months, but the culture remained viable in terms of RNA, protein and carbohydrate production.

Freshly isolated asparagus cells were seen to produce an exudate by day 2; this was thought to be partly responsible for the cells sticking to the bottom of the Petri dishes. Exudate production continued during the culture’s growth cycle, to increase dramatically as the culture reached the stationary phase of its growth after 3 months. The products were harvested and analysed by hydrolysis followed by chromatography (S. Fry, Edinburgh University). The exudate was seen to be qualitatively very complicated but a number of interesting components were detected:

a) high levels of galacturonic acid, galactose and arabinose suggesting the presence of pectic material;

b) all common amino acids were detected, indicating the presence of proteins in the exudate. This was further confirmed by running soluble exudate on SDS-PAGE gels;

c) extremely high levels of esterified phenolic acids were found, higher than reported in any cell wall. High diferulate levels were also found; the compound is involved in the cross-linking of polysaccharide molecules during cell wall formation.

The cultured cells therefore seem to have differentiated into a state where the metabolism leading to the production of substances contained within this exudate is grossly amplified. As will be discussed in Section 5.2.5, this property of the culture may be utilised in the study of cell wall matrix synthesis and establishment.
**Phases of dedifferentiation**

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
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<tr>
<td><strong>Day 0 - 2</strong></td>
<td><strong>Day 3 - 4</strong></td>
<td><strong>Day 4 - 6</strong></td>
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<tr>
<td>Little change in cell morphology or size</td>
<td>Slow cell expansion</td>
<td>Rapid cell expansion</td>
</tr>
<tr>
<td>Migration of nucleus</td>
<td>DNA replication</td>
<td>Continuation of cell cycle</td>
</tr>
<tr>
<td>Increase in size of nucleus and nucleolus</td>
<td>Mitosis</td>
<td>Further changes in protein composition</td>
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<td>Changes in protein composition</td>
<td>Further changes in protein composition</td>
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*Figure 5.3 Phases of dedifferentiation in mechanically isolated Asparagus cells.*
Figure 5.4 *Asparagus* cultures after 2 weeks (A), 4 weeks (B), 2 months (C), and 6 months (D).
5.2.3 Extraction and Purification of RNA

The RNA extraction method used in this project has been modified from Covey and Hull (1981) to counteract some of the problems associated with plant RNA extraction. The method is discussed in detail in Section 3.2.3.

Nucleic acid extracted from asparagus cells (especially cladodes) contains a large amount of soluble carbohydrates and other as yet unidentified impurities. The former show on a spectrophotometric scan as absorbing at 230nm (Fig. 5.5B), the latter shift the entire scan curve between 200-300nm by 0.1-0.3A units (Fig. 5.5C). These impurities comigrate with mRNA on a cellulose oligo dT column, and if allowed to remain, they will inhibit both \textit{in vitro} translations, and reverse transcriptase-mediated DNA synthesis. It has been found that reducing drastically (5 to 10 fold) the aqueous volume during the phenol/chloroform extractions causes the greater part of these impurities to flocculate. Therefore, in the RNA extraction, after 2 phenol/chloroform extractions, the nucleic acid in the aqueous phase is ethanol precipitated, and the pellet resuspended in 1/5 vol. with 100mM Tris-HCl pH 8.4. This resulted in a suspension which, when spun at low speed (5K on an SS34 rotor) will separate into a pellet containing \textgreater 80\% of the impurities and aqueous phase containing \textgreater 70\% of the nucleic acid. This process is repeated until the pellet dissolves completely, and even though it may cause the loss if some of the RNA, it will result in material which can then be utilised in further manipulations. As can be seen from Fig. 5.5A, the RNA extracted by this method contains no protein impurities and is very low in carbohydrate contamination.

All RNA preparations were made from cultured cells at the defined stages, except for the cladode tissue. The cladode tissue is prepared by stripping decapitated fronds and contains no meristematic tissue; this is because, during mechanical isolation, cells at the apical tip are destroyed. The first time point in the sampling of extracted cells is termed day 0, as it represents gene expression of the cells when they are plated out. This process of sterilisation, mechanical isolation, filtration, washing of cells and plating out takes approximately 3 hours to perform.
Figure 5.5  Optical density absorption scans of RNA preparations. (A) High quality pure RNA. (B) Carbohydrate impurities. (C) Unidentified impurities. All samples were extracted from asparagus cladodes.

Key  A'  Relative absorption values
On average *Asparagus* cladodes yielded 0.5mg total RNA/g tissue. The poly A+ fraction represented 1.2% of the total RNA. Cultured cells yielded 10mg total RNA/g tissue, and poly A+ was 1.4% of the total RNA.

### 5.2.4 Steady State Proteins and Transcripts at Different Phases of the Cell Culture

The observed changes in cell morphology at the onset of asparagus culture, particularly nuclear division and cell plate formation, indicate an increase in metabolic activity during dedifferentiation. Therefore, nucleic acid and protein were extracted from the cells to determine changes in the content and constitution of macromolecules during culture initiation. A change in the protein composition occurred, as determined by SDS-PAGE (Fig. 5.6). There was an increase in complexity of the protein extracted, with additional bands present in extracts from cultured cells which were not detected in day 0 cells. For example, a band was seen to appear at 16kD, its relative intensity increasing between days 3 and 6. Bands at 56kD and 16kD decreased in relative intensity between days 0–6; protein in these bands were recognised by polyclonal antibodies raised against total Ribulose bisphosphate carboxylase.

The steady state levels of mRNA were studied via the use of *in vitro* translation followed by running the products on 2D polyacrylamide gels. For the first dimension, or IEF, a broad range ampholine was used in conjunction with cooling to 10°C, and focusing conditions of 400V for 12h followed by 1500V for 1h (see Methods); this enables a reproducible linear gradient extending from pH 3.6 to pH 8.0 to be obtained (Fig. 5.7). When these gels are combined with a second dimension SDS-PAGE, a very reproducible protein profile can be generated (Harikrishna, 1989a). The number of resolved polypeptides range from 100–300 (Fig. 5.8); this is similar to results obtained by other workers (Harikrishna, 1989a). When a rough estimate of the number of counts resolved for polypeptide spots of different intensities was made, it was calculated that a low abundance spot represented 0.018% (± 0.0082%), an average spot represented 0.024% (± 0.0071%) and an abundant spot between 0.04–0.09% (± 0.0058%) of total incorporated radioactivity (Harikrishna *et al*., 1989a).
Figure 5.6 PAGE of total protein extracts from Asparagus cultured cells, showing a band at 16kD.

Lane 0. Cladodes

1-6. Days 1-6 after isolation respectively.
Figure 5.7  pH gradient along the IEF rod gels.
Figure 5.8 Number of resolved polypeptides on 2D-PAGE of *in vitro* translated mRNA.

Key:
- nmc - cladodes
- wc - whole fronds
- 0-7 - 0-7 days in culture respectively
- 2 mths - 2 months in culture.
This resolution is not as good as that reported by O'Farrell (1975) of 0.001%. The value of radioactivity for the low abundance spot was the minimum detectable level for the scintillation counter. Autoradiography did reveal fainter spots than those corresponding to the low abundance signal, but it was not possible to quantitate their radioactivity. Visual examination allowed a rough estimation of these minor spots as 2-5 fold less radioactive than the low abundance polypeptides.

The number of detectable polypeptides in the 2D gels (Fig. 5.8) increase gradually with time in culture to a maximum achieved between 2 and 5 days in culture, decreasing after six days to a stable level, which appears to be maintained between 7 days and 2 months in culture. It was also observed that cladode samples including apical tips contained more resolvable polypeptides. This was probably as a direct result of mRNA contained in the metabolically active cells of the apical meristem.

The use of mirror image gels made intensity comparison of different polypeptides simple (Fig. 5.9). Polypeptides of molecular weight 16 kD, specific to or highly upregulated in dedifferentiating cultured cells, can be quite clearly identified. These polypeptides were present in all batches of translated mRNA, and were considered fit for further detailed analysis. They were termed DD1. The abundance and time of appearance of the various peptides within the group seem to vary (see Section 5.3.2). The common molecular weights also suggest the presence of isomeric forms, or otherwise related peptides. DD1, as seen in in vitro translations may coincide with the band at 16 kD seen to appear in total protein extracts from cultures cells (Fig. 5.6).

5.2.5 Summary and Conclusions

The process of dedifferentiation in isolated asparagus cells involves an ordered, reproducible sequence of events which can be initiated in a population of cells relatively uniform at isolation. It appears to be a useful model system to examine gene expression during this developmental switch. The quantitative changes shown to occur in mRNA (as mirrored by in vitro translation) during the initiation of culture show the initiation of culture to be a highly active process.
Figure 5.9 2D-PAGE of *in vitro* translated mRNA from cladodes (A) and 5 day cultured cells (B). (C) shows a mirror image of the acidic proteins of the *in vitro* translated mRNAs from cladodes (X) and 5 day cultured cells (Y). The M16 p5.6 a/b doublet of the DD1 group of proteins is highlighted (□).
The complexity of the protein composition increases further from day 2 to day 5, and there is a marked change in the level of several in vitro translated proteins in addition to DDL (Harikrishna et al., 1989a).

After a couple of months in culture, a new course of development is initiated, during which the culture, initially a friable callus, undergoes a redifferentiation resulting in the formation of very hard small spheres. These grow up to a diameter of circa 4mm; disruption of the balls by cutting them up with a scalpel results in the formation of more spheres. The number of detectable transcripts on 2D PAGE from in vitro translations also decreases in the long term cultures.

Biochemically, the most striking visual process occurring during dedifferentiation and subsequent redifferentiation is the production of large amounts of exudate. Culture initiation from intact tissue is known to cause significant changes in the expression of several enzymatic activities (Masuda et al., 1988), and to bring about quantitative and qualitative differences in extracellular glycoproteins (Satoh et al., 1986). For example, glycosidase (Asamizu et al., 1981) peroxidase and acid phosphatase (Satoh et al., 1986) are released into the medium in carrot cell suspension, as are peroxidase (Gasper et al., 1983) and glucoamylase (Masuda et al., 1988) in cultured sugarbeet cells. A number of these proteins are involved with cell wall synthesis or degradation. For example, polyphenol oxidase is rapidly secreted in sycamore cell cultures (Bligny and Douce, 1983); the protein is involved in the synthesis and deposition of cell wall substances (mainly lignin) and makes up circa 2% of the total protein synthesised in culture. β-galactosidase and β-glucosidase activities are also correlated with cell growth in both sycamore and carrot cell suspension (Masuda et al., 1985); these cell wall bound glycosidases are involved in the degradation of wall polysaccharides to bring about cell wall loosening and allow cell elongation. In Medicago sativa the cellular localisation of β-galactosidase seems to dictate its substrate utilisation (Chaubet and Parcilleux, 1982). While the extracellular and cell wall bound enzyme is involved in the turnover of arabinogalacane, the internal isoform is responsible for the utilisation of lactose.

Asparagus cells produce a large amount of exudate; approximately 38% has been shown to be made up of sugars (S. Fry), while ferulic acid,
p-Coumaric acid and diferulic acid make up a further 7% of the exudate; these are all cell wall components. The plant cell wall matrix has been under study for some time, to try and clarify its structure and properties. In particular, it is known that important properties of cell walls, such as extensibility, digestibility and adherence are dictated by wall cross links (Fry, 1986). These are the result of the presence in the matrix of compounds such as extensin, pectins, hemicelluloses, various wall enzymes and lectins. Extensins have hydroxyproline rich peptides backbones; as the exudate does not contain any considerable amount of this amino acid residue, extensins do not seem to be excreted in any appreciable amounts. Pectins are based on a galacturionate (15% of total exudate from TFA and Driselase hydrolysates, S. Fry) backbone, and contain discrete domains rich in rhamnose residues many of which bear neutral arabinose- and/or galactose-rich side chains. These side chains sometimes carry ferulic or p-Coumaric acid residues. Cross linking of pectin occurs through oxidative coupling of phenolic substituents (diferulate) or esterification of phenolic acids. Both esterified phenolic acids and diferulic acid have been found in very large amounts in the asparagus exudate. This may therefore probe an interesting model to study the cross linking mechanisms of pectin and eventually the process by which new cell walls are constructed.

Finally, many events occur during dedifferentiation, including DNA synthesis and movement of the nucleus during reactivation of the cell cycle (Paul et al., 1989), a loss in photosynthetic capacity (Harikrishna et al., 1989b), and an increase in cell wall synthesis. The cells are exposed to several environmental stimuli, such as wounding and addition of culture medium containing hormones, calcium and sucrose. It is hoped to distinguish a particular aspect of dedifferentiation with which DDL is associated.
5.3 Characterisation of the DD1 group of proteins

5.3.1 Introduction

As outlined in Section 5.2, changes in the composition of *in vitro* translated proteins were detected throughout the initiation of the asparagus cultures. For example, more than 40 proteins are detected at day 5 that were not seen with *in vitro* translated protein synthesised from cladode mRNA (Fig. 5.8). The most striking of these changes is the presence of a group of proteins designated DD1, at 16 kD and pI 4-6. DD1 was not initially detected in translation products of mRNA isolated from cladodes. Further analysis revealed some components of the group at very low levels (<0.018%) in mRNA isolated from whole fronds, i.e. containing apical meristematic cells. The abundance and time of appearance of the various polypeptides within the group seems to vary. The common molecular weights suggest the presence of isomeric forms, or otherwise related peptides; this was confirmed by later results.

Once the DD1 group of proteins was targeted for study, the system had to be characterised further. This involved a more detailed analysis of the appearance of the various mRNA species (as mirrored by *in vitro* translation products) and changes in the steady state levels of transcript. The method relies on two major assumptions:

a) all transcripts are translated at equal efficiencies, and

b) the nascent peptides contain only one methionine residue.

If both assumptions hold, the amount of radioactivity on each spot is directly related to the quantity of RNA present. If either statement does not hold true, the signal will be amplified and any upregulation will appear artificially high. Because of this, Northern blots of the transcript should always be used to confirm the increased levels of mRNA.

It was also hoped to find a system producing significant amounts of mature protein resulting from these mRNA transcripts for microsequencing; this would only be possible if the mature protein is not post-transationally modified to any great extent, so that it can be
recognised by its position on a similar two-dimensional total protein gel. Glycosylation, for example, would bring about an upward shift in molecular weight, while removal of a large leader sequence would result in both a downward shift in molecular weight, and possibly a change in the pI value of the protein. Modification resulting in a block to the N-terminal in the peptide would also render the microsequencing reaction impossible.

Microsequencing enables the investigation of minute quantities of biologically active proteins. The approach is based on the work carried out by Edman in the 1950's (Edman, 1950; Edman and Bess, 1967). The Edman degradation relies on the high chemical reactivity of a protein's amino-terminal amino acid to butylchloride, and the ability to remove the derivatised amino acid from the protein while leaving the rest of the peptide chain intact. The reaction is performed in an inert atmosphere (Argon) to avoid inhibition by oxygen, and uses gas phase coupling and cleavage reagents to allow retention of the sample on a solid support. At the end of each degradation cycle a derivative, the anilinothiozolinone of the terminal amino acid, is removed from the sample, and automatically transferred from the cartridge to a second heated flask for conversion to the more stable phenylthiohydantoin (PTH) amino acid. The PTH-amino acid is then identified via separation on HPLC reverse phase chromatography by comparing its retention value to the spectrum of known amino acid markers. The protein sequence can be used to construct a DNA oligomer corresponding to the expected coding sequence. This can then be employed as a probe to select the corresponding transcript from a cDNA library (Wallace and Miyada, 1987).

Another method of cDNA library screening is antibody screening of expression libraries (Mierendorf, Percy and Young, 1987). Antibodies have the advantage of being able to respond to very small amounts of antigen, with a high degree of specificity. Unfortunately, relatively large amounts of protein are needed to induce the immune response in rabbits, and the asparagus cell culture system produces DDL in very low abundance. A novel technique, that relies on separating the immunogen on 2-D PAGE and transferring the peptide on nitrocellulose to concentrate
the protein sample and maximise the delivery of low abundance antigen was used (Chiles et al., 1987). This method also relies on implanting the nitrocellulose subcutaneously; the presence of the nitrocellulose amplifies the immune response, while the antigen remains undegraded for longer because of its solid nature, and therefore has a greater effect on the immune system.

5.3.2 Presence of DD1 Transcript in Steady State mRNA

The DD1 group of proteins comprises a number of peptides whose abundance and time of appearance during cell culture seems to vary. A system was devised by K. Harikrishna to differentiate between the various polypeptides. The proteins are labelled with an M value, depending on their molecular weight, and a P value, depending on their pl. A graphic representation of the various peptides and their respective labels can be seen in Fig. 5.10. Only those polypeptides which can be quite clearly identified and are clearly present in all batches of translated mRNA are discussed here. It is quite possible that other minor components of the DD1 group exist.

Two-dimensional gels of \textit{in vitro} translated mRNA from day 0 to day 8 in culture, and 2 months and 6 months in culture were run, to qualify the appearance and abundance of the DD1 proteins. From the \textit{in vitro} translation time courses (Fig. 5.11) it is possible to determine that M16 P5.32a only appears at medium intensity on days 4-6 in culture. The polypeptide M16 P5.32b appears within a day of culture, its intensity peaking at day 5, and being reduced to low levels by day 7. A low level expression of the polypeptide is maintained in culture through to 6 months. M16 P5.6a is initially expressed at day 0 and hence is possibly stress-induced. The expression increases and is maintained at high levels to day 7, reaches a low intensity by 2 months, and is maintained at such intensity through to 6 months. M16 P5.6b displays a different expression profile. The polypeptide is first observed at low density by day 1, whereupon it increases and is maintained at high levels until day 5; at days 6 and 7 its intensity is decreased, until at 2 months in culture it
Figure 5.10 Graphical representation of labelling for the DD1 polypeptides.

(A) *In vitro* translated proteins from 5 day cultured cells mRNA.

(B) Graphical labelling.
**Figure 5.11** Changes in abundance of DD1 during culture. (1) Whole fronds. (2) 3 hours, (3) 1 day, (4) 2 days, (5) 5 days, (6) 7 days, (7) 2 months, and (8) 6 months in culture. M16 P5.6 a and b are marked by the closed triangle.
is no longer detectable. M16 P6.34 is detected immediately at day 0 and is maintained at high levels to day 7. After day 7 the intensity of the polypeptide falls to low abundance levels by 2 months and remains so at 6 months.

5.3.3 Microsequencing

DD1 appears to accumulate in cultured cells, so that by 6 months there is a sufficient amount of the most abundant DD1 protein (M16 P5.6a) to enable purification from a 2D gel of total protein extract (Fig. 5.12). *Img of total protein extract from 6 months cultured cells was run on a two-dimensional polyacrylamide gel and Western blotted onto a PVDF membrane where the polypeptides could be viewed by Comassie staining. M16 P5.6a was then cut out and placed in an Applied Biosystems 407A sequencer for microsequencing (Fig. 5.13); 28 amino acid residues were identified. The sequence resulting from this (Fig. 5.13) showed the spot to contain two polypeptides, in a 3:1 ratio. The sequences were identical except for two residues; as one of the replacements would bring about a change in the pI value of the peptide, at least one more discrepancy in sequence must exist further down the proteins. It may be presumed that the secondary, less abundant, peptide is M16 P5.6b; this was confirmed by later results (see Section 5.3.4).

The N-terminal amino acid sequence was used in a homology search within the NBRF database, via the PSQ package. No homologous sequences were found, though when the nucleotide sequence of the full length DD1 cDNA is available a more complete search could be made.

5.3.4 Production of the Antibody

Total protein extract from 8 week old cultures was separated by 2D-PAGE and transferred to nitrocellulose by Western blotting (Fig. 5.14). M16 P5.6a is circled. The peptide was excised, and implanted into a rabbit (see Methods) to induce the production of antibody; a test bleed
Figure 5.12  2D gels showing the M16 P5.6 peptide used in microsequencing. (A) Total \textit{in vitro} translated proteins from 5 day cultured cells. (B) Total protein extracts from 6 months cultured cells. The peptide is indicated.
8 week cultured cells
Total protein extraction
2-dimensional gel

Transfer to PVDF membrane
Cut out DD1 protein

N-terminal sequencing

1  10  20  30

VKAVAVVAGDVVKGQVFFSQECDGPTTV
E  H

Figure 5.13 Purification of M16 P5.6a and b, and microsequencing. The differences in amino acid residues between the two polypeptides are shown.
Figure 5.14  Purification of DD1 from total protein extracts of 8 week *Asparagus* cultures; M16 P5.6a is circled.

(A) Whole gel. (B) Detail of gel showing M16 P5.6a protein.
was taken at the same time. Each bleed was allowed to coagulate at 37°C 2h, spun and the serum (supernatant) stored at 4°C.

20μg of total protein extract from 8 week old cultures was run on a 15% PAGE, Western blotted and immunoblotted with various dilutions of the serum. Preimmune serum did not respond to the protein extract. By the 6th boost a 1/100 dilution resulted in bands appearing at 56kD and 16kD. The larger peptide is thought to bind the antibody non-specifically, and may be the same as is recognised in the immunoprecipitation of in vitro translated proteins from 5 day mRNA (see Section 6.1.5.2). By the 8th boost, the signal at 56kD had disappeared if a serum dilution of 1/400 was used, while the signal at 16kD remained strongly positive. The rabbit was bled by cardiac puncture at this point, and the serum stored in 100μl aliquots at -20°C.

Total protein extracts from 5 days and 6 months in culture were run on 2D-PAGE, Western blotted and immunoblotted with the antibody (Fig. 5.15). The spot of M16 P5.6a in the 6 months total protein extract is clearly seen to comprise two polypeptides; the shape corresponds to that in in vitro translation products from days 6 and 7, and the minor spot is probably M16 P5.6b. The immunoblotting explains the microsequencing resulting in a double sequence (see Section 5.3.3). In the 5 day total protein extract, 5 polypeptides respond to the antibody, strongly suggesting the presence of isomeric forms of a single protein.

5.3.5 Conclusion

The DD1 polypeptides are expressed during the early stress phase of gene expression through the intermediate phase (ie. the period between stress-induced gene expression and cell division) to the late phase (ie. at days 4-7) of gene expression, when nuclear division and cytokinesis occur. It is possible that they are somehow involved in the production and maintenance of a callus phase, and could be involved in the maintenance of high metabolic rates encountered during wounding and cell division. Once again, as dedifferentiation within this system involves responses to wounding, media composition, the reactivation of the cell cycle, cell expansion and cell division, the appearance of the various
Figure 5.15 2D gels showing the DD1 peptide used in microsequencing and to raise antibodies. (A) Total *in vitro* translated proteins from 5 day cultured cells. (B) Total protein extracts from 6 months cultured cells. (C and D) Westerns of 6 months (C) and 5 day (D) cultured cells total protein extracts immunodetected with DD1 antibody. The peptide M16 P5.6a is indicated.
DDI transcripts at different times (and stages) during dedifferentiation may suggest separate functions of the polypeptides.

Gene expression during wounding in plants is an extremely complex phenomenon. Wound sites are often used as entry sites for infection by pathogens, and contribute to dehydration. Therefore, the associated physiological, cytological and biochemical changes observed after wounding are involved in several functions: wound healing, prevention of pathogen entry and prevention of desiccation. A further reason for the complexity observed in gene expression is that wounding is a very broad term. Wounding can range from the damage of individual cells to complete severance and separation of the shoot from the root (eg. as caused by grazing herbivores). Further complications arise from the different responses of dicotyledenous and monocotyledenous plants, different plants tissues, and plant organs to wounding. For instance, wound-induced cell proliferation is common in many herbaceous and woody dicots, but in monocots the first layer of intact cells adjacent to the wound is often modified by the accumulation of anti-microbial and water impermeable substances such as lignin and subarin (Moon et al., 1984).

Two main groups of polypeptides, synthesised as a result of wounding, have been extensively studied in the last few years. Neither of the two are wound specific, but their induction is brought about by a range of stress factors including wounding and pathogen attack. One such group is termed pathogenesis-related (PR) proteins. These were initially associated with necrotic reactions in host plants (Van Loon, 1985) but have since been shown to occur during initiation of flowering in tobacco, and leaf senescence in tomato. Some PR proteins have been shown to possess 1,3-β-glucanase or chitinase activities (Kombrik et al., 1988). They have acidic pI's and molecular weights around 14-21kD, properties also shared by the DDI polypeptides. The time course of appearance of PR proteins seems to vary from 20 minutes (with a peak of activity at 30min) in parsley (Somssich et al., 1986), to 4 days (with a peak of activity at 6 days) in tobacco (Antoniw et al., 1987).

Another group of wound induced proteins are the proteinase inhibitors (PI). These are induced systemically in plants as a response to wounding in leaves (Graham et al., 1986). They are also of small molecular weight, with acidic pI's, and seem to appear at different times
after wounding (Logemann et al., 1988; Graham et al., 1986). Again, as
with DD1, a number of polypeptides of common molecular weight but
different pI values can be seen on 2D gels. At the moment the only major
difference between these two groups of proteins, in terms of their
regulation of synthesis, general pI and molecular weight properties, or
substrate specificities, lies with the latter property. The PR
polypeptides characterised so far break down carbohydrate bonds in fungal
and bacterial cell walls, while P'Ts inhibit pathogen or insect peptides.

A number of genes coding for PR or P'T proteins have been identified
and characterised (An et al., 1989; Cornelissen et al., 1987; Keil et
al., 1989). The gene wun-l, for example, was found by differential
screening of cDNA libraries from wounded and unwounded potato tubers
(Logemann et al., 1988). The transcript was seen to appear 30min after
wounding, and peaked between 4 and 24h after wounding. The gene product
is an 18kD protein. Further characterisation of wun-l (Logemann et al.,
1989) shows similarities to PR proteins. For example the protein was
shown to be involved in general physiological reactions to stresses
correlated to cell death, rather than simply the wound response. The
expression of the gene was shown to be controlled at the level of
transcription. Chimeric constructs utilising the wun-l 5' upstream
sequences and the CAT or NPTII coding sequences were used in transient
expression experiments; these showed that the transcription-activating
functions of the wun-l 5' upstream sequences are maintained in different
host plants, including the monocot rice. As a trans acting factor is
thought to be involved in activation of PR proteins (Sanches-Serrano et
al., 1987), this must be conserved in monocot and dicot plants,
suggesting a similarity in the stress related responses between the two
systems. If PR proteins in monocot and dicot plants show common
characteristics, it will prove easier to ascertain whether DD1 belongs
to this category of polypeptides.

Another example of wound induced transcripts found in potato are the
win-l and win-2 genes (Stanford et al., 1989). DNA sequencing of these
two highly homologous (97%) genes was used to deduce the relative protein
sequences. Homology was shown to exist between win-l and win-2, and
proteins involved in plant defence such as chitinase (a subset of PR
proteins) and lectin. Interestingly, the two transcripts show
differential organ-specific expression in response to wounding, the level of wound-induced expression of each of the two genes also varying between the different organs of the potato plant. This is reminiscent of the DD1 group of proteins, where the different isoforms are expressed at varying levels in the cultured cells.

Wounding induces the synthesis and activities of a number of other polypeptides such as catalases, peroxidases, PAL, extensins and chalcone isomerase (see Section 1.2). Some of these have been extensively characterised and protein sequenced. As the microsequence of M16 P5.6a did not show homology to any known protein sequence, nor a high proline content, it is unlikely that DD1 correspond to any well known group of proteins.

Other stresses may cause the activation of DD1 transcription, such as the presence of growth regulators or other components in the media. Auxin has been shown to have a direct effect on protein and nucleic acid synthesis (Jacobsen, 1983). For example, it is known to activate transcription of specific mRNAs in auxin-starved tobacco protoplasts (Mayer, Aspart and Chartier, 1984), and soyabean and bean cell cultures (Bevan and Northcote, 1981b). Changes in the carbohydrate status have also been demonstrated to affect protein profiles and gene expression, for example in Pennisetum americanum (Baysdorf and Van der Wounder, 1988). Again, in Nicotiana plumbaginifolia, wounding causes only a slight induction in the synthesis of manganese superoxide dismutase (MnSOD), while a massive induction of MnSOD occurs in the presence of sucrose (Bowler et al., 1989). This protein is a nuclear encoded mitochondrial enzyme which catalyses the dismutation of superoxide radicals to oxygen and hydrogen peroxide. The radicals are produced by enhanced respiratory oxidation of sugars in the mitochondria. This increase in mitochondrial sugar respiration is often stress related occurring, for example, in the pathogenesis response (Bowler et al., 1989). The very high calcium content of the asparagus media (1.5mM) may also trigger a stress response. There is evidence that Ca++ ions inhibit cytoplasmic streaming in higher plants (Minorsky, 1985). For example, application of calcimycin to tomato suspension cells causes rapid inhibition of streaming, while raising Ca++ levels in the external medium of Elodea cultures, results in a decline of the cytoplasmic streaming rate. Increases in cytosolic
calcium also promotes microfilament disassembly (Minorsky, 1985), which eventually disrupts cell division. We know that the high calcium content of the asparagus media does not affect cell division. Therefore, a mechanism may exist by which Ca\(^{++}\) is either not allowed to enter the cells, actively extruded, or sequestered (presumably by association with a protein) to a specific compartment within the cells, where it will have no biological activity. Again, DDl may be involved in such responses.

One of the most striking processes during dedifferentiation in asparagus is the production of exudate. This may be involved initially with the binding of cells to the bottom of the Petri dishes. The production of exudate increases at 3-6 months in culture as the cells reach the end of their growth stage. As the DDl transcripts actually decrease to basal levels by this time, the proteins are unlikely to be involved in exudate production.

DDl may also be involved in the growth response of culture initiation. As the process is dependant on the exogenous supply of auxin (Lindsey and Yeoman, 1985), it should be possible to differentiate between the wound and growth responses in asparagus, and the appearance of DDl during either processes.

The question also arises of whether DDl is a group of isomers. Microsequencing shows a very high sequence homology (>90%) between M16 P5.6a and M16 P5.6b. A number of the DDl polypeptides also bind the antibody, suggesting common antigenic properies. Finally, work investigating the isomeric patterns of proteins during dedifferentiation of explants into callus and subsequent redifferentiation and organogenesis, show that polypeptide isoforms exhibit a characteristic cycle (Reiniert, Bajaj and Zbell, 1977). Alterations in the activity of peroxidases in cultivated *Sinapsis alba* and tobacco stem sections gave been shown to be connected with dedifferentiation, but the original state is restored at the beginning of redifferentiation. This is very reminiscent of the pattern seen in Fig. 5.11, where at 2 months in culture, when differentiation resulting in the production of the spherical entities is taking place, the DDl transcript pattern (Fig. 5.11 number 7) is identical to that in whole fronds (number 1). At this point we are confident that the DDl group of proteins are a set of polypeptide isoforms.
CHAPTER SIX

ISOLATION OF A DD1 cDNA CLONE AND CHARACTERISATION OF THE DD1 FAMILY OF PROTEINS
6.1 Construction and Screening of a cDNA Library

6.1.1 Introduction

One of the best methods available to date to view steady state pools of mRNA is \textit{in vitro} translation in the presence of radiolabelled amino acids. In this way, the gene products of picogram amounts of mRNA can be visualised and quantitated. The main advantage of the procedure is its sensitivity, so that meaningful results can be obtained with minimal amounts of tissue. At the same time, an amplification of the molecules giving a specific signal has to occur before further characterisation can take place.

Copy DNA (cDNA) molecules can be made from an mRNA template using a viral reverse transcriptase, and the molecules attached to a vector such as bacteriophage \( \lambda \) which can replicate in \textit{E.coli}. The phage is then packaged and introduced into its \textit{E.coli} host, where each individual cDNA molecule (clone) can be amplified \textit{ad infinitum} and reach levels high enough for experimental purposes.

Bacteriophage \( \lambda \) is a genetically complex but extensively studied virus of \textit{E. coli}. Its DNA, in the form in which it is isolated from the phage particle, is a linear duplex of about 47 kbp. At each end are short single stranded 5' projections of 12 nucleotides which are complementary in sequence and by which the DNA adopts a circular structure when it is injected into its host cell. Functionally related genes within \( \lambda \) are clustered together, except for two positive regulatory genes, N and Q. The region of interest for vector construction is the central one, where genes concerned with recombination and the process of lysogenation are found. Much of this central region is not essential for phage growth and can be deleted or replaced without seriously impairing the infectious growth cycle of the phage.

The main advantages of \( \lambda \) vectors over plasmid vectors are the high efficiency of introducing recombinant DNA into \textit{E. coli} by \textit{in vitro} packaging followed directly by infection, and the efficiency of screening bacteriophage at high plaque densities using either nucleic acid or
antibody probes. The phage used in this project is λ-ZAP (see Section 4.2.1 and Fig. 4.11).

As a portion of the amino acid sequence of the M16 P5.6a polypeptide is known, an oligonucleotide probe can be designed based on this information. Wallace (Wallace and Miyada, 1987) has shown that oligonucleotides hybridise to their complementary sequence with a high degree of specificity. Under appropriate conditions only duplexes will form in which all of the nucleotides are base paired, while mismatched duplexes will not. Oligonucleotides of unique sequence are useful for screening recombinant DNA libraries. The hybridisation specificity of oligonucleotide probes allows the use of unique sequence probes to screen for genomic clones of cDNAs encoding a specific member of a multigene family, to screen for a specific region of a gene, or specific mutants created by site-directed mutagenesis, and to screen libraries with probes whose sequence represent a consensus coding sequence.

The minimum length of oligomer used to confer specificity is very important. The asparagus genome has been shown to have a 1C value of 1.2pg (Paul et al., 1989). This is roughly equivalent to 1.2x10^9bp. If a random assignment of base pairs is calculated to occur along the genome, an oligomer of 15bp would occur every 1.0x10^9bp, while an oligomer of 16bp would only occur once every 4.3x10^9bp. Sixteen base pairs is therefore the minimal oligomer length that would confer specificity within the asparagus genome. This is probably an overestimate, if we consider that a large proportion of plant genomic sequence is repetitive.

Oligomers have often been used as probes by end labelling them with a reaction catalysed by a kinase (Wallace and Miyada, 1987). This is useful when the exact hybridisation conditions resulting in maximum stringency without loss of signal can be calculated from the known nucleotide sequence. Unfortunately with a mixed oligomer, such as the one used in this project, two problems arise: a decrease in hybridisation specificity, and a decreased abundance of the single correct probe in the mixture. A method was therefore devised that would amplify the probe, so that a high stringency temperature could still be used without significant loss of signal. This method also has the added advantage that it does not require the N-terminal end of the transcript, bearing the sequence complementary to the oligomer, to be represented in the clone
thus allowing non-full length clones to be picked up as well. Although
the advantage of this may not be apparent with the relatively small
transcript needed to code for the Ddl protein, it is evident when
considering transcripts longer than 2kb (or coding for polypeptides
exceeding 60kD).

The method involves the synthesis of first-strand cDNA from an mRNA
sample equivalent to that used in the synthesis of the cDNA library. The
nascent DNA is then hybridised to an oligomer equivalent to the coding
strand, which serves as primer to a Klenow-catalysed synthetic reaction
containing $^{32}$P-labelled dCTP. The probe thus made is then used in a
hybridisation reaction to screen plaque lifts from the cDNA library
(Fig. 6.1).

6.1.2 Construction of Asparagus cDNA Libraries

RNA poly A$^{++}$ extracted from day 5 cultured asparagus cells was used
to synthesise double stranded cDNA. This was ligated into $\lambda$-ZAP which had
been previously cut with EcoR I and dephosphorylated. The recombinants
were packaged and titrated. The library was seen to contain $1.05 \times 10^5$ pfu
at a concentration of $2 \times 10^5$ pfu/ml. This gave a cloning efficiency of
$7.2 \times 10^5$ pfu/$\mu$g cDNA. The cloning efficiency is simply a ratio between the
number of recombinants within the library, and the amount of cDNA used in
the ligation. It gives an estimate of the purity of the sample and
whether the DNA molecules are intact; ideally the cloning efficiency
should be $1.0 \times 10^6$ or greater. Twelve plaques were picked at random, grown
for 4h and the DNA extracted. This was cut with EcoR I to excise the cDNA
insert and run on a 2% agarose gel for sizing. The average size of insert
was seen to be 700bp.

RNA poly A$^{++}$ extracted from 6 months cultured asparagus cells was
also to produce a cDNA library. The library was seen to contain $3.5 \times 10^5$
pfu at a concentration of $6.5 \times 10^5$ pfu/ml. This gave a cloning efficiency
of $1.9 \times 10^5$ pfu/$\mu$g cDNA. Twelve plaques were picked at random, grown for 4h
and the DNA extracted. This was cut with EcoR I to excise the cDNA insert
and run on a 2% agarose gel for sizing. The average size of insert was
seen to be 750bp.
Figure 6.1 Synthesis of DDI probe and selection of clones.
(1) RNA poly(A)$^+$ from cultured asparagus cells

dT 14-18
Reverse Transcriptase

(2) RNA:cDNA hybrid

NaOH digestion

(3) cDNA first strand

Oligomer DD1
Klenow
$^{32}$P dCTP

(4) Oligo-primed 2nd strand synthesis to give DD1-homologous radioactive DNA

Plaque Hybridisation

Autoradiography
Although the 6 months cDNA library is of better quality, the 5 day cDNA library was used to attempt to purify the DDI transcripts as these are present at considerably higher levels in the mRNA latter sample.

6.1.3 Oligomer Design

Codon usage was first unravelled in *E. coli* by H.G. Khorana in 1964 (Lehninger, 1975) using synthetic oligoribonucleotides. The genetic code is now regarded as biologically universal. This has been shown in all species tested to date such as tobacco mosaic virus, man and other vertebrates, plants, and a large number of bacterial viruses. However, the frequency of use of the different codes for a given amino acid may vary from one species to another (Lehninger, 1975).

If we presume that asparagus uses the universal coding sequence, it should be possible to construct an oligomer specific to the transcript to be purified. Unfortunately there is the additional difficulty that most amino acids can be coded for by two to four different codons, with some, such as Leucine being coded for by six different codons. The oligomer specific to any peptide sequence would therefore have to contain all sequence permutations necessary to code for such a peptide.

As already discussed in section 6.1.1, the minimum length conferring specificity to an oligomer is 16bp. A stretch of 6 amino acids, with the least codon degeneracy, was therefore chosen, and an oligomer mixture synthesised (Fig. 6.2). This oligomer contained 128 variants.

6.1.4 Screening the Library

As outlined in section 6.1.1, the design of the probe for screening involved the use of an oligomer to specifically prime DDI-first strand cDNA. Different temperature conditions were therefore experimented with to select one that allowed primer binding while precluding non-specific priming such as snap-back effects of the single-stranded DNA. The results are shown in Table 6.1. 30°C was chosen as the temperature at which the
Figure 6.2 N-terminal sequence of M16 P5.6a showing the amino acid residues used in the construction of the oligomer
### TABLE 6.1

<table>
<thead>
<tr>
<th>T'</th>
<th>with oligomer</th>
<th></th>
<th>w/out oligomer</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>counts</td>
<td>% +ve</td>
<td>total</td>
<td>inc.</td>
</tr>
<tr>
<td>25°C</td>
<td>84087</td>
<td>1161</td>
<td>0.1</td>
<td>74987</td>
</tr>
<tr>
<td>30°C</td>
<td>73797</td>
<td>112</td>
<td>0.01</td>
<td>81396</td>
</tr>
<tr>
<td>37°C</td>
<td>94582</td>
<td>57</td>
<td>0.00</td>
<td>62081</td>
</tr>
</tbody>
</table>

Table showing the extent of second strand DNA synthesis dependant upon the incubation temperature conditions, and the number of plaques lighting up when these DNA probes were used to hybridise plaque lifts from the asparagus cDNA library.

### TABLE 6.2

<table>
<thead>
<tr>
<th>Plate</th>
<th>DD1 +ves</th>
<th>DD1 +ves</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cladode -ves</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>15</td>
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<tr>
<td>2</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
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<td>4</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>

Table showing the results of the primary screen.
Klenow-catalysed reaction would take place with minimum non-specific priming.

Plaque lifts in three replicas were taken from six 15cm Petri dishes containing 1000 plaques each. One replica from each dish was probed with either:

a) the DDL-specific probe;
b) $^{32}$P-labelled first strand cDNA from 5 day mRNA (positive control);
c) $^{32}$P-labelled first strand cDNA from cladode mRNA (negative control).

The results from the probing are summarized in Table 6.2 and plate 6 can be seen in Figure 6.3. The data suggested that several clones lighting up with the DDL probe were also expressed in cladodes, which may suggest some non-specificity of the probe, or of the priming reaction during synthesis of the probe.

Plaques corresponding to 45 clones lighting up with the DDL probe, which did not bind to first strand cDNA from cladode mRNA, were picked into phage dilution buffer. A proportion of the sample was incubated with host cells and helper phage, to excise the pBluescript, followed by plasmid minipreps. These were used in a series of replica DNA dot blots. This is because DNA dot blots are more sensitive than plaque lifts, because of the larger amounts of DNA present in dot blots, and the removal of cell debris and proteinaceous material present in plaque lifts. The replica dot blots were probed with $^{32}$P-labelled first strand cDNA from cladode mRNA and day 1 culture mRNA respectively. The latter mRNA sample was chosen as day 5 mRNA contains a larger number of transcripts resulting in polypeptides of molecular weights between 14 and 18kD. It was not known how many of the minor polypeptides were homologous to the DDL oligomer, and it was hoped the day 1 probe would be able to differentiate between the abundant and rare transcripts, as only the first type was present. Of the 45 clones examined by dot blotting, two responded strongly to the cultured cells probe, while appearing negative with the cladode probe. These were clones 34 and 39 and they were seen to cross hybridise with each other.
Figure 6.3 Screenings for DDL clones. Replicas plaque lifts probed with first strand cDNA from 5 day cultured cells (A), and cladode (B) mRNA, and DDL probe (C). Plaques lighting up with both the DDL probe and cladode probe (▲), or with the DDL probe but not the cladode probe (△) are shown.
6.1.5 Confirmation of Clone 34

Confirmation of the nature of a clone can arise from the examination of a number of known characteristics of the expected transcript:
a) the time of appearance of transcripts homologous to the clone within the steady state mRNA pool of a tissue may be known. In this case, RNA dot blots, or for greater accuracy Northern blots, of nucleic acid extracts from tissues where the transcript's presence has already been proven, may be used;
b) the antigenic properties of the target protein may be utilised in the production of polyclonal antibodies. If the clone to be examined is placed in an expression vector, it can be seen whether the \textit{in vitro} translation product has the expected antigenic properties;
c) the clone can be used to purify homologous transcript sequences from a total mRNA sample expected to contain such transcript. This can be then translated \textit{in vitro} and its product run on PAGE to confirm its nature;
d) if the expected sequence, or partial sequence, of the clone is known, DNA sequencing may be used.

6.1.5.1 RNA Dot Blots

RNA dot blots can serve a dual purpose: qualitatively to ascertain the presence of a particular transcript in the steady state RNA pools of a tissue, and quantitatively to estimate the level of transcript present. For this purpose, a blot was designed containing 500, 50 and 5ng of poly A\textsuperscript{+} RNA from each sample, so that the relative amounts of transcript present could be assessed (Fig. 6.4). Clone 34 was seen to bind to poly A\textsuperscript{+} RNA from 1 and 5 days in culture but not to cladode poly A\textsuperscript{+} RNA. This confirms the clone to be differentially transcribed in dedifferentiating and quiescent mesophyll cells.

6.1.5.2 Antigenic Properties

Even though the DDL antibodies have been shown to bind polypeptides in total protein extracts from asparagus cell cultures, it is not known if they will recognise the same polypeptide when synthesised \textit{in vitro} or in an \textit{E. coli} host. This has been shown to happen with some monoclonal antibodies which do not react with the denatured antigen (Smith \textit{et al.}, ...)
Figure 6.4 RNA dot blots showing the presence of transcript 34 in the steady state mRNA populations of *Asparagus* cladodes and cultured cells.
Day 1

Day 5

Cladode
This is because the antibodies may be responding to an antigenic moiety resulting from post-translational modification or specific folding of the protein. To ensure that the antibody able to recognise the unmodified peptide, it was used immunoprecipitate in vitro translation products from cultured cell mRNA. The result of the precipitation can be seen in Fig. 6.5A. The antibody was indeed able to precipitate a translation product of molecular weight 16kD; a second product of molecular weight 56kD was also seen. As the antibody is polyclonal, it is not uncommon for it to bind to seemingly unrelated polypeptides. The result of the immunoprecipitation is consistent with the appearance of a low abundance signal at 56kD in some Westerns from total protein extracts.

The recombinant vector in this study is pBluescript. The plasmid has its cloning site within an active lacZ gene, which can be induced by IPTG. Recombinants in which the insertion of DNA has not caused a frame shift mutation will produce a lacZ-fusion protein. Production of the native protein is dependent upon the presence of an intact ribosome binding site within the cDNA clone. E. coli host cells containing non-recombinant pBluescript and clone 34 respectively were grown in the presence of IPTG. Total protein extracts from the cells were then run on SDS-PAGE and Western blotted. Immunodetection showed the presence of a doublet at 16kD (Fig. 6.5B) in total protein extract from clone 34, but not in the pBluescript extract. Fig. 6.5C showed the lower band to have a molecular weight identical to that from mature Ddl in cultured cells. The upper band is probably the lacZ-34 fusion protein. This would have to be confirmed by immunodetection with antibodies to the lacZ gene product.

6.1.5.3 Hybrid Release

Any recombinant DNA sequence is able to form a stable hybrid with its corresponding mRNA. This property can be utilised for the unambiguous identification of a cloned gene and in the screening of a large number of recombinant DNA molecules. Recombinant DNA can be used to select a specific mRNA from the total mRNA population for translation in a cell-free protein synthesising system. This process is termed "hybrid release".
Figure 6.5 PAGE gels showing the nature of clone 34.

(A) Autoradiograph of *in vitro* translated proteins from cladode mRNA (Cl), and 5 day cultured cells mRNA (5d) with *in vitro* translated proteins from day 5 cultured cells mRNA immunoprecipitated with preimmune (P) and immune (Im) serum.

(B) Western of protein from induced *E. coli* cells containing clone 34 (p34) or unrecombinant pBluescript (pB) immunodetected with DDL antibody.

(C) Western of clone 34-transformed induced *E. coli* cells (p34) and 5 day cultured cells (5 days) total protein extracts, immunodetected with DDL antibody.
Clone 34 was used in the hybrid release experiment. The hybrid released mRNA was translated \textit{in vitro} and the reaction run on SDS-PAGE. The transcript thus selected coded for a peptide of molecular weight 16kD (data not shown). This is of the same molecular weight as the DDL peptide.

6.1.5.4 DNA Sequencing

The cDNA clone was digested with EcoR I/Hind III, EcoR I/Sma I, and Hind III, and subcloned into M13mp18 and M13mp19. Unfortunately, because of lack of time, the sequencing project was abandoned.

6.1.6 Conclusion

The DDL group of proteins, which is now thought to consist of at least 5 isoforms, was deemed interesting because of its major transcription upregulation during the process of dedifferentiation, coupled to a clear reduction in the relative amounts of mRNA in the steady state pool once redifferentiation takes place. Purification of the transcript(s) coding for DDL is one facet of the research which was undertaken. This was achieved by using the known peptide sequence to construct an oligonucleotide that could selectively bind the cDNA clone. The nature of the clone was then confirmed by RNA dot blots, immunoblotting and hybrid release translation. Unfortunately, due to lack of time, it was not possible to run the \textit{in vitro} translated hybrid released mRNA on 2-D PAGE to try and pinpoint which isoform corresponded to clones 34 and 39. The one-dimensional gel of the \textit{in vitro} translated hybrid released mRNA was not fit for reproduction. The cDNA clone can now be used to confirm the \textit{in vitro} data by Northern blotting.
6.2 Characterisation of Clone 34 and the DD1 Group of Proteins

6.2.1 Introduction

A large amount of information can be collected using both the cDNA clone and antibodies to DD1. The clone can be used to confirm steady state mRNA levels at different stages during culture; unfortunately it is not expected to be able to differentiate between the various DD1 transcripts when a full length clone is used, and we still do not possess the necessary information (DNA sequence) to pinpoint the possible regions of differential sequence between such transcripts (e.g. Dean et al., 1985).

The antibody will be used to clarify the biology of the DD1 proteins. Production and accumulation of DD1 proteins in culture will be investigated, as will the presence of the proteins in various tissues of the asparagus plant. Finally, an attempt will be made to pinpoint the role of DD1 in dedifferentiation; this will include experiments to separate the wound and growth response during culture initiation.

6.2.2 Characterisation of Clone 34

Clones 34 and 39 have been shown to cross hybridise. At the same time, DD1 is a family of polypeptides, with similar antigenic properties and probably coded for by different transcripts. Restriction mapping was carried out to see whether clones 34 and 39 were identical or showed sequence differences. Incubating the clones with restriction enzymes recognising specific 6-nucleotide sites (BamHI, BglII, HindIII, PstI, SmaI, XbaI) resulted in the restriction map shown in Fig. 6.6. The probability of an enzyme finding a specific 6-nucleotide site on a stretch of DNA, presuming random assignment of nucleotides, is 1/4096, while the probability of a 4-nucleotide site being found is 1/256. As a result the use restriction enzymes recognising specific 4-nucleotide sites is more likely to yield a significant number of internal fragments. Restriction
Figure 6.6  Restriction map of clone 39.

Key:  H - Hind III
      S - Sma I
digestion with Hinf I showed the presence of a number of internal sites. The resulting pattern was too complicated to draw a restriction map, but all the fragments were of the same size when the two clones were compared. This showed that the clones were very similar; DNA sequencing is still needed to see whether the clones are indeed identical.

Clone 34 was also used in a series of time course experiments to ascertain the appearance of the transcript in the steady state mRNA pools, and to confirm that the relative levels of DDl transcripts are mirrored by *in vitro* translated proteins. Poly A\(^+\) RNA from different stages in the cell culture, cladodes and whole fronds was run on a denaturing 2\% agarose gel, Northern blotted, and probed with clone 34 (Fig. 6.7). Cladode and apical tip tissue were shown to lack the transcript. The apical tip was used as it contains a large amount of meristematic (actively dividing) tissue, to ascertain whether the transcript is directly related to cell division. The probe produced a signal in culture from day 1. Replica Northerns from different time courses showed similar results; the apparent inconsistency in the two blots is a result of differences in RNA loading. The signal was still present, although at a considerably reduced level, after 6 months. Unfortunately, as DNA does not enter the formaldehyde gel used in the Northern, spiking of the RNA to quantitate the level of transcript was not possible.

The Northern was also used to estimate the length of the transcript. Comparison with the migration values of the ribosomal RNA subunits showed DDl to have an approximate sedimentation value of 11.2S. This is equivalent to a length of 900bp. Clones 34 and 39 are therefore probably full length.

6.2.3 Characterisation of the DDl Group of Proteins

Total protein was extracted from various tissues within the asparagus plant, and at different times in culture. This was run on PAGE and immunoblotted to ascertain the relative amount of DDl in the protein samples. Fig. 6.8 shows two such experiments. The time courses were repeated, and different extractions from the various plant tissues were
**Figure 6.7** Northern blots with poly A\(^+\) (A) and total RNA (B), and RNA dot blots (C), probed with clone 34.

- **Lanes A.** Whole fronds
- **C.** Cladodes.
- **0-8.** 0-8 days in culture.
- **II and VI.** 2 and 6 months in culture.
Figure 6.8 Western blotted total protein extracts from *Asparagus* cells immunodetected with DDI antibody. The time courses refer to cultured cells.
A

<table>
<thead>
<tr>
<th>Cladodes</th>
<th>0 days</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
<th>5 days</th>
<th>6 days</th>
<th>2 months</th>
</tr>
</thead>
</table>

-16kD

B

| Apex | Cladodes | 5 days | 8 weeks | 10 weeks | 12 weeks | 6 months | Root tips |

-16 kD
also utilised. The results shown here are fully reproducible. DD1 was detected in only one type of asparagus tissue assayed. Cladodes, apical tips, main roots, floral buds from one year old plants (E. Paul), and seedling mesocotyls (H. Bailey) did not contain DD1 when 20μg of total protein was assayed. DD1 was however detected in root tips (Fig. 6.8B). The time course showed that DD1 appeared in extracts from mechanically isolated cells after 24h in culture. The amount of DD1 relative to other polypeptides in the sample increased during the first 6 days in culture (Fig. 6.8A). The thickness of the band on the 15% PAGE would suggest the presence of polypeptides of slightly different molecular weight. This is more obvious if we look at Fig. 6.8B, where, by week 8 the DD1 band is more compact, even though a similar amount of DD1 seems to be present. The relative amounts of DD1 had decreased by 8 weeks in culture and remained constant until 16 weeks (not shown). The apparent increase in DD1 seen at 6 months remains unconfirmed, as only one time course was continued over 16 weeks.

The DD1 group could be candidates for hormone-induced proteins. This possibility was tested by E. Paul (Fig. 6.9) using immunoblots of protein extracts from cells grown in auxin-medium, cytokinin-medium, and hormone-free medium. The asparagus cells remained viable but failed to divide in these media. The DD1 proteins were detected in all extracts, including cells incubated in hormone free medium for a week. This effect precludes the possibility of DD1 being hormone induced, as well as it having an involvement in the growth response of tissue culture initiation, as this process has been shown to be dependant upon the presence of exogenous auxin (Yeoman and Street, 1977). The dependence of the growth response on hormones was further shown by the following experiment. Asparagus cells were isolated as per in the Methods section, except that all washes were done in hormone-free media. The cells were then plated and the appropriate amount of hormones added to 3 Petri dishes only. Hormones were next added to a further 3 Petri dishes on day one, and so on until day 6. 92% of viable cells where hormones had been added on day 0 proceeded to divide, but no cell division was seen to occur in any of the other Petri dishes. Therefore, it seems as though the growth response in mechanically isolated asparagus cells is dependant upon hormones being added to the media from the onset of culture.
Figure 6.9  Hormone independence of DDL protein synthesis.

Key:  
N - auxin+ media  
B - cytokinin+ media  
O - hormone-free media.
H. Bailey found that cutting asparagus seedling mesocotyls in 1 cm pieces induced the production of DD1. A number of time courses to study this induction were undertaken. This involved cutting the mesocotyls of 2 week old seedlings and placing them in the dark at 25°C on damp filter paper discs in Petri dishes. The mesocotyl slices were collected at various time intervals, the protein extracted in 2DMH, run on PAGE, and immunoblotted. A number of time courses were repeated, showing DD1 to be detectable 7-8 h after wounding; DD1 reached its maximum level after approximately 3 days. Thus, DD1 may be induced in asparagus by wounding intact seedlings.

6.2.4 Conclusion

DD1 has been shown to be a group of proteins whose synthesis is initiated at the onset of culture. The polypeptides are the translation products of transcripts synthesised de novo under such conditions, rather than mRNA molecules previously unavailable to translation.

There is some discrepancy between the results attained by Northern analysis and two dimensional PAGE of in vitro translated products, in terms of the presence of DD1 transcripts in the steady state mRNA population of whole fronds, or the time of appearance of DD1 mRNA after mechanical isolation (see Section 5.3.2). This may be simply a reflection of the differences in sensitivity between the two methods. The signal on the Northern blot does not reveal the presence of more than one transcript size; all the DD1 polypeptides have similar molecular weights and would therefore be presumably coded for by transcripts of similar length. The transcripts could only be resolved by 2D-PAGE of in vitro translated products of hybrid released mRNA; this could be used to confirm that clone 34 indeed clones for the polypeptide M16 P5.6a, rather than one of the other related polypeptides. But the full length clone will not be able to differentiate between the transcripts if, as it appears from the protein sequencing and serological data, they have sequences in common. If this is the case, the 5' or 3' untranslated sequences may show differences in sequence between the DD1 transcripts and could therefore be used to differentiate between the transcripts in a
Northern blot. This kind of work has already been done by other researchers, for example with the small subunit ribulose bisphosphate carboxylase gene family (Dean et al., 1985).

It has also been shown that the polypeptides are produced as a result of the wound response during culture initiation. This prompted an investigation as to whether DD1 might be induced in other plant tissues by wounding. Seedlings were chosen because of the ease with which the tissue can be grown and harvested in large amounts in a relatively short time. The fact that seedlings produced DD1 when wounded by cutting the mesocotyls, has opened the possibility of further investigating the biological role of DD1 and its appearance as a result of a number of disparate stresses (see Chapter 7).
6.3 General Conclusions to Chapters 5 and 6.

Three main questions arise concerning the structure and activity of the DDl group of proteins:

1) is DDl a family of isoforms, or a group of unrelated proteins of common molecular weight;
2) are DDl isoforms specific to the dedifferentiated cultured state, or can they be found in other tissues within an asparagus plant;
3) are DDl isoforms involved in the wound response or the growth response during culture initiation, and can we pinpoint further their role within the dedifferentiation process.

The definition of isoenzymes is: "The variants of a given enzyme, occurring within a single organism, having the same specificity for substrate, hence catalysing the same reaction, but with slight differences in molecular structure which makes it possible to separate them" (Abercrobie, et al., 1973). Unfortunately, we do not know if DDl is an enzyme (or indeed its substrate specificity) but circumstantial evidence exists pinpointing to the DDl group being a family of highly related polypeptides.

Work on protein isoform patterns during dedifferentiation of explants and subsequent redifferentiation and organogenesis has shown that isoforms exhibit a characteristic cycle (Reinert, Bajaj and Zbell, 1977). For example, alterations in the activity of peroxidases in cultivated Sinapsis alba and tobacco stem sections have been shown to be connected with dedifferentiation, but the original state is restored at the beginning of redifferentiation. This is very reminiscent of the pattern seen with the DDl \textit{in vitro} translation products, where the various peptides increase quantitatively during the dedifferentiation process, and at 2 months in culture, when redifferentiation resulting in the production of the spherical entities is taking place, the DDl steady state mRNA pattern is identical to that in the whole fronds.
The mature DDl proteins have also been shown to have sequence homology and similar antigenic properties. Microsequencing showed a very high sequence homology (>90%) between M16 P5.6a and M16 P5.6b in the first 28 amino acid residues. The proteins are thought to be made up of roughly 160 residues. Polyclonal antibody made against the two polypeptides also binds other members of the DDl family. Total protein extracts from 5 day and 6 month old cells run on 2D PGE and immunoblotted showed that at least 5 and 4 proteins respectively were recognised by the antibody.

DDl therefore is a group of proteins with similar antigenic properties, and sequence homology in at least 2 of the polypeptides. Any similarity in function and, if they have enzymic properties, substrate specificity will have to be investigated, but the evidence collected so far points to DDl being a group of related protein isoforms.

Immunohistochemistry was also used to answer the question of the tissue specificity of DDl. Immunoblotting of protein extracts showed DDl to be detectable only in the root tips of asparagus plants. This is not thought to be because of the meristematic cell content of root tips, as apical tips will not respond to DDl antibody. The presence of DDl in root tips could be a response to either the hormonal ratio within the root system, or the constant abrasion experienced by roots growing within the soil. From the work of E. Paul, we know that the production of DDl in culture is hormone independent, so the first hypothesis is probably discounted. DDl could therefore appear as a response to wounding in root tips; this is even more valid if we consider that the main root body of asparagus does not contain detectable amounts of DDl (results not shown).

Two responses are known to occur during dedifferentiation, a wound response and a growth response. The former is characterised by a rapid increase in metabolic activity and does not usually result in callus formation; it may be identified when, for example, explants are cultured on auxin-free medium. The growth response, resulting in cell division, is dependant upon an exogenous supply of auxin, and results in changes in the cell structure which may be viewed under the light and electron microscope (Yeoman and Street, 1977). It is this independence of DDl
induction from exogenous auxin that has allowed us to associate the proteins with the wound response. At the same time the term "wound response" is a very generic one, and, for asparagus cell culture initiation, includes responses to cell death (from cells being burst open), to pectic bridges between cells being disrupted, and to the culture medium, together with secondary (or delayed) wound responses and the process by which cells become competent to divide. For example, it is well established that the presence of pectic fragments can elicit defense- (Templeton and Lamb, 1988) and wound- (Ryan, 1988) regulated gene expression; the asparagus cells are exposed to cell wall fragments for a period of time up to 40min during mechanical isolation.

DDl may also be induced in response to a specific media component. We already know that DDL production is hormone independent, but other components may switch on gene expression. For example, changes in the carbohydrate status have been demonstrated to effect protein profiles and gene expression in Nicotiana plumbaginifolius (Bowler et al., 1989) and Pennisetum americanum (Baysdorfer and Van der Wounder, 1988), while Ca++ ions influences cytoplasmatic streaming and microfilament assembly in Elodea and tomato cells (Minotsky, 1985).

The finding that mesocotyls produce DDL as a response to wounding has also facilitated experimental design. The time taken for the tissue to produce DDL in response to wounding has also been investigated. If 20ug of total protein extract are assayed, DDL is detected after 7h and reaches maximum intensity after circa 3 days. This is quite a slow response and may be a secondary response, for example caused by ethylene production at the wound site (Boller and Kende, 1980).

Wounding has been shown to affect the profile of steady state mRNA populations. A number of genes have been shown to be induced as a result of wounding, including PAL (Borchert, 1978), peroxidases (Thompson et al., 1987), proteinase inhibitors (Graham et al., 1986), pathogenesis-related proteins (Van Loon, 1985), and hydroxyproline-rich glycoproteins (Chen and Varner, 1985). The first two have been extensively characterised and both the polypeptides and related transcripts sequenced. These sequences are remarkably conserved throughout the plant kingdom. But a search through protein sequence databases using the M16 P5.6a sequence showed no significant homology to
known proteins, and it is therefore unlikely that DDl are either PAL or peroxidases. Similarly, the microsequences showed no clusters of high proline content, discounting the possibility of DDl being a hydroxyproline–rich glycoprotein; this is further confirmed by the fact that the mature M16 P5.6a has the same molecular weight as its *in vitro* product counterpart, and is therefore probably not a glycoprotein. The possibility still exists of DDl being a P_R or P_I protein. All three groups of polypeptides have similar characteristics such as small molecular weights (14–20kD), and isoforms of acidic PIs and serological similarities. Other properties shown by P_R and P_I proteins include response to non-specific stresses, extracellular localisation (in the case of P_R proteins) and systemic reaction (for P_I proteins). More importantly P_R proteins have been shown to be induced in culture (Van Loon, 1985), and P_I proteins to be synthesised as response to the presence of pectic fragments such as cell wall fragments (Lamb *et al.*, 1989). Further investigation will be required to confirm whether DDl belongs to either protein group or represents an entirely new family of wound–induced proteins. This will be discussed in Chapter 7.
CHAPTER SEVEN

GENERAL CONCLUSIONS
Dedifferentiation has been defined as the process by which specialised quiescent cells give rise to heterotrophic, dividing cells. The stimulus for initiation of this process in plants is commonly wounding. Dedifferentiation also occurs in explants placed into culture (Aitchison *et al.*, 1977), where it is the first step in the expression of totipotency, which enables the regeneration of whole plants from cultured cells. Three questions were posed at the beginning of this thesis:

a) does dedifferentiation arise as a result of a breakdown in cell structure and function, or does it occur via a reproducible, ordered sequence of events;
b) what are the similarities and differences in gene expression between differentiated cells and those maintained in a dedifferentiated state by cell culture;
c) is the process of dedifferentiation and maintenance of the fast-dividing, dedifferentiated state dependant upon major changes in gene expression.

The approach used in this thesis has been to investigate the role of transcription in these putative changes in gene expression. This is a limited approach, mainly dictated by the technology and methodology available in the laboratory (see Section 3.2.1), and no attempt was made to examine the post-transcriptional changes involved in gene expression control. The aim of this thesis was to qualify major changes in transcription occurring during dedifferentiation and the maintenance of the dedifferentiated state. An attempt was also made to identify a number of cDNA clones specific to certain cell types with a view to isolating their promoter regions. These could then be utilised in future applied work concerned with the regulation of secondary metabolism and plant *in vitro* transformation (see Sections 3.1.4.4 to 3.1.4.7).

It was argued that an examination of dedifferentiation within the intact plant would be difficult due to the relatively large amounts of uniform material required for molecular analysis. However, large quantities of material can be obtained by using a model cell culture system that will undergo dedifferentiation in a reproducible and controlled manner under defined conditions. The selected model system was mechanically isolated mesophyll cells from *Asparagus officinalis* (see
Chapters 5 and 6). Dedifferentiation in this system was shown to be a highly active process as seen by the qualitative changes in mRNA from *in vitro* translated protein profiles (see Section 5.2.4). The analysis was extended from 0 to 8 days in culture; the complexity of the protein composition increased further from day 2 to 5, and there was a marked change in the level of several *in vitro* translated proteins in addition to DD1 (Harikrishna *et al.*, 1989a). Morphological, cytological and molecular changes occurring in these cells during dedifferentiation were also shown to be highly reproducible (see Section 5.2), further proving that dedifferentiation in *Asparagus* is a highly regulated process. Work with chloroplasts (Harikrishna *et al.*, 1989b) revealed that while photosynthetic processes in the chloroplasts were shut down, their structure was retained, as shown by the lack of active chlorophyll breakdown and gradual turnover of major chloroplastic proteins; this would further discount the occurrence of a general breakdown in cell structure during dedifferentiation. Therefore, at least in asparagus, dedifferentiation seems to result from a change in cell function, occurring via a reproducible, ordered sequence of events, and is not accompanied by a detectable breakdown in cell structure.

The system chosen to study established dedifferentiated cells was that of *Petunia hybrida* suspension cultures (see Chapters 3 and 4). The culture is heterogeneous in that, even though it contains a single cell type (fast dividing, heterotrophic cells), they are all asynchronous in regards to their cell cycle. The system used as a comparison was 5 week old seedlings. Seedlings contain a range of different cell types resulting in a dilution of the transcription signals for specific cells. It was therefore hoped that in the cultured cell system, where one specific cell type is present, the transcripts (and other features) specific for that cell type would be amplified, as compared to the seedlings. The results presented in this thesis indicated that, in the case of the *Petunia* system, the approach was insufficient (see Section 4.3).
The last question posed in this thesis, on whether the process of dedifferentiation and maintenance of the fast dividing, dedifferentiated state is dependant upon major changes in gene expression, is probably the focal point of this study. Once again, the study was limited to the examination of changes in transcriptional activity within the various cell types. Dedifferentiation in cells of Asparagus officinalis was indeed accompanied by major changes in steady state mRNA levels (see Section 5.2.4). Some of the novel transcripts appearing in dedifferentiating asparagus cells are probably caused by events other than those intimately related to the dedifferentiation process itself. An example of this is the DDL group of transcripts, which was shown to be induced by wounding (see Section 6.2). Only minor differences were found between the steady state mRNA populations of Petunia hybrida cultured cells and seedlings, and these were mainly caused by transcripts disappearing in culture (see Section 3.2.5). No transcripts specific to the suspension culture system were detected.

It is at this point that it would seem appropriate to foward a hypothesis concerning the plastic response to wounding in plants. As a plant is wounded, tissue around the wound site responds by a massive change in gene expression resulting in the production of novel proteins and the structural changes leading to dedifferentiation. Both processes require either the production of novel compounds previously unavailable in the cell, or the activation of already existing preproteins; therefore de novo transcription may be one of the processes that has to occur to provide the cell with these new materials. Once the cell has dedifferentiated, if artificially kept in this condition by tissue culture, it reverts to a "steady state". In this steady state all unnecessary functions (such as photosynthesis) are lost, as can be seen by the qualitative loss of mRNA species as mirrored by in vitro translation (Fig. 3.8). Also, all functions necessary for the changes that have occured (ie. the process of dedifferentiation) to come about, are lost. It is at this point that the control of gene expression shifts from transcriptional control to later stages in the production of proteins. It could be argued at this point that the structure and function of the dedifferentiated cells is not caused by different proteins and enzymes
being present, but by a shift in priority within the biochemical pathways of the plant cells. This is energetically more economical to the cells.

Therefore, changes in gene expression involving \textit{de novo} transcription might only occur in response to major changes in environmental conditions. The basal transcription patterns for cells in an established state is probably common to all cell types with regards to primary cell functions such as growth, division and catabolism. In such established states, the control of metabolism probably resides within the biochemical pathways utilised by the cell at any moment in time.
7.1 Future Work

The Petunia hybrida suspension culture consists of fast dividing, heterotrophic cells. Although these cells are growing in an asynchronous manner, they all belong to the same cell type and are therefore homogeneous in terms of their overall cell structure and function. If the problem of asynchronous growth could be solved, the Petunia culture would be an ideal system for the study of the cell cycle.

The cell cycle in eukaryotic cells consists of an ordered sequence of events including DNA synthesis, organelle replication, and mitosis. These events are expected to be controlled by a number of genes, whose changes in expression should be mirrored by a change in the protein composition of the cells (Kodama et al., 1989). The literature available on this subject is fragmented and sometimes contradictory. In particular, the work that has taken place until now has suffered from two major setbacks:

a) The markers used to show changes in gene expression have sometimes been inappropriate. For example, reports of enzyme accumulation measured as enzyme activity, rather than enzyme synthesis will not allow for systems where there is a delay in the cell cycle between synthesis of the enzyme precursor and its activation. Furthermore, because the activities of many enzymes are regulated by feedback mechanisms, periodicity may be due to periodic changes in the synthesis of regulatory molecules affecting enzyme activity, rather than enzyme synthesis. Many of the papers published in the last decade utilise in vivo labelling and separation of extracted proteins by 2-D gels, followed by autoradiography. While this gives a very good indication of the timing of synthesis of a particular protein, it gives no information on the presence or lack of biological activity in the resulting peptide. This approach also will not show up proteins that were synthesised prior to labelling, and then modified (eg. by phosphorylation) during the labelling period. As phosphorylation is a known regulator of enzyme activity during the cell cycle (see Section 4.3), in vivo labelling experiments should not be used as the sole approach.

b) Many studies of periodic synthesis utilise synchronous cultures prepared by induction techniques involving changes in the media. These
methods may be expected to drastically affect the nature of periodic enzyme synthesis during at least the first synchronous division cycle (Elliott and McLaughlin, 1978). The problem has been circumvented in yeast, where cell size will change according to its position in the cell cycle, by using centrifugal elutriation (Elliott and McLaughlin, 1978). The cells are grown in a standard culture, where they do not experience stress caused by changes in media, and are separated according to size. Unfortunately this is not possible in plant cultures where cell size is not directly related to the cell cycle, and where cell microaggregates are usually present.

Work carried out on yeast cells (Elliot and McLaughlin, 1978) showed an exponential increase in the rate of mRNA and protein synthesis during the cell cycle, while the rate of DNA synthesis varied periodically. No stage specific proteins were detected and the increase in protein synthesis was an overall effect, rather than being limited to a particular subset of polypeptides. Work on HeLa cells (Bravo and Cells, 1980) reports that no polypeptides unique to a specific cell cycle stage were synthesised, while work on transformed human amnion cells (Celis and Nielsen, 1986) and Chinese hamster ovary cells (Westwood et al., 1985) report the presence of phase specific proteins which appear in the S phase and G2/M phase respectively. The apparent discrepancies resulting from the work with mammalian cells could be due to the sensitivity of the techniques or the different cell systems used in these studies. All three groups of researchers used the common technique of in vivo labelling followed by 2-D gel separation and autoradiography of protein extracts, so it may be assumed that they have similar sensitivities. But the different cell systems used may amplify these differences in stage specific protein synthesis. An example of this may be found with 'dividin' (or NEPHGE 10) which is present at all stages in HeLa cells, but is undetectable in the M and G2 stages in transformed human amnion cells. Celis and Nielsen (1986) state that it is easier to obtain pure populations of mitotic cells with amnion cells, which again might partly explain the discrepancies in the results gained from the different cell systems.

The main problem of cell cycle studies with plant systems has been that of synchronisation. It is important that the cells be allowed to
recover from the stresses introduced by the synchronisation technique before cell cycle studies take place. In Kodama's paper (Kodama et al., 1989), *Catharanthus roseus* cells are allowed some time to recover from the double phosphate starvation method used to synchronise them. Unfortunately this only represents the G1 phase and not the first mitotic division, which may be more desirable. The work is nonetheless a very complete one, as it includes 2-D gels of *in vitro* translated products of poly A+ RNA, *in vivo* labelled polypeptides, and total protein extracts from all stages in the cell cycle. The researchers show putative changes in protein synthesis in the cells at specific stages of the cell cycle, but no stage-specific proteins were identified. Not surprisingly, they show that transcription, translation, and post-translational regulation are all involved in controlling the range of proteins ultimately present in the cytoplasm of the cultured cells.

At this point in time, it would be interesting to design a synchronisation technique for the *Petunia hybrida* cells, using one of the methods outlined in Section 3.2.6. A preliminary study of the changes occuring during the cell cycle in *Petunia* should follow, probably in a structure similar to that in Kodama's paper. Specific polypeptides could then be targeted for further study, utilising methods discussed in this thesis, such as cDNA cloning, protein microsequencing and antibody production.

Collaborative work with K. Harikrishna showed that several genes were differentially expressed in cladodes and dedifferentiating cells. The work in this thesis concentrates on the DDL family but could be equally applied to other proteins.

Unfortunately both the cDNA clones and antibodies for DDL were only purified in the latter months of 1988. As a result, because of lack of time, a large number of experiments to clarify the biological role of DDL were not accomplished. Cultured cells may be used to investigate the cellular localisation of DDL. If, for example, DDL is released into the media in the preparation of protoplasts, the proteins are probably extracellular. Protoplasts can also be burst open and the organelles fractionated on a Percoll gradient, or the vacuoles purified. A vacuolar and/or extracellular location of members of the DDL family would be
consistent with DDL being a putative PR-type protein.

Both the cultured asparagus cells and seedlings may be used to ascertain whether DDL may be induced by stresses other than wounding. For example, cultured cells may be used to investigate the effect of subculturing, ethylene addition or changes in the media composition on DDL synthesis. The seedlings may be used for stress investigations such as heat and cold shocks, sodium chloride addition, dessication, anaerobis and water stress. The cultured cells may also be used to investigate addition of compounds such as salicylic acid, which are known to induce PR proteins.

Both PI and PR proteins seem to have serological similarities throughout the plant kingdom. Whether DDL belongs to one of these two groups of proteins or is unrelated, it would be interesting to investigate possible homologies to DDL in heterologous systems. These could be DNA sequence homologies or serological similarities. To ascertain the former, Southern blot analysis of heterologous DNA could be performed (see Section 4.1.5), for the latter total protein extracts from wounded plant tissues could be immunoblotted with DDL antibodies.

The question also arises on whether DDL may be coded for by a family of genes, and what copy number this gene, or family of genes, may have. Both questions could be answered by Southern blotting (eg. Fig 4.10). The size and number of bands on the Southern would be indicative of the number of different genes present, while spiking could be used to give an indication of the copy number. This study would have to be performed prior to any genomic cloning taking place.

Leaf specific mRNAs are found to be present in stem hnRNA, and vice versa. Developmentally regulated genes in tobacco are therefore divided into two classes, those that are constitutively expressed in the nuclear RNA of heterologous organs, and those who are not. A post transcriptional control must therefore exist whereby these hnRNAs never enter the cytosol as mature mRNA molecules; the sequences for this control must exist
within the transcribed molecule rather than the promoter itself.

The steady state levels of functional mRNAs are determined in part by their rate of decay in the cytoplasm. The determinants of messenger RNA stability have not yet been fully qualified, although sequences promoting mRNA decay in eukaryotes have been identified (Brawerman, 1987). Work on the 3' control region of the potato proteinase inhibitor II gene (An et al., 1989) has also shown that a specific DNA sequence surrounding the polyadenylation site is essential for efficient gene expression. The stability, or half life, of a specific mRNA can easily be investigated using actinomycin D (a transcription inhibitor) and propionamide (a translation inhibitor). Treatment with actinomycin D has sometimes been shown to cause superinduction. Superinduction is defined as an increase in the activity of an enzyme following application of an inhibitor to a system at such a time as the untreated system shows a decline in protein synthesis. Superinduction may be caused by either a decrease in protein degradation or an increase in protein synthesis. For example, the former effect is responsible for superinduction of tyrosine aminotransferase in hepatoma cells after cortisone treatment (Jones and Northcote, 1981) where the enzyme is inhibited and broken down by a second polypeptide. In the case of tyrosine aminotransferase the second polypeptide has an mRNA of very high turnover, which is preferentially inhibited by actinomycin D. If actinomycin D therefore preferentially inhibits the secondary polypeptide, inhibiting breakdown of the protein under study, the effect should be mimicked by propionamide. Superinduction by an increase in protein synthesis also occurs with PAL in bean cell suspension culture after hormone treatment (Jones and Northcote, 1981). It is explained by the fact that the PAL mRNA has a long 1/2 life; as the mRNA exists, it has to compete with newly synthesised mRNAs for translation but as actinomycin D inhibits de novo transcription, mRNAs with longer 1/2 lives will be preferentially translated because of lack of competition. This effect is cancelled by propionamide, which inhibits novel translation. Therefore, if treatment with actinomycin D caused superinduction of DDL, and treatment with propionamide cancels this effect, the mRNA can be said to have a long half life in the steady state mRNA population. This property of the DDL mRNA would have to be known before its 5' and 3' untranslated sequences
are used to drive the expression of heterologous genes. Therefore, if the expression of a recombinant gene in host plants is to be controlled by DDL sequences, it is necessary to know the strength and specificity of the promoter, as well as the effect of 5' and 3' untranslated sequences on the stability of the mRNA species before constructs can be made. By the same token, it might be possible to change the characteristics of the DDL gene sequences by mutagenesis, to try and improve the expression of the gene.

A major conclusion is that dedifferentiation is an ordered process involving specific changes in the transcription rate of specific genes. However, once the dedifferentiated state has been established, the cell's steady state mRNA population reverts to a profile very similar to that of "normal" cells. A cDNA clone (clone 34) has been individuated, which could be used to isolate a promoter with useful properties. If its putative role in switching on gene expression as a response to general or wound stress can be ascertained, it presents a number of interesting applications. For example, it could be used to investigate the claim by workers such as Logemann (Logemann et al., 1989) that cultured cells are under some form of stress. Alternatively, it could be used as a tool to control the synthesis of specific proteins in cultured cells, such as those involved in secondary metabolite production or embryogenesis (see Section 3.1.4) to manipulate the plant's metabolic functions and regeneration potential. A final use might be to utilise this type of promoter to transiently drive the expression of selectable marker genes during plant transformation. Such genes would not be switched on in regenerated plants and therefore will not impose a drain on metabolism or produce unwanted proteins in crop plants.

This thesis provides possibly the first insight into the molecular biology of dedifferentiation and the dedifferentiated state and it is hoped that the data will stimulate interest in this very important but often overlooked "plastic" developmental response.
REFERENCES


Birnboy HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid Res. 7, 1513-1523.


Carmin A, Van Staden J (1983) Role of roots in regulating the growth rate and cytokinin content in leaves. Pl. Physiol. 73, 76-78.


Chen J, Varner JE (1985) Isolation and characterisation of cDNA clones for carrot extensin and a proline-rich 33kDa protein. PNAS USA 82, 4399-4403.


Elliott SG, McLaughlin (1978) Rate of macromolecular synthesis through the cell cycle of the yeast Saccharomyces cerevisiae. PNAS 75, 4384-4388.


Keil M, Sanchez-Serrano JJ, Willmitzer L (1989) Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. EMBO 8, 1323-1330.


Koymbrink E, Schroder M, Hahlbrock K (1988) Several "pathogenesis-related" proteins in potato are 1,3-\beta-glucanases and chitinases. PNAS USA 85, 782-786.


Sanger F, Wicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. PNAS USA 74, 5463.


Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. PNAS USA 14, 4767-4771.


Dedifferentiation is the process by which specialised quiescent cells give rise to heterotrophic, dividing cells. This process may be initiated in vivo as a response to wounding, or in vitro during culture initiation. This thesis is concerned with evaluating whether the process of dedifferentiation and maintenance of the fast-dividing, dedifferentiated state by culture, is dependant upon major changes in gene expression. In particular, the role of transcription, as mirrored by changes in steady state mRNA levels, in these putative changes in gene expression has been investigated. Mechanically isolated Asparagus officinalis mesophyll cells were used to study dedifferentiating cells, and suspension cultures of Petunia hybrida to investigate the established dedifferentiated state.

This thesis shows that dedifferentiation in Asparagus officinalis is accompanied by major changes in the steady state mRNA profiles of the cells. A group of novel transcripts appearing in dedifferentiating asparagus cells were termed DD1, and targeted for further study. Two cDNA clones coding for DD1 transcripts were isolated and characterised, and antibodies to DD1 raised for serological work. Only minor differences were found between the steady state mRNA populations of Petunia hybrida cultured cells and seedlings, and these were mainly caused by transcripts disappearing in culture; no transcripts specific to the suspension culture system were detected.

The results presented in this thesis are used to forward the hypothesis that changes in gene expression involving de novo transcription may only occur in response to major changes in environmental conditions. It is suggested that the basal transcription pattern for cells in an established state is probably common to all cell types with regards to primary cell functions such as growth, division and catabolism. In such established states, the control of metabolism probably resides within the biochemical pathways utilised by the cell at any moment in time.