A MOLECULAR STUDY OF DEDIFFERENTIATION AND CELL CYCLE REACTIVATION IN MECHANICALLY ISOLATED ASPARAGUS CELLS.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Kepada Bapak, Emak dan Nenek.
Correspondence

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<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>rubisco</td>
<td>ribulose bisphosphate carboxylase/oxygenase</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>soin</td>
<td>solution</td>
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<td>SSU</td>
<td>small subunit rubisco</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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<td>tRNA</td>
<td>transfer RNA</td>
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<td>vols</td>
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General Introduction

1.1 Plasticity in Plant Development

Land plants are sessile and therefore cannot easily avoid environmental variation or predation. Plants can actively change their structure and their mode of development in response to environmental change. This form of response to environmental stimuli has been called a "plastic response". These plastic responses involve changes in morphology, physiology and biochemistry of the plant. Since the development of form involves many interacting factors (e.g. environmental factors, genotypic factors and developmental-stage specific factors) it has proved to be very difficult to examine and understand fully. Plasticity has an effect on the distribution of plants, as plants that are more adaptive will be at a selective advantage in unstable environments.

Examples of such plastic responses can be found in natural responses such as the alteration in the size of annual rings in perennials, leaf size and variegation. There are more extreme responses such as those resulting from wounding (callus formation) and grazing or experimental decapitation (shoot formation from axillary buds and their subsequent rooting via the production of adventitious roots).

The leaf is an organ that displays obvious structural and biochemical changes in response to the environment. Environmental stimuli such as temperature, photoperiod, light quality and quantity, mineral nutrition and water stress; can cause a change in leaf size by altering either cell numbers or cell volume (Dale, 1986). The leaf shape is altered when *Mesembryanthemum crystallinum* is subjected to salt stress (Winter, 1985). Other stimuli can also induce a change in leaf size, for example the primary young leaves of *Phaseolus vulgaris* increase in size when one of the pair of young leaves is removed, or when the stem is decapitated above the primary leaf node (Dale, 1986).

It is well established that light quality can affect gene expression as well as the activity of photosynthetic enzymes (see chapter 4). Environmental stresses such as salt stress and heat shock are known to elicit specific changes in gene expression (Ostrem *et al.*, 1987; Lindquist, 1986). Salt stress will induce crassulacean acid metabolism (CAM) in
Mesembryanthemum crystallinum plants as well as many other CAM plants, and will change the water-relation characteristics and levels of organic cytoplasmic solutes within the plants (Heun et al., 1981). All plants will synthesize a particular set of proteins in response to heat shock (reviewed in Nagao et al., 1986).

The environment can also induce a switch in the developmental pathway of higher plants. One of the more well characterised switches of developmental pathways is the development of the floral meristem. Floral meristems arise from a redetermination of shoot meristems. In some plants, flowering can be induced by a change in photoperiod (Sussex, 1989). The conversion of a vegetative meristem to a floral meristem involves a loss of cytohistological zonation and an equalisation of cell cycle times throughout the meristem (Sussex, 1989). All the cells in the meristem would be fully differentiated by the completion of floral organogenesis.

Plastic responses can be invoked both in vivo and in vitro. Tran Thanh Van (1986) observed that in vitro systems exhibit an even greater plasticity in morphogenic expression and in the use of metabolic pathways than in vivo systems. Tissue culture explants can be induced to form embryos, shoots, roots and callus (Tran Thanh Van, 1986); cells capable of organogenesis or embryogenesis are often referred to as "totipotent". Tran Thanh Van (1986) has stated that the effect of wounding, coupled with the ability to use signals such as carbohydrates, oligosaccharides, growth substances, minerals, pH changes and cell to cell signals, to generate a well-defined morphogenic pathway, makes in vitro systems a powerful tool for the study of development.

The wound response is a complex plastic response which displays an interaction between the environment, genotype and the developmental stage. Furthermore, there are different types of wounding which in turn induce different responses in the plant; severe wounding in dicots, such as decapitation, will cause a reactivation and the subsequent development of quiescent lateral buds. However, it is also possible to wound small regions of tissue, such as in feeding of some insects, or to wound individual cells such as in fungal pathogen attack. In both these latter examples the wound response is more localised and there is no reactivation of lateral buds.
Gene expression during wounding is an extremely complex phenomenon as wound sites contribute to dehydration and act as entry sites for infection by pathogens. Therefore the associated physiological, cytological and biochemical changes observed during wounding are involved in several functions; wound healing, prevention of desiccation and prevention of pathogen entry. As mentioned earlier wounding is a broad term; injuries range from damage to individual cells, to complete severance and separation of the shoot from the root system. Further complications arise from the different responses of dicotyledonous and monocotyledonous plants, different plant tissues and plant organs to wounding (Moon et al., 1984; Davies et al., 1986; Thiellet et al., 1982). For instance wound-induced cell proliferation is common in many herbaceous and woody dicots (Wilson and Grange, 1984), but in monocots, the first layer of intact cells adjacent to the wound is modified by the accumulation of anti-microbial and water impermeable substances such as lignin and suberin (Moon et al., 1984). These responses all serve to isolate the damaged area of the organ and to prevent desiccation and infection of the plant by opportunistic pathogenic fungi and bacteria (Lipetz, 1970). The cells formed by wound-induced cell division are callus cells which proliferate at wound sites and contain large vacuoles. Calli have been found to contain several more antigens when compared to the donor organ tissue (Raff et al., 1979). It is postulated by Raff et al. (1979) that these *extra* antigens are involved in recognition reactions in vegetative grafts and in certain host epiparasite interactions. So plant plastic responses and in particular wounding is a very large topic but this thesis will concentrate on one aspect of the wound response, which is wound-induced cellular dedifferentiation as a prelude to reactivation of the cell cycle. In particular, the cellular, molecular and physiological aspects are examined.

1.2 Dedifferentiation.

Dedifferentiation is a developmental pathway which leads to the formation of callus or cells which are capable of greater levels of plasticity. Hence dedifferentiation is often the first step in the expression of totipotency, which enables the regeneration of whole plants from cultured cells. In this thesis dedifferentiation is defined as the process by which specialised cells that are division cycle arrested give rise to heterotrophic, dividing cells. Dedifferentiation normally
occurs as a result of wounding and often results in the formation of a callus plug which seals off the wound site from the environment. Cells around wound sites are often involved in complex biochemical activity which is geared up towards the prevention of infection by microbes and in wound healing. However, dedifferentiation has been observed to occur without prior wounding during floral development of Catharanthus roseus; Verbeke and Walker (1986) found that during postgenital fusion of the distal adaxial surfaces of 2 originally separate carpel primordia of Catharanthus roseus approximately 400 epidermal cells underwent rapid dedifferentiation into parenchymatous cells. Dedifferentiation is however, more commonly associated with the wound response than with floral development.

1.3 Why Examine Dedifferentiation?

Dedifferentiation is a developmental pathway that is worthy of further investigation because of the following reasons. Callus formation is often the first step in many tissue culture manipulations and results from wound-induced cellular dedifferentiation and cell cycle reactivation. It is generally only developmentally undetermined non-dividing cells that are able to dedifferentiate and undergo cell division. It would be interesting to understand this phenomenon as it might prove possible to manipulate it.

Fully dedifferentiated suspension cultured cells are widely used model systems which have been used to examine gene expression during the cell cycle (Kodama et al., 1989), growth cycle (Wylegall et al., 1985; Amileni et al., 1979) and in response to changing environmental conditions (Bevan and Northcote, 1981 b) and various elicitors (Tempelton and Lamb, 1988). It is well established that suspension cultured cells have altered gene expression particularly in regard to secondary metabolism, so an understanding of dedifferentiation can enable more accurate comparisons of gene expression to be made with intact plants. The majority of explants that have been successfully transformed by Agrobacterium tumefaciens undergo a brief callus phase prior to transformation; an examination of dedifferentiation may explain why this cell type is more competent. A phenomenon called somaclonal variation occurs in tissue cultures that have undergone dedifferentiation (Kikuchi et al., 1987; Hussey, 1983). This term is used to describe a higher than background mutation rate, and is normally associated with
chromosomal aberrations that often result in polyploid cell formation (Bennici and Caffaro, 1984). Ideally, transformed plants are regenerated from single, undifferentiated cells (Draper et al., 1988). Therefore, if genetic engineering is to become routine technology for future plant breeding, the control of genetic variability arising from tissue culture will be important. An examination of the cellular and molecular biology of dedifferentiation may help to explain why the genome is more unstable in undifferentiated tissue.

1.4 Problems with Examining Dedifferentiation in Intact Plants

The processes involved in dedifferentiation are not easy to study in plants for many reasons. Firstly, the size of the tissue layer involved in dedifferentiation may be very small; large quantities of physiologically and morphologically similar material is required for molecular analysis and small tissue layers will make analysis difficult. These dedifferentiating cell layers are very often enclosed within wounded or dead tissue and are therefore difficult to isolate and examine. There is a complex interaction between wounded cells at the wound site, adjacent non-wounded cells and the rest of the plant. For example a wound can cause the formation and subsequent transport of a systemic molecular signal, to tissues a long way from the wound site where this signal induces biochemical changes to occur. This type of systemic response is responsible for the induction of proteinase inhibitor gene expression in wild tomato species (Ryan and An, 1988). Furthermore, diffusible compounds with either anti-pathogen activity or involved in wound-healing are often produced in damaged tissue (Tempelton and Lamb, 1988; Lipetz, 1970; Misaghi, 1982). These diffusible compounds are transported to non-damaged cells via plasmodsmata and differ from a systemic signal in that only cells in the vicinity of the wound site respond to their presence.

The wound response has a transient nature as there is often a great deal of associated cell death, and wound healing can generally be separated into three distinct phases (see section 5.1.7; Wilson and Grange, 1984). Furthermore these dead cells accumulate compounds that interfere with molecular analyses (Logemann et al., 1987). The inconsistent timing also makes accurate analysis of the various phases of the response difficult. For these reasons it was decided to study cellular dedifferentiation in vitro.
1.5 The use of In Vitro Systems for an Efficient Molecular Analysis of Dedifferentiation

Since in vitro systems show a larger degree of plasticity, it is relatively easy to induce dedifferentiation in culture. Such in vitro systems however, have their own inherent problems and cannot be directly compared to the intact plant. For example, high levels of plant growth regulators often used in culture make it difficult to separate effects induced by plant growth regulators from phenomenon associated with dedifferentiation. Tissue culture media very often contain various nutrients and salts that affect gene expression. Nutrients such as sucrose in the media will induce a change in the explants mode of respiration, from photoautotrophic to heterotrophic (Dalton and Street, 1977; see Chapter 4). This in turn will affect the function of chloroplasts, as photosynthesis will be no longer required.

The concentrations of salts used in the media may be different from within the plant and thus contribute to altered gene expression, which may otherwise be attributed to dedifferentiation. There is very often a component of tissue culture media that has osmotic potential. This will cause an altered gene expression profile (Fleck et al., 1982) and again will be difficult to distinguish from dedifferentiation. The change in osmotic potential of the medium and a high environmental humidity will cause a change in water relations of the tissue and therefore effect both the physiology and the morphology of the explant. A tissue explant has a larger surface area for gas exchange than the intact plant. Culture vessels are sealed and often accumulate biologically active gases such as ethylene. This could also make a contribution to an altered gene expression profile which may be unrelated to dedifferentiation.

1.6 In Vitro Systems that have been used in the past to Examine Wounding/Dedifferentiation

The in vitro systems that have been used to examine wounding/dedifferentiation or cell division include artichoke and potato tuber slices, carrot tap root explants, many other types of stem, leaf or embryo explants, suspension cultured cells and protoplasts. Each of these systems have their own inherent short comings. Artichoke tubers have been used to study wound-induced DNA replication and the cell cycle. Wounding was used to induce synchronous cell division in a thin layer of cells below the cut surface (Harland et al., 1973). The major disadvantage of this system was that synchronous cell division occurred only in a
few cell layers and that there were seasonal variations in the response of tuber tissue to excision and culture (MacLeod et al., 1979). Therefore molecular analysis is difficult to perform on such a system.

Explanted immature embryos of maize have been used to examine callus formation at the ultrastructural level (Franz and Schel, 1987). Although this system was satisfactory for electron microscopic examination of callus formation, only very small quantities of dedifferentiating tissue could be obtained which would not be sufficient for a molecular study.

Suspension cultured cells have been used to study the cell cycle and the growth cycle. Such tissue is the product of several sub-cultures and is thought to be undifferentiated. The passage of tissue through several sub-cultures will result in the selection of only quick-growing cell lines that may not bear close resemblance to cells in the intact plant. Cells in such cultures also contain higher percentages of nuclei with aberrant chromosomes. It is important for a high degree of synchronisation to be achieved before molecular analysis can be contemplated. If this cannot be achieved, minor differences in gene expression cannot be detected. The methods used for cell synchronisation include starvation methods and the use of DNA synthesis inhibitors. Such methods in themselves cause alterations in gene expression and can change cell cycle duration. For these reasons, as well as the fact that the cells are already undifferentiated, the system is not ideal for the examination of dedifferentiation or the cell cycle.

Protoplasts have been used to study the initiation of DNA synthesis and mitosis using $^{3}H$ thymidine incorporation (Zelcer and Galun, 1976). The effects of plant growth substances on the cell cycle and gene expression of protoplasts has also been investigated (Cooke and Meyer, 1981, Meyer et al., 1984 a & b). Dedifferentiation has been examined by molecular methods in protoplasts of tobacco and mungbean. However, only a small amount of information was obtained in these studies; in tobacco, large sub unit ribulose bisphosphate carboxylase oxygenase (rubisco) reduced in abundance with protoplast formation (Fleck et al., 1980) and lipid composition and isoperoxidase profiles changed with dedifferentiation in mungbean protoplasts (Goldberg et al., 1986). The advantage of protoplasts over the previously mentioned systems is that single cells are used, and most of the cells respond (up
to 60% or more) to the media. The disadvantage of protoplasts is that they are extracted harshly, using cell wall degrading enzymes, are under a high osmotic pressure (Hypertonic medium) and are physiologically abnormal, the cell wall is absent and it is difficult to generate large quantities of viable cells from intact plants.

1.7 The Ideal In Vitro System

The ideal in vitro system should be able to generate large quantities of material, be as near to single cells as possible to facilitate microscopic examination, should be simple and quick to prepare, should be as physiologically normal as possible and should have a high synchrony of cell dedifferentiation, expansion and cell division. The only available systems that can satisfy some or all of the criteria are protoplasts and mechanically isolated cells. As mentioned earlier protoplasts do not satisfy all of the criteria as they are not structurally or physiologically normal. However, protoplasts can be examined microscopically within their culture vessels and have some degree of inherent synchrony. Therefore molecular analysis can be performed without the presence of synchronising agents.

Mechanically isolated cells are however, superior to protoplasts as such cells retain their cell walls, are cultured in hypotonic medium and are therefore less physiologically different from cells in the intact plant. Other advantages of mechanically isolated cells are as follows: unlimited numbers of cells can be isolated and cultured with ease (Jullien and Guern, 1979); a high natural synchrony of cell expansion and cell division can be achieved (Rossini, 1972); the cells are physiologically and morphologically similar, have high viabilities, and can be examined microscopically. However, there are disadvantages associated with any in vitro system. Care has to be taken when comparing mechanically isolated cells with the intact plant. Firstly, the components of the culture medium can affect gene expression. Secondly, there is little cell to cell contact unlike in the intact plant and the cells are exposed to the same environmental conditions, unlike the intact plant where there are nutritional and hormonal gradients. Thirdly, cultured cells are generally heterotrophic, unlike a callus on a plant which may contain a mixture of photoautotrophic and heterotrophic cells. The in vitro system chosen
to examine wound-induced dedifferentiation was cultured mechanically isolated *Asparagus officinalis* mesophyll cells (see Chapter 3 for details).

### 1.8 Summary of Approaches used in this Thesis.

Cytological, physiological and the molecular aspects of wound-induced dedifferentiation are examined in this thesis. Chapter 3 is concerned with the optimisation of the cell culture system and with cytological analysis to identify markers for the dedifferentiation process. Fluorescent dyes were used to identify stages during dedifferentiation. The initiation of DNA synthesis and the continuation of the cell cycle were examined by Feulgen staining followed by microdensitometry. Common methods for extraction of macromolecules were tested and modified where necessary.

Limited physiological aspects of dedifferentiation relating to photosynthesis were examined (chapter 4) by monitoring oxygen consumption and oxygen evolution; such parameters could be used as early markers for dedifferentiation. An analysis of chloroplast integrity was also carried out. Nuclear and chloroplast encoded genes involved in photosynthesis were monitored by Northern and Western analysis during the switch from photoautotrophic to heterotrophic growth.

General changes in gene expression during dedifferentiation are examined in chapter 5. The approach involved the isolation of poly (A)$^+$ RNA followed by *in vitro* translation in rabbit reticulocyte lysate to produce radioactive proteins. These proteins were separated by two dimensional (2-D) polyacrylamide gel electrophoresis and up-regulated dedifferentiation/wound-induced proteins identified. Overall gene expression was examined and categorised into distinct phases during dedifferentiation. A cDNA library was constructed to isolate genes coding for some of the identified up-regulated transcripts.

### 1.9 Aim of Project.

The project is based on the use of an *in vitro* mechanically isolated cell system to examine certain aspects of plant plastic responses. After a week of culture asparagus mesophyll cells form microaggregates of dividing callus cells. I was interested to examine the switch in
development of the mesophyll cells to give rise to callus cells. This switch has not been well characterised, though much is known concerning the properties of callus resulting from this developmental switch. A series of experiments were devised to examine dedifferentiation using cytological, physiological and molecular approaches. The cytological analysis was concerned with the identification of markers for dedifferentiation and used both non-invasive techniques as well as specific staining techniques. It was important to determine the feasibility for molecular analysis by measuring the yields of extracted macromolecules. Dedifferentiation also involves a change in mode of respiration from photoautotrophic to heterotrophic. This switch was examined in more detail with an oxygen electrode to identify further markers for dedifferentiation. Markers enabled dedifferentiating cells of the same stage to be harvested for molecular analysis. The molecular approach was based on an examination of mRNA complexity by \textit{in vitro} translations followed by 2-D gel electrophoresis. A cDNA library could be constructed from any stage of dedifferentiation that appeared interesting. Differentially expressed clones could be isolated from such cDNA libraries and can be used to determine if \textit{in vitro} dedifferentiation is the same as \textit{in vivo} dedifferentiation in intact plants.

In this thesis chapter 3 is concerned with the optimisation of the tissue culture system as well as the cytological characterisation of dedifferentiation for the identification of markers. Chapter 4 is concerned with the examination of the loss of photosynthetic potential during dedifferentiation for the further identification of dedifferentiation markers. Chapter 5 is concerned with the analysis of mRNA complexity and the construction of cDNA libraries from stages of interest.
Chapter 2 Materials and Methods

All chemicals were obtained from SIGMA unless otherwise stated.

2.1 Growing Conditions for Asparagus Plants

Seeds of Asparagus officinalis var Connovers Colossal (Asmer seeds) were soaked in water for 3 hr, sterilised for 3 min in ethanol and then rinsed in water prior to sowing in 1:1 Levingtons compost (Fisons Agricultural Division, Bramford, Ipswich, England) / vermiculite mixture. The seedlings were raised in a Fison cabinet at 27°C, 12 hr day, and an irradiance of 90 μmol m⁻² s⁻¹. At 3 weeks after sowing, plants were transferred to a controlled environment room at 25°C, 12 hr day, and irradiance of 200-280 μmol m⁻²s⁻¹. The plants were watered once a week with Hoaglands solution (Epstein, 1972; see section 2.2.2). The cladodes were harvested after 5 to 6 weeks growth, when the side branches were not very well developed.

2.2 Tissue Culture

2.2.1 Asparagus Tissue Culture

The Asparagus fronds were sterilised in 10 % bleach, thick/thin (1:1) for 20 min, and then washed 4 times with sterile water to remove any bleach. Cladodes were stripped off by hand using a pair of sterile gloves (Microtouch, autoclaved). The sterile cladodes were held under sterile tap water (10-15 ml) and the cells scrapped off using a sterile plastic scraper (same dimensions as a microscope slide except with some flexibility) in a 240 mm Petri dish (Sterilin). The resultant cell suspension was poured through a 64 μm mesh filter and collected in a 240 mm Petri dish. The sieved cells were collected using an electrical pipette (Pipette Boy Company) and transferred to a 250 ml centrifuge bottle (Sorval). The cells were pelleted by centrifugation for 2.5 min, at 800 rpm in a Sorval RT 5000B centrifuge in a H 1000B rotor. The pelleted cells were resuspended in 100 ml of water. An aliquot was removed and viable numbers determined by the appearance of cell contents in a haemocytometer. The cells were repelleted and resuspended in asparagus medium (see section 2.2.2) and then repelleted and
resuspended at a concentration of 4 X 10^5 viable cells per ml together with glutamine
(1 g l\(^{-1}\), filter sterilised). The cells were plated out in a 10 ml volume in 90 mm Petri dishes
(Sterilin) and sealed with "Nescofilm". The plates were incubated in the dark at 25 \(\text{C}\) on an
orbital shaker (36 rpm).

2.2.2 Asparagus Medium

Asparagus medium consisted of modified Nagata and Takebe (1971) salts and its components
are as follows.

<table>
<thead>
<tr>
<th>component</th>
<th>conc (\text{mg}\ l^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{NH}_4\text{NO}_3)</td>
<td>825</td>
</tr>
<tr>
<td>(\text{KNO}_3)</td>
<td>950</td>
</tr>
<tr>
<td>(\text{CaCl}_2\cdot2\text{H}_2\text{O})</td>
<td>220</td>
</tr>
<tr>
<td>(\text{MgSO}_4\cdot7\text{H}_2\text{O})</td>
<td>1233</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>680</td>
</tr>
<tr>
<td>(\text{Na}_2\text{EDTA})</td>
<td>37.3</td>
</tr>
<tr>
<td>(\text{FeSO}_4\cdot7\text{H}_2\text{O})</td>
<td>27.8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10 (\text{g}\ l^{-1})</td>
</tr>
<tr>
<td>Myo Inositol</td>
<td>100 (\text{mg}\ l^{-1})</td>
</tr>
<tr>
<td>(\text{H}_3\text{BO}_4)</td>
<td>6.2</td>
</tr>
<tr>
<td>(\text{MnO}_4\cdot4\text{H}_2\text{O})</td>
<td>22.3</td>
</tr>
<tr>
<td>(\text{ZnSO}_4\cdot7\text{H}_2\text{O})</td>
<td>10.58</td>
</tr>
<tr>
<td>(\text{KI})</td>
<td>0.83</td>
</tr>
<tr>
<td>(\text{NaMoO}_4\cdot2\text{H}_2\text{O})</td>
<td>0.25</td>
</tr>
<tr>
<td>(\text{CuSO}_4\cdot5\text{H}_2\text{O})</td>
<td>0.025</td>
</tr>
<tr>
<td>(\text{CoSO}_4\cdot7\text{H}_2\text{O})</td>
<td>0.03</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1</td>
</tr>
<tr>
<td>NAA (naphthylacetic acid)</td>
<td>1</td>
</tr>
<tr>
<td>6-BAP (6-benzyl aminopurine)</td>
<td>0.3</td>
</tr>
<tr>
<td>pH 5.8 with KOH</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>30 (\text{g}\ l^{-1})</td>
</tr>
<tr>
<td>Glutamine (filter sterilised,\n frozen stock added just before plating out)</td>
<td>1 (\text{g}\ l^{-1})</td>
</tr>
</tbody>
</table>
### Hoaglands Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>6mM</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>4mM</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>2mM</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1mM</td>
</tr>
<tr>
<td>KCl</td>
<td>0.05mM</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.025mM</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>2nM</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>2nM</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.5nM</td>
</tr>
<tr>
<td>H₂MoO₄</td>
<td>0.5nM</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>2mM</td>
</tr>
</tbody>
</table>

### 2.3 Cytology

#### 2.3.1 Tinopal Staining

Tinopal BOPT (CIBA-Geigy U.K. Ltd.) was made up as a saturated solution in distilled water and stored at 4°C in the dark. For staining cells, 0.1 ml of this solution was mixed with 20 ml of asparagus medium (see section 2.2.2), and one drop of this solution was mixed with an equal volume of cells on a clean microscope slide. After 5 min, the preparation was viewed by Ultra Violet (UV) microscopy using a Nikon phase contrast microscopy (model Diaphot-TMD), fitted with a UV excitation filter cassette, and auxiliary excitation filter (IF 395 425 ) and an eyepiece-side absorption filter (470K) with dichroic mirror (DM 455).

#### 2.3.2 Acridine Orange Staining of Cells

A solution of acridine orange was prepared by dissolving 10.0 mg of acridine orange (Sigma) in 4 ml of 50 mM glycine/NaOH buffer at pH 8.5, and mixing with 6 ml of asparagus medium (see section 2.2.2). Two drops of this solution was mixed with 1.5 ml of cells, and the mixture kept in the dark for 20 min. The suspension was spun in a microfuge (13000 rpm) 20 sec and the pelleted cells resuspended in fresh media. A drop of this preparation was viewed by UV microscopy using a Nikon phase contrast microscope (model Diaphot-TMD), fitted with a
blue excitation filter cassette (code B, 495 n.m), an auxiliary excitation filter (I.F420 485) and an eyepiece-side absorption filter (520W) with a dichroic mirror (DM510).

2.3.3 DAPI Staining

The method of Sellden and Leech (1981) was used to study DNA replication in *Asparagus* cells. An equal volume of DAPI (4, 6-Diamidino phenyl indole; Sigma, D-1388) solution (0.5 µg DAPI + 0.9 gm NaCl + 1 ml 1M Tris-HCl pH 7.5 made up to 100 ml) and cell suspension was placed on a microscope slide and viewed under UV illumination (Nikon phase contrast microscope as section 2.3.2).

2.3.4 Cell Size Measurements

The average size of cells on each day in culture was measured using a Zeiss drawing tube to project a blown up image of the cells onto an Apple microcomputer graphics pad. The cell size was then calculated using Apple graphics software.

2.4 Physiology

2.4.1 Oxygen Electrode Measurements

The oxygen electrode was turned on 1 hr before use to ensure complete equilibration of the apparatus. The apparatus was calibrated by filling the electrode chamber with air saturated water (distilled water, 25°C: 0.237 µmol O₂ ml⁻¹) and setting the recorder on 90. This was left for a few minutes and any readjustments made. Gaseous nitrogen was bubbled through the chamber to remove any traces of oxygen from the water. When the trace on the recorder levelled out it was set on zero. The water was removed by aspiration and the chamber refilled with fresh distilled water and the recorder trace readjusted to 90 if required. The samples were prepared by pelleting cultured cells (from a Petri dish, 4 x 10⁶ cells) in a 20 ml Universal bottle (Sterilin) by centrifuging for 2.5 min at 800 rpm (Sorval, RT6000B, H1000B rotor). Only 4 ml of medium supernatant was retained, the rest discarded. The cells were resuspended in the remaining 4 ml of medium, resulting in 1 x 10⁶ viable cells per ml. The cells (2 x 10⁶ in 2 ml) were placed into the electrode chamber and oxygen evolution rates measured under
illumination and in the dark. This was repeated after the addition of 10 μl 0.1 M sodium bicarbonate. The oxygen evolution rates can be expressed as either gross rates or net rates. Gross photosynthetic rate is the oxygen evolution/sec without any adjustments to take into account the oxygen consumed by respiration. Net photosynthesis is when the dark incubated oxygen consumption measurements are taken into account by addition to the measured oxygen evolution rate. The respiratory rate is the measured oxygen consumption in the dark.

2.4.2 Electron Transport Chain Measurements

The activity of the whole electron transport chain excluding ferredoxin and FNR can be measured in an oxygen electrode (Coombs et al., 1985) using methyl viologen as a terminal oxygen receptor. The oxygen electrode was set up and measurements made as in section 2.4.1. The cells (2 x 10^6) were placed in the oxygen electrode chamber together with 100 μM methyl viologen, 5 mM NH₄Cl and 2 mM sodium azide. Measurements were made after 10 min, when most of the cells had absorbed some methyl viologen. The end product of the reaction is hydrogen peroxide which detected by the oxygen electrode. It is very important for catalase activity to be removed, hence sodium azide or potassium cyanide is added to the reaction mix. The stoichiometry of the electron transport chain is four electrons transported per O₂ consumed.

2.4.3 Chlorophyll Extraction

Chlorophyll was extracted according to the method of Inskeep and Bloom (1985). N,N-Dimethylformamide (5 ml) was added to Asparagus cells (2 x 10^6) in a centrifuge tube and incubated in the dark for 48 hr at 4°C. Absorbance was determined at the wavelengths 664.5 nm and 647 nm in a spectrophotometer. The total chlorophyll, chlorophyll a and chlorophyll b was calculated as follows; total chlorophyll = 17.90 A₆₄₇ + 8.08 A₆₆₄.₅, chlorophyll a = 12.70 A₆₆₄.₅ - 2.79 A₆₄₇, and chlorophyll b = 20.70 A₆₄₇ - 4.62 A₆₆₄.₅.
2.5 Macromolecule Extraction and Quantification

2.5.1 Protein Extraction

The protein extraction was carried out using the Mayer method (Mayer et al, 1987) with a modified extraction buffer. Plant material was pulverised into a fine powder by grinding with liquid nitrogen using a pre-chilled mortar and pestle. To this tissue (0.5 ml g\(^{-1}\) tissue) 2 DMH extraction buffer (2 % Ampholines pH 3-10 [BDH PLC, Poole UK] + 300 mM NaCl + 1 mM EDTA + 1 mM EGTA + 2 % Triton X-100 + 5 mM Ascorbic acid + 100 mM DTT + 10\(\mu\)g ml\(^{-1}\) Leupeptin) was added and 20 \(\mu\)g g\(^{-1}\) tissue 100 mM PMSF (phenylmethyl sulphonyl fluoride made up in propan-2-ol) was 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 for 10 min, and the supernatant collected. To this, 100 \(\mu\)l ml\(^{-1}\) of 3 mg ml\(^{-1}\) DNase I was added, and the tube incubated at 37\(^\circ\)C for 15 min. The tube was spun (5 min) once more, and solid urea (Aristar, BDH PLC Poole UK) was added to the supernatant to 9 M (0.1g). The samples were stored at -70 \(^\circ\)C and quantified using the following techniques.

2.5.2 Protein Quantification, Bradford Assay

Protein was quantified according to the method of Ramagli Rodriguez (1985) which is a modified Bradford (1976) method. This modification had to be employed as the extraction buffer contained detergent and urea. The reagent was made up with 100 mg Coomassie brilliant blue G-250 in 50 ml 95 % ethanol and 100 ml of 85 % w/v phosphoric acid (BDH PLC, Poole, UK) in 1 litre of distilled water. 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 100 \(\mu\)g BSA in 0.1 ml. A sample of 10 \(\mu\)l was acidified by the addition of 10 \(\mu\)l, 0.1 M HCl and made up to 100 \(\mu\)l with distilled water, 900 \(\mu\)l Bradfords reagent was added and the solution scanned at 595 nm in a spectrophotometer. This method was used until March 1988 when it was superseded by the dot binding assay.
2.5.3 Protein Quantification, Dot Binding Assay.

Protein was quantified according to the method of Ghosh et al. (1988) after March 1988. This protocol offers advantages over the Bradford method as it is not affected by the presence of detergents, protease inhibitors or biological molecules such as chlorophyll. Protein standards (BSA 1 mg ml\(^{-1}\)) and protein samples in equal volumes of buffer (10 \(\mu\)l) were spotted out on 1 cm squares drawn on Whatman 3 MM paper. After air drying for 15 min, the filter was stained in fresh stain (0.2 % Coomassie Brilliant Blue R250 in methanol: water: acetic acid. 50:40:10) for 20 min. The filter was destained in several changes of methanol: water: acetic acid (20:60:10) for 1 hr. After air drying, 1 cm squares were cut out of the filters and the stain eluted for 1 hr in 1 ml of 1 M potassium acetate in 70 % ethanol on a rocking platform. The absorbance was read at 590 nm in a Lambda 5 spectrophotometer (see section 2.5.7).

2.5.4 DNA Extraction, CTAB method

Plant material was freeze dried and ground with alumina (1: 0.7g, tissue: alumina) in a mortar and pestle. The optimal quantities of tissue for a miniprep are 0.1 g callus or 0.07 g leaf. The powder was then placed in an Eppendorf centrifuge tube and 600 \(\mu\)l of extraction buffer (50 mM Tris-HCl pH 8.0 + 0.7 M NaCl + 10 mM EDTA + 1% CTAB + 1% 2-mercaptoethanol) added. After mixing, the suspension was incubated for 10 min at 56 °C and then extracted with 600 \(\mu\)l of chloroform:isoamyl alcohol (24:1). Denatured protein interface on the organic layer was washed with 100 \(\mu\)l extraction buffer which was pooled with the initial aqueous phase. CTAB (hexadecyltrimethylammonium bromide) was then added to a concentration of 1 % and the extraction repeated. One volume of precipitation buffer (50 mM Tris-HCl pH 8.0 + 10 mM EDTA + 1 % CTAB) was added to the aqueous phase in a clean Eppendorf tube and left at room temperature for 20 min. The precipitate was collected by centrifugation (microfuge, 13000 rpm, 1 min), the supernatant removed, and the pellet dissolved in 1 M NaCl. Two volumes of ethanol at -20°C were added to precipitate the nucleic acids, which was collected by centrifugation and then 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 quantified using the diphenylamine method (section 2.5.5).
2.5.5 DNA Quantification via the Diphenylamino Assay

Note it is important that all work is done in a fume cupboard.

The DNA sample (10 µl DNA + 140 µl H₂O) was added to 150 µl 3 N perchloric acid (20 µl paradehyde + 198 ml glacial acetic acid + 8 gm diphenylamine) mixed well in an Eppendorf tube and incubated in the dark 16-20 hr 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 5-50 µg ml⁻¹. Distilled water replaced the DNA sample in the control and was used each time to zero the spectrophotometer (section 2.5.7).

2.5.6 RNA Extraction

The method used was a derivation of Covey and Hull (1981), altered so as to reduce contamination by soluble carbohydrates. An appropriate amount of tissue was ground in liquid nitrogen in a precoolied pestle and mortar. Grinding medium (6 % phenol + 4-aminosalicylate + 1 % Triisopropyl naphthalene sulphonate + 6 % phenol + 50 mM Tris-HCl pH 8.4) was then added at 2 ml gm⁻¹ of tissue. When ground into a fine powder, the mixture was thawed and poured into a 50 ml polypropylene tubes (Sorval, GSA rotor). One volume of phenol:chloroform (1:1) was added (stored at 4°C in the dark), and the tubes shaken thoroughly and centrifuged at 5 K for 10 min. The aqueous phase was removed and reextracted once with one volume of the phenol:chloroform mixture. Sodium acetate pH 6.0 was added to 0.2 M, together with 2.5 volumes of absolute ethanol at -20°C. The total nucleic acids were collected by centrifugation (10 K for 10 min). The pellet thus recovered was resuspended in 1 ml 50 mM Tris-HCl pH 8.4/gm tissue, and reextracted with the phenol:chloroform mixture until the interface between the aqueous and organic phase was clear. Sodium acetate pH 6.0 was added to 0.2 M and another ethanol precipitation effected. The pellet was dissolved in 0.5 ml water gm⁻¹ tissue. If the solution appeared cloudy, it was then spun at 5K for 5 min. The pellet contains some nucleic acids but is mainly carbohydrate. Three volumes of 4 M sodium acetate pH 6.0 was added to the supernatant...
to precipitate the RNA. The solution was placed on ice for 3 hr, and spun at 10 K for 30 min. The RNA pellet was dissolved in water and sodium acetate added to 0.2 M and ethanol precipitated. The RNA was then spun down, the pellet rinsed in 70% ethanol once, dried in a vacuum desiccator, dissolved in water to 5 mg ml⁻¹ and stored frozen (-20°C) in aliquots of 0.5 ml.

2.5.7 Spectrophotometry
A dual beam recording spectrophotometer (Perkin-Elmer Lambda 5 UV/ VIS) was used routinely for DNA and RNA concentration determination at OD₂₅₀ and OD₂₈₀ for chlorophyll measurements at OD₆₆₄.₅ and OD₆₄₇ and for protein quantification at OD₅₉₀.

2.5.8 RNA Poly (A)⁺⁺ Purification
The appropriate amount of oligo-dT cellulose (5 mg cellulose mg⁻¹ RNA) was equilibrated in sterile loading buffer (20 mM Tris-HCl pH 7.6 + 0.5 M LiCl + 1 mM EDTA + 0.1% SDS) and poured into a Pasteur pipette previously plugged with autoclaved fish tank wool. At the same time 1 volume of 2X loading buffer was added to the RNA sample, the mixture heated to 65°C for 7 min and then placed straight on ice for 5-10 min. The RNA was now loaded onto the column in a volume of 1000 µl and the eluate reloaded 6 times. The column was washed with 50 ml of loading buffer to remove any tRNA and rRNA. The elution buffer (10 mM Tris-HCl pH 7.5 + 1 mM EDTA + 0.05% SDS) was heated to 45°C and added in 300 µl aliquots (5 aliquots) to the column. Poly (A)⁺⁺ RNA was collected in sterile Eppendorf tubes, quantified and then precipitated with 0.2 M sodium acetate pH 6.0 and 2.5 volumes of ethanol. If required for cDNA synthesis, the RNA was repurified to yield poly (A)⁺⁺ mRNA.

2.5.9 Nucleic Acid Extraction
The total nucleic acids were extracted as in section 2.5.6 except that the purification was stopped at the differential solubility step. Two Petri dishes of cultured cells (8 x 10⁶) from the same tissue culture extraction were harvested by filtration and stored under liquid nitrogen until a time course of 1-6 days were available. The nucleic acids were extracted in equal volumes
of extract buffer for each time point. The nucleic acid extraction was run on a 0.8 % agarose gel (see section 2.7.1) and photographed. The total extracted DNA was calculated by comparing band intensity with Lambda markers. For the DNA dot blots the RNA was removed by the addition of RNase I (10 µg) and its efficiency tested by running an agarose gel.

2.6 Biochemistry

2.6.1 In Vitro Translation of mRNA

The method used commercial rabbit reticulocyte lysate (RRL - Amersham No 90) and \[^{35}\text{S}\]-methionine (> 21.62 GBq mol\(^{-1}\) - Amersham SJ 204) both stored in 50 µl aliquots under liquid nitrogen. The translation mixture consisting of 7 µl RRL + 1 µl \[^{35}\text{S}\] methionine + 2 µl poly (A)\(^+\) RNA was mixed gently and incubated at 28 °C for 40 min. RNase 1 (1 µl of 10 mg ml\(^{-1}\)) was added to the mixture for 20 min at 28 °C to digest all RNA and stop the translation. If the sample was to be run on a one dimensional PAGE gel then 11 µl of "cracking" buffer (2% SDS + 5% mercaptoethanol + 10% sucrose 0.002% bromophenol blue in distilled water) was added; if the sample was to be run on an IEF rod gel, then 11 µl of 2 DMH (see section 2.5.1) was added instead and solid urea added to 9 M (0.01 gm).

The isotope incorporation into protein was checked by first spotting 1 µl of the mixture onto filter paper squares (Whatman No 1) and incubating them in 10% TCA + 0.5% casein hydrolysate (0 °C, 10 min), 5% TCA (100 °C, 10 min), 10% TCA (0°C, 10 min) and washing twice in absolute ethanol. The radioactive filters were then counted in a scintillation counter (see section 2.6.2).

2.6.2 Scintillation Counting

The sample was placed in 6 ml of scintillation fluid (4.0 gm PPO + 0.8 gm POPOP in 1000 ml toluene) and counted in a LKB 1217 Rackbeta liquid scintillation counter. The channels used were 8-110 for \[^{35}\text{S}\] and 110-225 for \[^{32}\text{P}\]. Cherenkov counting for \[^{32}\text{P}\] used the 110-225 channel but no scintillation liquid was added to the sample.
2.6.3 Copy DNA Synthesis

The synthesis was carried out using the Gubler and Hoffman method (1983), with alterations enabling it to be carried out as a one-tube reaction. The poly (A)$^+$ RNA was boiled for 2 min, then placed on ice 5 min. The first strand reaction comprised 2.5 $\mu$l poly (A)$^+$ RNA (0.2 $\mu$g $\mu$l$^{-1}$), 1.0 $\mu$l RT-1 buffer (250mM Tris-HCl pH 8.3 + 50mM MgCl$_2$ + 6.25mM dA, dT, dGTP + dCTP), 0.5 $\mu$l 100mM DTT, 0.5 $\mu$l Oligo dT$_{14-18}$ (1mg ml$^{-1}$) and 0.5 $\mu$l (10U) of Reverse Transcriptase (New England Biolabs). This reaction was usually set up as three replica's, with a total of 1.5 $\mu$g initial poly (A)$^+$ RNA. A further tube was also set up containing 0.5 $\mu$l $^{32}$P-dCTP (0.37 MBq $\mu$l$^{-1}$) to monitor yield and to run on an acrylamide/urea gel. The tubes were incubated at 43 °C 1 hr, then set on ice.

The second strand reaction comprised of 20.0 $\mu$l RT-2 buffer (100mM Tris-HCl pH 7.5 + 25mM MgCl$_2$ + 50mM (NH$_4$)$_2$SO$_4$ + 500mM KCl + 0.2mM dA, dT, dG, dCTP), 2.0 $\mu$l BSA (BRL, 2.5mg $\mu$l$^{-1}$), 5.0 $\mu$l cDNA/RNA hybrid, 10U DNA Polymerase I (Pharmacia), 0.8U RNase H (Pharmacia), and H$_2$O to 100 $\mu$l. $^{32}$P-dCTP (0.5 $\mu$l) was also added to one of the tubes. The tubes were incubated at 12°C for 1 hr, followed by 22°C for 1 hr and 70°C for 10 min.

If cloning into Lambda vectors was to follow, 0.5 $\mu$l T4 polymerase (Pharmacia) was added to the tubes and these were incubated at 37 °C for 10 min to blunt end the cDNA. To stop the reaction EDTA pH 8.0 (1/10 vol) was added to the cDNA (whether blunt ended or not) and the reaction loaded onto a Sephadex G50 fine column in a Pasteur pipette, previously equilibrated in TE.1 (10mM Tris-HCl pH 7.5 + 0.1mM EDTA). The column was eluted with 100 $\mu$l 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 carried out by the addition of 1/10 vol 2M sodium acetate pH 5.5 and 2.5 vol ethanol.

2.6.4 cDNA Yield Estimation and Product Analysis

The yield for both first and second strand reactions was estimated by TCA precipitation. This involved determining the input radioactivity by spotting a 1 $\mu$l aliquot of the reaction onto a 2.5 cm GFC filter (Whatman) disc (T). At the same time a 1 $\mu$l aliquot was added to 0.5 ml
of 0.5mg ml\(^{-1}\) carrier DNA (Herring or salmon sperm DNA), 125 \(\mu\)l of 50% TCA was then added and the tube incubated on ice for 5 min. The precipitated DNA was then collected on a GFC filter (I) on a filter tower. The filter was washed with 2 x 5 ml of 10% TCA and 5 ml absolute ethanol. Both T and I GFC filters were then counted in a scintillation counter. The ratio between total and incorporated counts was then used to estimate the amount of DNA synthesised.

The cDNA products were analysed by running them on a 7 M urea, 5 \% acrylamide gel made in 0.5 X TBE (5.4 g Tris base + 2.75g boric acid + 2 ml 0.5M EDTA pH 8.0 in 1000 ml \(H_2O\)). An aliquot of the sample (5 x 10\(^4\) cpm) was 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 2 min. The gel was run in 0.5 X TBE at 45 mA until the xylene cyanol dye was 3/4 down the gel. The gel was then transferred onto 3 M Whatman filter paper backing and autoradiographed.

2.6.5 Cloning into Lambda Vectors

The Amersham 'cDNA cloning into gt 10' 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 cloned into Lambda-ZAP (Stratagene).

2.6.6 Ligation

The ligations were performed in 20 \(\mu\)l volumes, in a buffer containing 50mM Tris-HCl pH 7.8, 10mM MgCl\(_2\), 1mM DTT, 1mM ATP, 50\(\mu\)g ml\(^{-1}\) BSA (BRL), 10mM spermidine. If a circularisation of the DNA was required, the reaction contained 1pmol DNA ends ml\(^{-1}\); if recombination was to be favoured, the reaction contained 5 pmol DNA ends ml\(^{-1}\) (1\(\mu\)g of 1kbp linear DNA=3.0pmol ends). Sticky end ligation utilised 0.1 U ligase, blunt ended ligation 1 U ligase (Pharmacia).

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.

2.6.7 Infection of Cells with λ-Zap and Excision of pBluescript from λ-Zap

200μl of BB4 plating cells, 200μl of phage stock and 10μl (10^7 pfu) of R408 helper phage were combined in a 15 ml polypropylene centrifuge tube and incubated at 37°C 15 min. 5 ml of 2X YT media (16 gL^-1 tryptone, 10 gL^-1 yeast extract, 5 gL^-1 NaCl, pH 7.2 with 5M NaOH) added, and the cells incubated 4-6 hr at 37°C with constant shaking.

The tubes were heated at 70°C 20 min to neutralise the helper virus, spun 5 min 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 200 μl of BB4 plating cells to 100 μl of phage stock, incubating at 37°C 15 min, and plating on L-agar (see section 2.9.1)/ampicillin plates. Recombinant colonies were grown overnight at 37°C.

The BB4 cells were prepared by picking a colony from a master plate and growing overnight at 37°C in L-broth/tetracycline (12.5 μg/ml) supplemented with 0.2% maltose and 10 mM MgSO_4. 1 ml of overnight culture was then added to 50 ml of pre-warmed L broth + 0.4% maltose + 10 mM MgSO_4 and the cells incubated at 37°C with vigorous shaking until an O.D_600 = 0.5. The culture was cooled on ice, spun at 3K 10 min at 4°C, and the pellet resuspended in 15 ml of ice-cold 10mM MgSO_4. Aliquots of 150 μL were used for transfection.

An appropriate amount of phage mixture in phage dilution buffer (5.8g NaCl + 2g MgSO_4·7H_2O + 5 ml 2% gelatin per 1000 ml water) was added to 150 μl of competent cells and the mixture incubated at 37°C 15 min. Top agar (LB + 7% agarose + 10mM MgSO_4) was melted and placed at 42°C in a water bath. To each cell aliquot 4 ml of liquid top agar was added, mixed quickly by inversion and poured onto a dry 90mm Petri dish containing L-agar.
2.6.8 Oligo Labelling of DNA Fragments

This method uses very small amounts of DNA (10ng). 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 and Volgetstein, 1984).

The buffer was made out of three separate components: solution A (625 µl 2M Tris-HCl pH 8.0, 25 µl 5M MgCl₂, 350 µl H₂O, 18 µl 2-mercaptoethanol, 5 µl each 0.1 M dATP, dTTP, dGTP), solution B (2M HEPES pH 6.6) and solution C (hexadeoxyribonucleotides - Pharmacia 27.2166.01- suspended in 3mM Tris-HCl pH 7.0, 0.2mM EDTA at 90 OD units ml⁻¹). The solutions were mixed in the ratio 2:5:3, A:B:C.

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

The labelling mix had a final volume of 15 µl and contained 3 µl buffer, 6ng BSA, 10 ng DNA (volume known from weight of slice and size of band), 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 hr or more. It was stopped by the addition of 85 µl stop solution (20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA, 0.25% SDS) and boiled for 5 min prior to addition to the hybridisation solution. The method gives specific activities of about 10⁹ dpm µg⁻¹ of DNA.

2.6.9 Autoradiography

DNA and RNA dot blots were set up on Amersham film (Hyperfilm™-MP, RPN 6) with an intensifying screen at -80°C. The length of exposure depended on the intensity of the signal, but was usually 2 days. In vitro translation one and two dimensional gels were placed on high
performance autoradiography film (Amersham Hyperfilm™-MP, RPN 6) without an intensifying screen for two weeks.

2.6.10 Small Scale Plasmid Preparation

This is based on the method of Birnboim and Doly (1979). The cells containing the plasmids of interest were grown overnight in 3 ml L-broth with the relevant antibiotic. An aliquot (1.5 ml) of cultures were then transferred to Eppendorf tubes and spun 15 sec in a microfuge (13000 rpm). The liquid was poured off and the tubes placed on ice. A volume (100 μl) of solution A (10mM EDTA + 25mM Tris-HCl pH 8.0) was added, the pellets resuspended by vortexing, and 200 μl of solution B (27 ml H₂O + 2 ml 3M NaOH + 1.2 ml 25% SDS) added. The tubes were spun for 10 min in a microfuge (13000 rpm) and 430 μl of supernatant removed into a second Eppendorf tube and 1 ml ethanol added. The tubes were incubated at -70°C for 30 min and spun 10 min (13000 rpm). After drying the DNA pellet in a vacuum desiccator, the DNA was resuspended in 80 μl of TE. Restriction digests required 5-10 μl of DNA in a final reaction volume of 20 μl.

2.6.11 Restriction Digestion of DNA

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

2.7 Electrophoresis and Nucleic Acid Hybridisation

2.7.1 Agarose Gel Electrophoresis of DNA Fragments

Electrophoresis was carried out at 80 V for 2 hr or 18 V overnight in 1 X Elfo buffer (4.84gm Tris base, 2 ml 0.5M EDTA per 1000 ml dH₂O at pH 7.7 with glacial acetic acid + 5μg ml⁻¹ of ethidium bromide). The gels contained 0.6-2 % agarose made up in 1 X Elfo buffer (see above). Restricted DNA samples were mixed with a fifth volume of loading buffer( 25%
glycerol + 1% SDS + 0.01% bromophenol blue (Fisons) + 0.01% xylene cyanol (Fisons)), and electrophoresed at 80 V. DNA bands were visualised on a FOTO Dyne UV (300 nm) transilluminator and photographed with a Polaroid MP4 Land Camera through an orange Kodak "Wratten" filter.

2.7.2 Agarose Gel Electrophoresis of RNA

An RNA denaturing gel (agarose 2g in 73ml H₂O) cooled to 60°C [10ml 10X MOPS (2.09g Na-MOPS, 0.37g Na acetate, 0.18g Na₂EDTA at pH7.0 in 500ml H₂O) + 16.6ml formaldehyde] was poured in a fume hood. The denatured sample (RNA + 9 vols denaturing buffer(1M LiCl, 0.05M EDTA, 2% SDS, 0.01M Tris-HCl pH 6.5 + 1vol deionised DMSO), heated to 55°C for 15 min was loaded onto the gel, together with dye (2μl/lane, bromophenol blue:xylene cyanol, 1:1). The gel was run at 150mA for approximately 10 cm. The buffer used was 1X MOPS after running the gel was rinsed briefly in 20X SSC (30 min) prior to Northern blotting and illumination on UV transilluminator. The gel was placed on 2 sheets of pre-wetted (20X SSC) Whatman 3MM filter paper on a blotting sponge partially immersed in 20X SSC, overlayed with Hybond-N (Amersham) pre-soaked in 3X SSC, followed by Whatman 3MM paper and a stack of paper towels weighed down with a glass plate, ensuring that all air bubbles were excluded. After blotting overnight, the filter was rinsed briefly in 3X SSC and baked for 10 min at 80°C prior to UV cross-linking for 45 sec on a UV transilluminator. Prehybridisation/hybridisation was carried out as section 2.7.6.

2.7.3 Preparation of DNA Dot Blots

The method used was from the Hybond-N product hand book (Amersham). The DNA samples were heated to 95°C for 5 min then chilled on ice for an additional 5 min. An equal volume of 20 X SSC (3M NaCl + 0.3M Na₃ citrate) was added to the DNA samples. The DNA solution was spotted onto Hybond-N membrane prewetted in 10 X SSC in 2 μl aliquots. The spots were allowed to dry between the addition of each aliquot of DNA in order to keep the spots as small as possible. A positive reconstruction was prepared as above with the number of copies based on the DNA content for a haploid genome of Asparagus (1.2pg, Paul et al.,
The dry membrane was placed DNA side up on a filter pad moistened in denaturing solution (1.5M NaCl + 0.5M NaOH) for 5 min. The membrane was then placed DNA side up onto a filter pad moistened in neutralising solution (1.5M NaCl + 0.5M Tris-HCl pH 7.2 + 0.001M EDTA) for 1 min. The membrane was then blotted dry and placed DNA side down onto a UV transilluminator for 45 sec to crosslink the DNA onto the membrane. The prepared filter was then hybridised with the relevant probe (see section 2.7.4).

### 2.7.4 Preparation of RNA Dot Blots

The RNA (total and poly (A)+) was added to 3 volumes of RNA denaturing buffer [formamide (deionised) 500 µl + formaldehyde (37% soln) 162 µl + 10 X MOPS (0.2 M 3-[N-Morpolino]propane-sulphonic acid + 0.05M Na acetate pH 7.0 + 0.01M Na₂ EDTA) 100 µl and warmed to 65°C for 5 min. The denatured RNA was chilled on ice and 1 volume of cold 20 X SSC added. The RNA was spotted onto Hybond-N membranes (prewetted in 10 X SSC) in 2 µl aliquots, the spots allowed to dry prior to addition of further aliquots of RNA. The membranes were dried at 80°C in an oven for 10 min, the membranes were placed RNA side down on a UV transilluminator and exposed for 45 sec. The membranes were then pre/hybridised with the appropriate probe (section 2.7.6).

### 2.7.5 DNA Pre/Hybridisation

The DNA filter was prehybridised in a 140 mm Petri dish (Sterilin) with 25 ml DNA pre/hybridisation solution [5 X SSPE (3.6M NaCl + 0.2M Na phosphate + 0.02M EDTA pH 7.7), 5 X Denhardtts solution (from 100 X stock, 2% [w/v] BSA (BRL) + 2 % [w/v] Ficoll™ (Pharmacia) + 2% [w/v] PVP [polyvinyl pyrolidone], 0.5% SDS, 0.5 ml 1mg ml⁻¹ sheared herring sperm DNA (boiled for 5 min and added to pre/hybridisation solution prior to filter addition)] in a water bath at 65°C for 1-2 hr. The radioactive probe (prepared as in section 2.6.6) was then added to the hybridisation mix through a hole in the top of the petri dish which was then covered with radioactive tape. The filter was allowed to hybridise for at least 12 hr. The radioactive hybridisation solution was poured down a radioactive disposal sink followed by copious amounts of water. The filters were transferred to a polypropylene
sandwich box, and rinsed 3 times with wash solution A (2 X SSC + 0.1% SDS) at 65°C. The filters were then washed 2 times in wash solution A for 30 min at 65°C. The filters were washed stringently in wash solution B (0.1 X SSC + 0.1% SDS) for 15 min at 65°C, the amount of radioactivity stripped off monitored using a Geiger counter. When no more radioactivity was stripped off the filters the washing was stopped (normally 4-5 washes). The wet filters were wrapped in 'Saranwrap' and placed onto X-ray film (as section 2.6.7).

2.7.6 RNA Pre/Hybridisation

The RNA filters were prehybridised in RNA pre/hybridisation buffer [50% deionised formamide + 6 X SSPE (section 2.7.5) + 5 X Denhardts (section 2.7.5) + 0.1% SDS + 100μg ml⁻¹ denatured Salmon sperm DNA + 6% PEG (6000-8000 mwt, polyethylene glycol)] for 1-2 hr at 42°C. Prehybridisation solution was poured off until the remaining solution just covered the blot. The probe (see section 2.6.6 for probe construction) was then added after boiling for 10 min at the concentration of 1-2ng ml⁻¹. The hybridisation was allowed to proceed for at least 12 hr at 42°C before the hybridisation solution was poured away and the filters washed twice with wash solution A (see section 2.7.5) at room temperature. The filter was then washed twice in prewarmed wash solution A for 20 min at 42°C. The filters were washed stringently in prewarmed wash solution B (see section 2.7.5) for 15 min at 42°C. This was repeated until no more radioactivity is stripped of the filters. The filters were then wrapped in 'Saranwrap' and placed down on film (see section 2.6.7).

2.7.7 Plaque Hybridisation

Phage was plated out, using BB4 plating cells, at an appropriate dilution (400pfu/90 mm Petri dish, 1500pfu/140 mm Petri dish) onto L-plates using top agarose (6.5g/L agar). The plates were incubated at 37°C overnight and then placed at 4°C 1hr.

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.
The filters were laid plaque side up on 3MM Whatman filter paper pre-wetted in denaturing solution (0.5M tris-HCl pH 7.0, 1.5M NaCl) 5 min, and 2X SSC 5 min. The filters were then air dried, baked at 80°C 1 hr, and used in filter hybridisations.

Prehybridisation solution consisted of the following: 50% formamide, 6X SSC, 25mM Na phosphate buffer pH 6.5, 100 μg/ml sheared denatured salmon sperm DNA, 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 3 hr, the solution poured off, and the hybridisation solution added. Hybridisation was at 42°C for 12-18 hr in a slow shaking incubator. The filters were then washed at 65°C with 2X SSC, 0.1% SDS twice for 15 min, and with 0.2X SSC, 0.2% SDS twice for 30 min, before exposure to X-ray film.

2.7.8 Probe Removal from Hybond-N™ Filters

The filter membranes (DNA and RNA) were washed in probe stripper solution (0.005M Tris-HCl pH 8.0 + 0.002M Na₂EDTA + 0.1% Denhardts solution) for 1-2 hr at 65°C until all radioactivity was removed from the filters. For optimum results the filter must never be allowed to dry out before probe stripping.

2.7.9 Polyacrylamide Gel Electrophoresis

The method used is as Laemmli (1970) and consisted of a stacking and a resolving portion. Both portions were made out of the relevant amounts of acrylamide/bisacrylamide (30:0.8% w/v) stock, 250mM Tris-HCl, 0.1% w/v ammonium persulphate and 0.1% SDS. Both gels were polymerised by the addition of TEMED (N’ N’ N’-tetramethylethylenediamine). The resolving gel was either a 12% acrylamide or a 10% acrylamide gel made up with Tris-HCl pH 8.8. The stacking gel was poured so that it extended 2 cm below the wells and was made up with Tris-HCl 6.8 and run at 125 V overnight on a vertical electrophoresis apparatus. The reservoir buffer was a glycine buffer (14.4gm glycine + 1.0gm SDS + 3.0gm Tris base in 1000 ml H₂O at pH 8.3).
2.7.10 Isoelectric Focusing Gels and Two Dimensional Gel Electrophoresis

The method used was a minor modification of O’Farrel (1975) equilibrium isoelectric focusing protocol. The following components were mixed in a Buchner flask and degassed for 1 hr on a bench vacuum line: Urea (Aristar grade) BDH, Poole, U.K., 3.4gm, acrylamide and bisacrylamide (ultra pure, BRL, Bethesda, 28.38:1.62 % w/v) 823 ml, "Nonidet" NP-40 (ICI, deionised, 10% v/v) 1.237 ml, double distilled water, 1.10 ml. This volume provided enough gel mix to pour ten gels.

After 1 hr, the remaining components of the gel mix were added: Ampholines pH 3-10 (BDH PLC, Poole, U.K.) 416.5 ml, ammonium persulphate (10%) 12.6 ml, TEMED 18.55 ml. The mixture was taken up in a 2 ml disposable syringe, a large (1 x 150 mm) needle was used to inject the mixture carefully into the glass rods up to 10 cm depth (13 cm glass rods with a bore of 1.5 mm). The gels were overlaid with 10 μl of 8M Urea with a Hamilton syringe, and left 1 hr to set. The overlay was then removed and replaced with 20 μl of 2DMH + 9M urea followed by 10 μl H₂O. The gels were left for a further 1-2 hr. The overlay was then removed and replaced with 20 μl 2 DMH + 9M urea containing coloured markers (congo red 0.1gm ml⁻¹, fast green 0.1gm ml⁻¹). The gel rods were fitted into the gel tank and top (0.02 M NaOH, degassed 4 hr) and bottom (0.01 M phosphoric acid) running buffers added. The gel tank was attached to a cooling water bath at 10°C. The gels were prefocused at 200 V for 15 min, 300 V for 30 min, 400 V for 30 min. After this prefocusing period the green coloured dye (fast green) should run half way down the gel and the red dye (congo red) should have just entered the gel. The top buffer and any solution overlaying the gel was discarded, the samples introduced, and overlaid with 5 μl 2 DMH. Fresh top buffer was then placed in the gel tank. The gel was run at 400 V for 12 hr, followed by 1500 V for 1 hr.

The gels were now removed from the rods (gels were removed by ringing and injecting the rim of the gel with water) equilibrated 2 hr in SDS sample buffer (75 mM Tris + 10% glycerol + 2.5% SDS + 5% 2-mercaptoethanol + 0.01% bromophenol blue (Fisons)) and stored at -70°C until further use. The second dimension was run on a standard PAGE gel (see section 2.7.7).
2.7.11 Protein Staining in PAGE and Equilibration of Radioactive Gels

Gels were soaked in Comassie solution (25% methanol + 10% glacial acetic acid + 100mg/l Comassie Brilliant Blue R-250 (Serva)) at 37°C for 30 min with gentle agitation. 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.

With radioactive gels the same procedure as above was observed except that destaining was carried out at 37°C for 5 hr with the destain solution replaced every hr. The gels were incubated with Amplify solution (Amersham) for 20 min prior to being dried on an LKB gel drier prior to autoradiography (Section 2.6.8).

2.7.12 Western Blotting

The method involved the use of a wet blotter from a Bio Rad "Protean" minigel kit. The transfer buffer consisted of 25mM Tris, 192mM glycine and 20% methanol. All components of the transfer "sandwich" were soaked in the buffer prior to assembly. 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.

```
.InputStreamReader Anode (+) [Grey]
$\text{Scotchbrite pad}$
# 3 mm filter paper
+++ Nitrocellulose
* Gel
# 3 mm filter paper
$\text{Scotchbrite pad}$
```

The electrodes were connected to a power supply and run at a constant current of 300 mA.
for 2 hr. After transfer the blot was transiently stained for protein using 0.2% Ponceau S in 3% TCA, for 5 min, followed by washing in water.

2.7.13 Immunoblotting

The method used alkaline phosphatase-conjugated secondary antibody. A Western blot was blocked with TBS-Tween (50mM Tris-HCl pH 7.4 + 192mM glycine + 0.1% Tween 20) containing 3% Marvel (Cadbury-§KHEEPLC) at 37°C for 30 min. It was then incubated with the primary antibody at the correct dilution, in a sealed bag for 1 hr at room temperature. If it was a high stringency Western blot, the appropriate dilution of antibody was added to TBS + 1M glucose, 10% v/v glycerol and 10% Marvel. A low stringency Western blot had the primary antibody incubated with the blot with TBS and 3% Marvel. Washes were with TBS-Tween for 10 min each. Three washes were generally used. A 1:500 dilution of alkaline phosphatase-conjugated antibody diluted in TBS with 3% Marvel was then used to incubate the blot with for 1 hr at room temperature in a sealed bag. Three 10 min washes of TBS-Tween then followed. The blot was then equilibrated (5 min) in substrate buffer (100mM Tris-HCl pH 9.5 + 100mM NaCl + 5mM MgCl), and then developed. The developing solution contained BCIP (5-bromo-4-chloro-3-indoyl-phosphate, 50mg ml⁻¹ in dimethylformamide) at 16.5 µl/5 ml substrate buffer.
2.8 Vectors and Host Strains

\( \lambda \)-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 except 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 ColE1 derivative that carries a gene for ampicillin resistance, an inducible lac promoter upstream of the N-terminal coding region of \( \beta \)-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 \( \beta \)-galactosidase. The insertion of the T7 promoter/multiple cloning site/T3 promoter sequence, which does not interfere with \( \alpha \)-complementation, is located in the proper reading frame within the N-terminal coding region of the \( \beta \)-galactosidase gene.

BB4

An E. coli strain:
Genotype supF58, supE44, hsdR514(\( \kappa_k \), \( \kappa_m \)), gaiK2, gaiT22, trp55, metB1, tonA, \( \lambda \)-, \( \Delta \)(arg-lac)U169 [F', proAB, lacI\(^{\beta} \)Z\( \Delta \)M15, Tn10 (tet\(^R \)]).
2.9 Bacterial Media

2.9.1 Luria Broth (LB)

Bacto tryptone at \(10.0\text{gL}^{-1}\)
Bacto Yeast extract \(5.0\text{gL}^{-1}\)
NaCl \(10.0\text{gL}^{-1}\)

pH 7.5 with NaOH
L agar plates contain 1.5% agar
L soft top contains 0.7% agarose

2.9.2 2X YT Broth

Bacto tryptone \(16\text{gL}^{-1}\)
Yeast extract \(10\text{gL}^{-1}\)
NaCl \(10\text{gL}^{-1}\)

pH 7.2-7.4 with NaOH
Chapter 3 Growth of Mechanically Isolated Asparagus Mesophyll Cells in Culture

3.1 Introduction

3.1.1 General Introduction

With the global increase in demand for crops, there has been a great interest in developing genetic engineering systems for crop plants. Transformed plants are often regenerated from undifferentiated tissues especially in monocots which are not susceptible to Agrobacterium infection, and thus the transformation of protoplasts offers the most effective route for gene transfer. In order for such an approach to work the progenitor tissue has to be totipotent. Totipotency is when a single somatic cell can be induced to produce a whole plant. Plants have a remarkable degree of flexibility over the control of their development. This is especially apparent in tissue culture as numerous metabolic pathways and developmental pathways can be induced. As has been mentioned in the introduction (chapter 1) a developmental pathway called dedifferentiation is often the first step in the expression of totipotency. This pathway represents a transition pathway from a differentiated state to a less differentiated state, and results in the formation of dividing callus cells from differentiated, cell cycle arrested cells. Callus cells are characterised by large vacuoles and an amorphous shape. In plant development dividing cells are found within meristems and in wound-induced callus.

Therefore dedifferentiation appears to be a biphasic response, involving a reactivation of the cell cycle and a change in the morphology and physiology of the subsequently formed cells. There is little known about dedifferentiation at the molecular level, although many morphological changes, physiological and biochemical changes that are induced by dedifferentiation have been described. Since dedifferentiation represents an important plant developmental pathway which is exploited in tissue culture, somaclonal variation, plant transformation and is responsible for wound healing in plants, an examination of this pathway is worthwhile to give a better understanding of plant plastic development.
3.1.2 Methods Used to Examine Dedifferentiation

For molecular analysis to be possible large quantities of physiologically and biochemically similar cells have to be available. As has been mentioned in chapter 1 this is only possible with in vitro systems. Four different in vitro approaches have been used to examine plant developmental pathways. One approach has been to use suspension cultured cells to study development. Such systems have been used to examine the cell cycle (Kodama et al., 1989), secondary metabolism (Fujita et al., 1983) and cell wall synthesis (Ammino and Komamine, 1985). Molecular analyses have also been performed on suspension cells, for example the study of the effects of fungal elicitors on plant cell gene expression (Tempelton and Lamb, 1988). This system is not suitable for use in the examination of dedifferentiation as the cells are already dedifferentiated and are dividing actively. Furthermore, as suspension cultures represent a mixed population of cell types, synchronising agents are required to ensure that minor differences in gene expression can be detected.

Jerusalem artichoke tuber slices have been used to examine the cell cycle and the biochemistry of DNA synthesis. However, only a few cell layers respond to wounding and the culture medium by exhibiting synchronous cell divisions (Harland, Jackson and Yeoman, 1973). An additional problem with this system besides the lack of sufficient responding tissue, is that there are seasonal variations in RNA metabolism of tuber tissue in response to excision and culture (MacLeod et al., 1979). This will make an accurate assessment of gene expression during the reactivation of the cell cycle difficult.

Explanted maize embryos have been used to examine wound-induced dedifferentiation at an ultrastructural level. Despite only a small cell layer responding to excision and growth regulator stimuli it was possible to perform electron microscopy on this material; there was however, insufficient material for molecular analysis. Such studies have provided some valuable information concerning the ultrastructural changes during dedifferentiation. A cytological study of callus initiation on maize embryos has demonstrated that dedifferentiation can be separated into two distinct phases 1 and 2 (Fransz and Schel, 1987). Phase 1 takes a day to complete, and during this phase a change in nucleolar morphology, an increase in vacuolation, a change in cell wall shape and an increase in certain organelle numbers was
observed (Fransz and Schel, 1987). Phase 2 is characterised by a recovery of mitotic activity (Fransz and Schel, 1987).

Protoplasts have been used to examine gene expression during cell cycle reactivation, DNA synthesis and response to cytokinin and auxin (Meyer and Chartier, 1981; Meyer et al., 1984 A & B). The lipid composition and isoperoxidase profiles changed during dedifferentiation of protoplasts derived from mung bean hypocotyls (Goldberg et al., 1986). The synthesis of rubisco decreased in dedifferentiating protoplasts of tobacco (Fleck et al., 1980). However, as mentioned in chapter 1 protoplasts are physiologically unnatural and have altered gene expression induced by their hypertonic culture medium. Osmotic shock "stress proteins" have been demonstrated in protoplasts of Nicotiana sylvestris where they represent the major component in altered gene expression of freshly isolated protoplasts (Fleck et al., 1982). This phenomenon, together with the lack of the cell wall and the inability to culture large quantities of material precludes this system from being used to examine dedifferentiation at the molecular level.

Mesophyll cells can also be isolated from some plant species mechanically. That is mechanical shearing forces can be used to disrupt plasmodesmatal contacts and cell adhesion components in the middle lamella with minimum damage to the cells. In this manner unlimited numbers of physiologically and morphologically similar cells can be isolated with high viability from certain species. Furthermore, methods exist for the large scale tissue culture of mechanically isolated cells. Mechanically isolated cells offer advantages over protoplasts in that the isolated cells have intact cell walls, are physiologically normal and may be cultured in hypotonic or isotonic media. "Osmotic shock" proteins and proteins involved in the extracellular secretion and synthesis of the reforming cell wall are not expected to contribute significantly to protein synthesis in mechanically isolated cells. Mechanically isolated cells have also been extensively used to examine various physiological phenomena such as photosynthesis (Hills, 1986) and amino acid metabolism (Jullien and Guern, 1979). Cytodifferentiation including some molecular aspects has been examined in detail with mechanically isolated Zinnia elegans mesophyll cells (reviewed by Fukuda and Komamine, 1985).
3.1.3 Dedifferentiation

Dedifferentiation was defined in chapter 1 and is a term which is often used loosely without any understanding of the process. However, various studies on different experimental systems have provided an insight on some processes involved in dedifferentiation. The following is a summary of dedifferentiation. There have been numerous studies discussing aberrant chromosomes formed during wound-induced dedifferentiation in both in vitro and in vivo systems. In *Vicia faba*, root decapitation-induced dedifferentiation results in extra DNA synthesis over normal levels, which is required for mitosis to occur (Cionini et al., 1985). Caryological behaviour during the first phases of dedifferentiation and habituation of *Nicotiana bigelovii* hypocotyls was examined by Bennici and Caffaro (1985) and indicated that a high degree of aneuploidy occurred. DNA copy number has also been observed to change during the formation of rice callus (Kikuchi et al., 1987). All this evidence suggests that the control of DNA replication during dedifferentiation may be different and maybe not as precise as DNA replication in meristems. This type of phenomenon is similar to that observed in animal tumours. The cause of a lot of these tumours is not known though viruses have been implicated with several.

In mimicking wound-induced dedifferentiation in culture the culture environment will contribute to some changes. For instance since there is sucrose in the medium the cells will switch from photoautotrophic to become heterotrophic; therefore changes are expected to occur in organelle activity and numbers. The cell wall composition of cultured cells is different from cell walls of the intact plant (Blaschek et al., 1981). The enzymes involved in the synthesis of such cell wall components will change, there may be alterations in the elasticity of the cell wall and therefore cell shape. It has been established that the isoperoxidase and phospholipid profiles change with dedifferentiation (Goldberg et al., 1986). However, this was examined in a protoplast system where cell wall regeneration occurs, so it is not known if this phenomenon will occur in mechanically isolated cells. From the above studies it can be concluded that dedifferentiation can result in morphological, physiological and biochemical changes. Therefore any good system for the examination of dedifferentiation should be amenable for cytological, physiological, biochemical and molecular genetic analysis.
3.1.4 The Mechanically Isolated Cell System

There are numerous species of plant that are amenable for mechanical isolation of mesophyll cells and subsequent tissue culture. Three species of plant which were initially tested for their large scale culture characteristics were *Asparagus officinalis*, *Arachis hypogea* and *Calystegia sepium*. Of the three asparagus showed the most promise as a large scale dedifferentiating cultured cell system.

*Asparagus officinalis* L. is a dioecious species with a sex ratio of 1:1; however, male plants have flower types that vary from staminate to hermaphroditic (Reuther, 1984). The variety used in this thesis is diploid, though it is possible to obtain tetraploid plants. It is possible to obtain callus from asparagus explants, protoplasts and mechanically isolated cells. The early culture methods for mechanically isolated asparagus cells used modified Murashige and Skoog media with auxin elements, NAA and BAP (Jullien, 1973). Survival and mitotic activity depended on the concentration of cells. A high plating density (3.5 X 10^5 cells/ml) allowed mitosis in up to 40% of the living cells that develop to very small colonies with approximately 10 cells in each aggregate (Reuther, 1984). With a reduction in the plating density to 5 X 10^4 cell/ml the percentage division decreased to 5% but the residual colonies increased in size (Reuther, 1984). Mechanically isolated *Asparagus officinalis* mesophyll cells have been cultured in large numbers (up to 5 X 10^6 cells) in 25 ml of culture medium in shake culture (Jullien and Guern, 1979). Jullien and Guern (1979) reported that up to 250 X 10^6 cells could be isolated mechanically with 60 % viability. It has also been observed by Jullien and Guern (1979) that survival and mitotic activity of the cells could be increased if mineral nitrogen in the media was replaced with glutamine (30 mM). This phenomenon was attributed to the lack of nitrate reductase in asparagus cladophylls (Jullien and Guern, 1979; Reuther, 1984). However, Jullien and Guern (1979) observed that the first cell division was asynchronous and that the time taken before cell division was dependent on glutamine concentration. Furthermore, organogenesis of callus derived from mechanically isolated cells and protoplasts has been achieved (Bui Dang Ha et al., 1975; Draper, personal communication). Taking these aspects into consideration it would appear that asparagus is a good candidate for a model cell culture system as it satisfies most criteria except strict synchrony of cell division. In addition, since
it is possible to genetically transform asparagus with *Agrobacterium tumefaciens* (Bytebier et al., 1987) further molecular analysis with different gene constructs could be examined in the future during dedifferentiation in transgenic asparagus. However, the system needs to be optimised to improve synchrony and numbers of dividing cells, before a molecular analysis of dedifferentiation can be successfully undertaken.

### 3.1.5 Which Parameters Can Be Examined Feasibly During Dedifferentiation of Mechanically isolated Cells?

Mechanically isolated asparagus cells have been examined physiologically (see chapter 4) and cytologically (Jullien, 1973), though a molecular analysis has not been carried out. Therefore it was assumed that it would be relatively simple to perform a cytological study of dedifferentiating cells and to analyse metabolic and molecular aspects of photosynthesis during cellular dedifferentiation. By these experiments it was hoped to be able to identify markers for various stages of dedifferentiation. The asparagus cell system was described by Jullien and Guern (1979) as exhibiting a limited degree of synchrony, so the probability of identifying low abundance transcripts would be low. For this reason markers had to be identified for dedifferentiation so that only populations of cells that were actively undergoing dedifferentiation would be used for molecular analysis. If synchrony could be improved, the probability of obtaining a population of cells, with a high proportion at the same stage of dedifferentiation would be increased. Markers would enable an assessment of dedifferentiation synchrony to be made and comparisons between individual cell cultures.

For these reasons the tissue culture system had to be optimised to increase reproducibility and synchrony. In order to do this the growth conditions of the donor plants had to be optimised. This would enable more uniform optimally grown plants to be used for cell cultures. Once the tissue culture system is optimised only then can an assessment of the system’s suitability for molecular analysis be made. This chapter is mainly concerned with attempting to resolve the suitability of the system for molecular analysis.
3.1.6 Aims of the Study

One main aim of the study was to optimise the tissue culture system to minimise the effects of a lack of synchrony and to develop routine methods for the determination of specific stages in cellular dedifferentiation, prior to the molecular analysis of cells. The ideal cells in culture would have high synchrony of cell expansion and cell division. Observations on and measurements of cell expansion, organelle movement, DNA synthesis, nuclear division and phragmoplast formation should provide useful parameters for defining phases of cell dedifferentiation. Techniques to monitor these processes should be quick, simple and non-invasive and therefore initial observations were made on large populations of cells in Petri dishes using a Nikon inverted microscope. Rapid fluorescent staining techniques were used for more detailed observations on cellular dedifferentiation. These could be applied to unfixed material allowing observations and measurements to be made immediately, prior to harvesting at particular phases in the dedifferentiation process.

Once such an examination was made, methods for macromolecule extraction and purification was optimised for the system. Approximate yields of extracted RNA were determined so that the feasibility of the system for an in vitro translation study of steady state transcripts and for the synthesis of complementary DNA (cDNA) libraries could be tested.

3.2 Results

3.2.1 Growth of Plant Material

A series of experiments were initiated to determine the optimum growing conditions to produce healthy plants that were amenable to both cell isolation, tissue culture and RNA isolation. The different growing conditions tested consisted of germinating seeds in a controlled environment cabinet at 27°C, then transferring seedlings after 2 weeks to different growth chambers. These conditions were as follows, a Fisons cabinet set at 24°C, a growth room set at 20°C and in a glass house with the temperature fluctuating between 18-24°C. Even though the plants from some of the conditions tested appeared to be morphologically similar, the behaviour of cells isolated from such plants in culture were different. For example, it was observed that cells from glasshouse-grown plants had a longer lag period prior to the
first cell division than cells obtained from plants grown in the growth room or Fisons cabinet. It was observed that the best quality plants giving rise to the most reliable cell cultures were consistently obtained from plants grown in a Fisons cabinet.

### 3.2.2 Determination of the Optimum Age of Cladodes for Cell Isolation and Culture

The optimum cladode age required to obtain the highest cell viability and subsequent cell division needed to be determined. Jullien (1972) used cladodes from plants after flowering for cell isolation. At Leicester there were no mature plants available and since it takes a long period of time for plants to reach maturity for flowering to occur, younger material had to be used. It was observed that material younger than 5 weeks after sowing yielded populations of cells with low viability, and therefore not amenable for use in a large scale culture system. Cells isolated from donor plants 5-6.5 weeks after sowing had very high viabilities (60-70%) and high rates of cell division. However, age is not a satisfactory criterion for determining when plants are at the right stage to provide useful material for cell cultures. The main reason for this factor is because the growth rate of plants tend to vary with the seed batch which effects the germination time. It was decided that the best criterion for the stage to obtain optimum cell viability, was morphological, i.e. plants which were dark green and had very fine fronds without side branches tended to produce the best cells. This normally coincided with an age of between 5-6.5 weeks after sowing with plants grown in a Fisons cabinet.

### 3.2.3 Scale up of Cell Isolation Procedure and the Optimisation of the Tissue Culture System

The published method for asparagus cell isolation for tissue culture (Jullien, 1973) used glass homogenisers which could not be scaled up to cope with the requirement for large numbers of cells. Secondly, the plating densities used by Jullien (1973) were relatively low \((2 \times 10^5)\) and large numbers of plates would have be used to obtain the required cells for molecular analysis.

A method using a plastic card (scraper method) to isolate cells had been used with great efficiency to isolate cells from asparagus for photosynthetic experiments (Foyer et al., 1982;
This method was modified to maintain sterility (see section 2.2.1) and cultured cells isolated using this method were compared to cells isolated by use of glass homogenisers. Cladodes were stripped off sterile fronds using sterile gloves, and cells were scraped off the cladodes with the plastic card (see section 2.2.1). This process was extremely quick and easy to perform. It was also observed that the percentage viability of extracted cells was comparable with either method, but higher numbers of viable cells per gram fresh weight were isolated using the scraper method. For this reason the scraper method was adopted and made it possible to obtain unlimited quantities of cells aseptically.

Jullien and Guern (1979) used 250 ml Erlenmeyer flasks as culture vessels, but this made direct observations of the cells difficult. An experiment was set up to evaluate which culture vessels would contribute to high cell division frequencies and facilitate rapid harvesting. The culture vessels tested included glass crystallising dishes, Erlenmeyer flasks, glass Petri dishes and disposable plastic Petri dishes. In general it was observed that up to 30 % of cells tended to adhere to the bottom of the culture dishes and consequently were lost at harvesting. Therefore an additional parameter was included into the experiment evaluating culture vessels, by testing the effects of different types of substratum on cell cultures. The different substratum tested included an agar underlay, siliconised glass, nitrocellulose underlay, cellulose underlay and plastic Petri dishes without substratum. The results from this experiment are summarised in Table. 3.1. It is quite clear from Table. 3.1 that the conditions giving the best cell growth and easy harvesting were plastic Petri dishes without substratum.

A cell harvesting method was developed which involved filtering the cell culture supernatant onto Whatman No. 1 filter paper on a Buchner funnel. The cells that were stuck down to the plastic Petri dish were also removed by using a "scraper" made from a glass pipette, and were added to the culture filtrate. The whole process took less than 2 minutes to complete per culture dish.

Additional experiments on the alteration of culture conditions were initiated to optimise the asparagus culture system. Two conditions which were tested out included the effect of agitation and plating density. A plating density of $1 \times 10^5$ cells/ml was used routinely in experiments to examine the effects of agitation on cell growth at 25 °C. A rotary shaker was
Table 3.1 Summary of asparagus cell vessel and substratum experiment.
**Summary of asparagus cell vessel and substratum experiment**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells growing in 9 cm dishes in liquid media over solid asparagus medium</td>
<td>Considerable clumping of cells compared to control</td>
</tr>
<tr>
<td>As above-except with purified grade agar</td>
<td>Less clumping than above but less than ideal</td>
</tr>
<tr>
<td>Control: cells grown in 9 cm Petri dishes in liquid asparagus medium</td>
<td>Cells expanded and divided (50%) by day 5 of culture. Some cell adhesion to bottom of Petri dish</td>
</tr>
<tr>
<td>Siliconised crystallising dishes</td>
<td>Cell adhesion to culture dish still observed with 20% less expansion and division than control</td>
</tr>
<tr>
<td>Siliconised crystallising dishes with nitrocellulose underlay</td>
<td>Cells died</td>
</tr>
<tr>
<td>Crystallising dishes with cellulose underlay</td>
<td>Cells grew very slowly</td>
</tr>
<tr>
<td>Crystallising dishes without underlay</td>
<td>Cell division &amp; expansion less than controls</td>
</tr>
<tr>
<td>Crystallising dishes with coverslip underlay</td>
<td>No cell adhesion to glass coverslips &amp; cell growth as above</td>
</tr>
</tbody>
</table>
used to agitate the cultures at 36 rpm. It was observed that the percentage cell expansion compared to inviable cells was 40.9% in control non-agitated cultures and 64.96% in agitated cultures.

Since agitation made it possible to obtain superior growth rates, an experiment was initiated to investigate the effects of a higher plating density than $1 \times 10^5$ on cell growth. The plating densities tested were $1 \times 10^5$, $2 \times 10^5$, $3 \times 10^5$, $4 \times 10^5$, and $8 \times 10^5$. If tolerated, higher plating densities would be extremely useful, as it would speed up plating out and harvesting of cultures, as less culture plates would be required. Cells from the same donor plants were used in all plating density experiments and all cultures were incubated under identical conditions (25 °C in the dark on a rotating platform set at 36 rpm). The cultures were examined cytologically (see chapter 2) after 7 days, when cell divisions normally first occur at the control ($1 \times 10^5$) cell density. It was observed that the plating density of $4 \times 10^5$ yielded the best compromise between growth rate (see Table 3.2) and the ability to observe single cells under an inverted microscope (see chapter 2). The highest plating density ($8 \times 10^5$) was discounted as the cell suspension was so dense that it was impossible to make accurate measurements on cell division and expansion of individual cells. It was also observed that with all cultures, cell aggregation occurred initially and within 3-4 days in culture all the cells had separated and were evenly distributed. This made microscopical examinations easy to make and plating densities of $4 \times 10^5$ were used routinely in all further work presented in this thesis.

3.2.4 Morphological Observations

Markers for dedifferentiation had to be identified, so that highly synchronous dedifferentiating cells could be harvested, pooled and used for molecular analysis. The morphology of asparagus cell cultures, after the initial homogenisation, through to the end of the first divisions, are shown in Fig. 3.1. Populations of freshly isolated cells (day 0) contain 50 to 70% viable cells, as determined from the appearance of cell contents, which contained intact chloroplasts and clear cytoplasm (Fig. 3.1 A). Initial observations made on the morphology of cultured cells identified two major distinct criteria that could be used to stage the cultures.
Table 3.2 Effects of cell plating density on cell division after 7 days in culture. Cultures were incubated at 25°C in the dark on a rotating platform set at 36 rpm.
Effect of cell plating density on cell division after 7 days in culture

<table>
<thead>
<tr>
<th>PLATING DENSITY</th>
<th>EXPANDING CELLS (% OF TOTAL CELLS)</th>
<th>DIVIDING CELLS (% OF TOTAL CELLS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 X 10^5</td>
<td>66.25%</td>
<td>36.2%</td>
</tr>
<tr>
<td>2 X 10^5</td>
<td>83.1%</td>
<td>55.4%</td>
</tr>
<tr>
<td>3 X 10^5</td>
<td>79.6%</td>
<td>46.3%</td>
</tr>
<tr>
<td>4 X 10^5</td>
<td>84.6%</td>
<td>61.9%</td>
</tr>
<tr>
<td>8 X 10^5</td>
<td>observations difficult to make</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Development of cell cultures. (A) Freshly isolated cells on day 0. (B) Expanding cells on day 3. (C) Divided, expanded cells on day 5. Scale bars 10 μm, X 300. (D) Finely dispersed cells in Petri dish following isolation. (E) Aggregated cell culture on day 2.
The criteria were cell expansion, based on the increase in cell size compared to inviable cells, and cell division, based on when the phragmoplast has completely bisected the cell. Stains such as tinopal and acridine orange (see chapter 2) were used as an aid to visualise fully formed phragmoplasts and nuclei respectively. More refined analysis of cell expansion was carried out by measuring cell area using a Zeiss drawing tube and an Apple microcomputer digitising pad (see chapter 2). Identical results on the timing of cell expansion was obtained by both the microcomputer method and the by visual comparisons.

Little change in morphology of unstained cells could be seen between day 0 (freshly plated out cells, 3 hr after mechanical isolation) and day 3 (Fig. 3.1 B) and there was a little increase in cell size (Fig. 3.2). Some cell expansion occurred from day 3 to 4 but the large increase in cell size, to form typical rounded, cultured cells, did not occur until after day 4 (Fig. 3.2). The increase in cell size was associated with an increase in size of vacuole, so that by day 6 the cytoplasm was in a thin layer at the periphery, with cytoplasmic strands crossing the vacuole (Fig. 3.3 D). At the macroscopic level it was observed that a proportion (35-40 %) of cells become attached to the bottom of the culture dish. The free-floating cells were usually aggregated for the first few days and then disaggregate at the same time as rapid cell expansion was initiated (Fig. 3.1 D & E). It was also observed after 2 days that an exudate is secreted from the cultured cells and appears to be responsible for attaching some of the cells to the bottom of the Petri dish.

The majority of cells are pear-shaped and so changes in the relative position of the organelles can be followed. In many cells the nucleus was situated in the "neck" of the cell prior to division (Fig. 3.3 A). The chloroplasts tended to cluster round the nucleus (Fig. 3.3 D). An idea of cell cycle timing was revealed by Feulgen staining. However, samples were only examined every 24 hr and therefore does not give accurate estimate of cell cycle timings. DAPI was used as a quick method to determine when nuclear division occurred.

3.2.5 Timing of Cell Division

The onset of various phases of the reactivated cell division cycle was determined in collaboration with Dr E. Paul (Paul et al., 1989). To determine the time of DNA synthesis cells were stained with Schiff's reagent. It was found not to be possible to obtain an even staining
Fig 3.2 Summary of cell expansion and division during culture. Increase in area of undivided (c) and divided cells (a) and the percentage of viable cells that have divided (b) in cultures from day 0 to 6. Bars represent SE.
Fig 3.3 A-E. Micrographs of cells from various stages of dedifferentiation. (A) Acridine orange stained cell on day 3 with brightly fluorescing nucleus situated in the "neck" of the cell, X 900. (B) Undivided (left) and divided (right) cells on day 4, X 700. (C) Cluster of 4 cells on day 6 following an initial unequal division and further divisions of the smaller, upper daughter cell have occurred, X 700. (D) Phase contrast view of a nucleus within a cell following the first division. Chloroplasts are situated around the nucleus and microfibers extend across the cell, X 2400. (E) Tinopal stained cells on day 5. Divided, expanded cells are brightly stained, especially along the cell plate. Small unexpanded cells are poorly stained, X 250. Scale bar A, B and C 10 μm, D 5μm and E 20 μm.
with different aged cultured cells. As a consequence of this isolated nuclei were used instead for Feulgen staining. The isolated nuclei from all stages appeared well stained. It was observed that there was a change in the appearance of Feulgen-stained isolated nuclei, and in stained nuclei within intact cells, from day 0 to 4. At isolation the nucleus stained uniformly and the nucleolus was small, but during culture the nucleus and the nucleolus increased in size, and the staining became less uniform (Fig. 3.4 A & B).

The microdensitometry values of nuclei from day 0, 1 and 2 each formed a single peak (Fig. 3.4). The results from day 3 contained a few higher values and those from day 4 nuclei included a second peak with values double the lower peak. The mean value of the lower peak on day 0 to 4 was 26.2 units and the mean of the higher peak from day 4 was 53.3 units. Freshly isolated cells appear to be in the G1 phase of the cell cycle, and DNA synthesis first occurs at day 3 of culture. The only mitotic phase present in the isolated nuclei at 3 days was early prophase; values for these nuclei were in the higher peak. By measuring the DNA content in total nucleic acid extracted from cells (Fig. 3.5) it was determined that a similar increase in DNA content occurred as visualised by the Feulgen staining method. To determine the C value (relative DNA content per nucleus) for asparagus, microdensitometry measurements from root tip squashes were compared to those from Allium cepa. The asparagus 1C value was determined to be 1.2 pg (Paul et al., 1989).

Nuclei could be visualised in intact unfixed cells by using DAPI (see chapter 2). An examination of DAPI-stained cells, showed that mitotic nuclei were present in 4% of cells on day 4, and cells in the final stage of cytokinesis (day 4) with a developing phragmoplast having daughter nuclei in late telophase. Divided cells (as defined by the presence of a completed cell plate visualised by tinopal staining) were first seen on day 4 (Fig. 3.1 C), and 91% of the cells had divided by day 6 (Fig. 3.2). The majority of divisions gave rise to daughter cells of unequal size with the cell plate formed across the neck of the cell (Fig. 3.3 B & E). Further divisions occurred rapidly and were primarily from the larger daughter cell (Fig. 3.3 C). The smaller daughter cell either did not divide or divided at a much slower rate.

The cytological analysis has identified two distinct criteria, cell expansion and cell division that can be used to stage cultures. Many nuclei were observed to migrate to the *neck* of
Figure 3.4 Frequency distribution of microdensitometry measurements of Feulgen stained isolated nuclei from day 0 to 4 cultures. Inset shows micrographs of Feulgen stained isolated nuclei on day 0 (A) and day 4 (B), X 2200. Scale bars 5 μm.
the cell together with the aggregation of plastids around the nucleus prior to mitosis. No reliable cytological markers for dedifferentiation were identified prior to day 3 when cell expansion was first prominent. There is a discrepancy between the percentage of cell division and DNA content data from Feulgen staining. This discrepancy is thought to be due to variability of different cultures.

3.2.6 Synthesis of Nucleic Acids During Dedifferentiation

The observation of changes in cell morphology, and in particular nuclear division and phragmoplast formation together with oxygen consumption measurements (see Chapter 4), indicated that an increase in metabolic activity occurred during dedifferentiation. Much of this could be due to an increased nucleic acid and protein synthesis required for cell division. Therefore, nucleic acids and proteins were extracted from the cells to determine quantitative and qualitative changes in the content of macromolecules during dedifferentiation. Ribosomal RNA and high molecular weight DNA can be separated into distinct bands by gel electrophoresis of total nucleic acids (Fig. 3.5 A, & Fig. 4.12). This enables an estimation of the increase in quantity of nucleic acids from extracts prepared from equal numbers of different aged cultured cells. It is also possible from Fig. 4.12 (chapter 4) to observe that the relative abundance of the chloroplast rRNA decreases with time in culture. The data on Fig. 3.5 B was obtained from a gel scan of Fig. 3.5 A, and shows that there is an increase in both RNA and DNA between 2 to 3 days in culture. The rise in RNA abundance between day 2 and 3 is very dramatic and appears to follow the measured increase in respiration rate (see Chapter 4; Fig. 4.7). Therefore this rise in RNA synthesis is likely to contribute to a genuine increase in the cells metabolic activity, rather than a change in extractability of nucleic acids. It is therefore possible to conclude that the amount of extracted RNA increased from day 2 to 3 (before cell division) and from day 4 to 6 (after the first division). Even though the DNA content measurements (Fig. 3.5) included both nuclear and organellar DNA, the same trend observed with Feulgen stained nuclei (Fig. 3.4) was followed. That is, an increase in DNA content which occurred between 2 to 3 days of culture.

Up to 0.4 μg of poly(A)$^+$ RNA is required for optimum in vitro translations. Depending on the metabolic activity of donor cells poly(A)$^+$ RNA normally makes up approximately 2-4% of the total RNA. The figures for RNA content from Fig. 3.5 was derived using an extraction
Fig 3.5 Nucleic acid content of cultured cells. (A) Agarose gel of total nucleic acids. Lane (M) DNA size markers. (0) is freshly isolated cells. (1) is 1 day in culture. (2) 2 days in culture. (3) 3 days in culture. (4) 4 days in culture. (5) 5 days in culture. (6) 6 days in culture. DNA is the band that comigrates with the 23130 bp marker and rRNA are bands below 2027 bp marker. (B) Data derived from gel scan of DNA and 25S & 23S rRNA (upper RNA band) from agarose gel (A). Units are μg RNA/DNA per million viable starting cells.
DNA and RNA content with time in culture

A

B

Days in culture

(ng DNA)

(μg RNA)

(μg RNA)

(μg DNA)

(M) M 0 1 2 3 4 5 6

23130

9416

6557

4361

2322

2027

564

μg million cells

0 2 4 6 8
method that did not yield high quality RNA. The high quality RNA extraction method yields less RNA per cell. Therefore, at day 2, 100 X 10^6 viable cells yield approximately 290 \(\mu g\) of RNA of high purity. Thus mRNA extracted from approximately 14 X 10^6 viable cells will be required for a single \textit{in vitro} translation.

3.2.7 Protein Synthesis During Dedifferentiation

A change in the total extractable protein content (Fig. 3.6) and protein composition (Fig. 3.7) of the cells occurred during dedifferentiation. This was determined by protein extractions of fixed quantities of tissue, and by 1-D SDS PAGE of the extracted protein samples. It can be observed from the crude protein content measurements (Fig. 3.6) that there was an increase in protein content with time in culture as would be expected. These measurements were useful in deciding on the amount of tissue cultured material required to yield adequate quantities of protein for antibody production and protein purification. From the 1-D SDS PAGE it was observed that there was an increase in the complexity of the total protein extract (Fig. 3.7). Additional bands were present in extracts from cultured cells which were not detected in day 0 cells, for example the band at 16 kD. The relative intensity of this band increased from day 3 to 6. Bands at 56 and 14 kD decreased in relative intensity from day 0 to 6. Protein in the 14 kD band was identified as being small subunit ribulose bisphosphate carboxylase oxygenase (SSU) as it co-electrophoresed with purified spinach SSU. The 56 kD band was identified as large subunit ribulose bisphosphate carboxylase oxygenase (LSU) as it reacted with a polyclonal antibody prepared against total spinach rubisco and asparagus LSU (see chapter 4).

3.3.1 Summary and Discussion

This chapter has been concerned with the optimisation of the asparagus mechanically isolated cell culture system, as well as with the identification of cytological markers for dedifferentiation. Initially the system was optimised by improving growth conditions. Maximum cell viability and growth was achieved by germinating seeds at 27 °C and transferring germinated seedlings after 2 weeks of growth to a Fisons cabinet set at 24 °C (see section
**Fig 3.6** Protein content of cultured cells. Protein was extracted from $4 \times 10^6$ viable cells in equal volume of 2DMH extraction buffer (see section 2.5.1). The protein extracts were quantified by the dot binding assay (see section 2.5.3). Units are $\mu$g protein per million viable starting cells.
Protein content of cultured cells

μg protein

Days in culture

50
40
30
20
10

0 2 4 6 8
Fig 3.7 Composition of total protein from dedifferentiating cells. SDS-polyacrylamide gel electrophoresis of protein extracted from cell cultures on days 0 to 6. 25 μg of protein loaded per lane.
3.2.1). These plants were watered once weekly with Hoagland's solution (Epstein, 1972), as it was observed that this reduces contaminating carbohydrate (see chapter 5) in extracted RNA samples. Furthermore, it was observed that age of the seedlings was not a good parameter as choice of tissue for culture. The frond morphology was a better parameter as a marker for tissue culture competence. Asparagus fronds which were optimum for reproducible cell culture contained unrolled cladodes and the fronds lacked lateral branches. Seedlings normally reached this stage under the standardised growth conditions between 5 to 6.5 weeks after sowing (see section 3.2.2). Age was also an unsatisfactory criterion for cladode choice because there was variation in germination time within and between separate seed batches. The asparagus cell isolation procedure was optimised by modifying a plastic scraper method used by other labs (Foyer, et al., 1982; Cockburn, personal communication). Of the various culture dishes tested, plastic disposable Petri dishes were optimal.

From the observations made in this chapter it can be concluded that during dedifferentiation a complex series of events occurs, which results in a change from quiescent, photosynthetic, mesophyll cells to heterotrophic, dividing, cultured cells. From the initial observations reported here it appears that dedifferentiation of a population of asparagus cells can be divided into three phases, based on the events occurring in the majority of cells (Fig. 3.8). During the first phase, from day 0 to 2, there was little change in appearance of the unstained cells. However, at the molecular level there were considerable changes in ribosomal RNA and total protein composition. The rRNA content per million cells appeared to increase with time in culture, particularly after 2 days. This increase in rRNA followed a similar increase in respiration rate (Chapter 4). However, the protein composition changed both qualitatively and quantitatively. A new protein band at 16 Kd was visible after 3 days in culture and increased thereafter; there was also a concomitant decrease in the abundance of protein in a 14 Kd band (SSU, see section 4.2.3).

DNA synthesis and the first mitosis occurred during the second phase of dedifferentiation, followed by formation of the cell plate. From day 5 to 6, the third phase occurs, in which the cells progress rapidly through the cell cycle. At this stage, and only following the first cell division, the cells expand and assumed a morphology typical of most cultured plant cells.
Fig 3.8  Summary of the phases of dedifferentiation. Determination of phases by cytological observations.
Differentiation Mechanical Isolation Dedifferentiation

Meristematic cell → Mesophyll cell (quiescent) → Cultured cell (dividing)

Cell and nuclear size

Cell cycle reactivation

Phases of dedifferentiation

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 - 2</td>
<td>Day 3 - 4</td>
<td>Day 4 - 6</td>
</tr>
<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 increase in size of nucleus and nucleolus</td>
<td>DNA replication Mitosis</td>
<td>Continuation of cell cycle</td>
</tr>
<tr>
<td>Changes in protein composition</td>
<td>Further changes in protein composition</td>
<td>Further changes in protein composition</td>
</tr>
</tbody>
</table>
Dedifferentiation is thus a highly active process, as would be expected from the many different metabolic pathways (such as those involved with genome replication, cell plate formation and cell wall synthesis) that are activated or modified during this phase of plant cell development. The increase in ribosomal RNA content, increase in size of the nucleolus and the change in chromatin staining are perhaps indicative of an increased gene expression associated with this metabolic activity (Paul et al., 1989), and have been observed previously during dedifferentiation of artichoke tuber cells and protoplasts (Yeoman and Davidson, 1971; Jordan et al., 1987; Bergounioux et al., 1988).

Dedifferentiation of mechanically isolated cells is similar to certain aspects of processes which occur in the intact plant and in particular the response to wounding. Reactivation of the cell cycle and mitosis in dedifferentiating cells may also parallel such phenomena in quiescent and active meristems in intact plants. Thus, mechanically isolated asparagus cells may provide a useful model system with which to study these processes, as relatively large amounts of uniform material can be prepared reproducibly for molecular analysis and the cells are more accessible for microscopic observation. However, differences from the situation in the intact plant do preclude a direct comparison. In culture the cells are randomly dispersed and surrounded by a uniform medium, whereas in the plant, meristematic cells occupy specific locations in organised tissue and are exposed to complex and variable gradients of nutrients, phytohormones and other material. For example, a change in auxin supply is known to result in altered gene expression of established cell suspension cultures (Bevan and Northcote, 1981b; Ulrich and Key, 1988; section 5.1.3). Thus, the quantitative alterations in protein composition of the mechanically isolated cells may be in part a consequence of the difference in auxin supply between the intact plant and the culture medium. In addition to differences in the cellular environment, there are differences in the structure of cultured cells which complicates a direct comparison with the intact plant. For example, mechanically isolated cells contain a large vacuole, and so mitosis and cytokinesis may differ from that in the microvacuolated meristem cells.

A callus of dedifferentiated cells is formed in response to wounding in the intact plant. Several aspects of this process resemble dedifferentiation of mechanically isolated cells. For
example, movement of the nucleus and reactivation of the cell cycle occur in both processes (Schnepf and Volkmann., 1974). Proteins, such as extensin, phenylalanine ammonia-lyase and chitinase are induced rapidly by wounding of plant tissue (see section 5.1.11-5.1.16; Templeton and Lamb, 1988). In mechanically isolated asparagus cells also there were qualitative changes in protein content during the first day in culture. As a tissue culture system, mechanically isolated asparagus cells have several advantages. The cells are uniform and, apart from the non-viable cells, appear to form a homogeneous population. The distribution of the microdensitometry values from Feulgen stained nuclei in a single peak on day 0, and the presence of a second peak (including mitotic nuclei) in values from dividing cells indicated that the cells in the fronds are G1 arrested (Paul., et al., 1989). This is in contrast to the mixed populations of G1 and G2 arrested cells found in some other material, in which G2 cells are though to have greater potential for reactivation of the cell cycle (Bergounioux, et al., 1988).

Analogous tissue culture systems, in which reactivation of the cell cycle is stimulated in differentiated cells, are callus formation on the cut surface of storage tissue and protoplast development. Dedifferentiation of cells on the cut surface of artichoke tubers is similar to asparagus cells in that the nuclei are arrested in G1, but molecular analysis of this material would be difficult as cells within the tissue do not develop uniformly (Yeoman et al., 1968). Dedifferentiation of protoplasts is probably a more complicated process than that of mechanically isolated cells as there is greater disruption of the cell environment, which results in a greater change in cell form (see section 4.1.6). Protoplasts are subjected to osmotic stress and the cell wall is synthesised extracellularly de novo, an unusual process in plant development. Changes in the composition of protein synthesised by in vitro translation of mRNA isolated from protoplasts as compared to the source leaf cells have been noted (Zelcer and Galun, 1976; Fleck et al., 1980). The increase in some proteins is though to be due to the imposition of osmotic shock (Fleck, et al., 1982).

The only obvious cytological markers for dedifferentiation are cell expansion and cell division. Both of these events occurred quite late in the process of dedifferentiation. Processes such as nuclear migration and chloroplast migration occurred when cell expansion
was observed, and so are also late markers. Therefore chromosome and nuclei stains would only highlight changes that occur late during dedifferentiation. Although the RNA content of the cultured cells increases dramatically by day 2 it was not feasible to use this as a marker, as this analysis method is complicated and time consuming. However, it was determined that the respiration rate increased at day 2 (see Fig 4.7). Respiration can be easily measured in an oxygen electrode and so can be used to determine if cultures of 2 days age are dedifferentiating normally. However, it is not possible to obtain any cytological markers concerning dedifferentiation of cultures aged less than 2 days old. For molecular analysis it was important to determine that cultures harvested between day 0-3 would dedifferentiate normally i.e. conform to the established cytological criteria. Therefore the only available method to do this was to keep several culture dishes in a harvested batch until an estimation of dedifferentiation could be estimated by using the cytological and physiological (see chapter 4) markers.

The results reported here suggests that dedifferentiation is a highly active and reproducible process that can be separated into 3 different phases (see Fig. 3.8). A reactivation of the cell cycle where there is little cytological or physiological change. This is followed by DNA synthesis, the first mitosis and phragmoplast formation, and finally the continuation of the cell cycle. The cytological markers chosen for dedifferentiation were cell expansion and cell division. The cell culture system is reproducible and synchronous enough for molecular analysis of each of the different phases to be possible. Approximately 14 X 10^6 viable 2 day old cultured asparagus cells are required to yield sufficient high quality poly(A)^+ RNA for a single *in vitro* translation. Since large numbers of viable cells can be reproducibly cultured, this asparagus mechanically isolated cell culture system appears to yield adequate quantities of macromolecules for molecular analysis.
Chapter 4 Photosynthetic Characteristics of Dedifferentiating Asparagus Mesophyll Cells

4.1 Introduction

4.1.1 General Introduction.

Over the years mechanically isolated asparagus mesophyll cells have been used to examine various aspects of cytology, physiology and biochemistry, mainly because single cells have a reduced permeability barrier compared to intact tissues and that all cells can be exposed to the same external environment. For example, mechanically isolated asparagus cells have been used to examine orthophosphate fluxes (Foyer et al., 1984), nitrogen metabolism (Walton et al., 1984), enzyme activity measurements (Fraser and Ridley, 1984), pH regulation (Foyer et al., 1982), cell division (Jullien, 1973), amino acid transport (Cheruel and Jullien, 1979), photosynthesis (Hills, 1986) and photoautotrophism in cultured cells (Peel, 1982). During tissue culture (see chapter 3) of dark incubated asparagus cells a switch in metabolism from photoautotrophic to heterotrophic has to occur. The cytological examination of dedifferentiating cells did not identify a useful early marker (between day 0-day 3) for dedifferentiation (see chapter 3). Thus, a search for a physiological marker for dedifferentiation which occurred prior to cell division was launched. Since, the techniques for monitoring photosynthesis are simple, and chloroplast proteins are highly conserved and abundant, it was decided to combine the search for a physiological marker for dedifferentiation with an examination of chloroplast components and the switch in primary carbohydrate metabolism. Eventually it was hoped to be able to determine the effects of cellular dedifferentiation on plastid structure, physiology and function. This chapter is concerned with the examination of this switch in metabolism, as well as the components likely to lead to the loss of photosynthetic potential.

4.1.2 Plastid Interconversions

Chloroplasts are derived from small undifferentiated proplastids which are normally maternally inherited by the plant zygote (Mullet, 1988). There are various types of plastids which are interconvertible, and since every cell type has its own range of plastids it is
impossible to give a valid scheme for plastid interconversion during the differentiation of a single cell (Schnepf, 1980). These interconversions are a relatively slow process when compared to cellular metamorphosis during leaf development (Bergmann and Berger, 1966). In culture, the state of existing plastids depends on external factors such as light and growth regulators, and not on the initial cell type. The plastids in cultured cells are normally chloroplasts, or proplastid-like leucoplasts which are easily interconverted (Schnepf, 1980). Interconversion of plastids to form proplastid-like structures occurs after a series of cell divisions and is connected with remeristemisation of cells, which can be induced when a wound cambium is formed, or during callus formation or embryo development in vitro (Schnepf, 1980).

Sjolund and Weier (1971) carried out an ultrastructural study of chloroplast structure and dedifferentiation in tissue cultures of Streptanthus tortuosus. They observed that subculturing of green cultures resulted in a bleaching of the chloroplasts initially, and after 90 days the chloroplasts recovered their chlorophyll content to levels equal to the original inoculum (Sjolund and Weier, 1971). Dark incubated intact plants develop etioplasts that contain characteristic dark-induced prolamellar bodies. These are not found in dark incubated cultures which instead contain proplastids which lack grana (Sjolund and Weier, 1971). Another characteristic of cultures is that changes in plastid ultrastructure are paralleled by a dedifferentiation of vacuolate cells to a less differentiated, meristematic state (Sjolund and Weier, 1971). Sjolund and Weier (1971) concluded that the high level of nutrients encountered during subculture at the start of tissue culture may either act directly to cause chloroplast dedifferentiation, or that the high growth rate induced by fresh medium may cause a degradation of chloroplast membrane proteins. However, it is not true in all cases that cell growth is antagonistic to chloroplast differentiation, especially with cells whose continued survival depends on their ability to photosynthesise (Peel, 1982).
4.1.3 Proplastids

Proplastids are the progenitors of chloroplasts and are characterised by their small size (0.5-1.0 \( \mu \text{m} \)), spherical shape and lack of prominent internal membranes (Mullet, 1988). Proplastids contain low amounts of plastid DNA, RNA, ribosomes and soluble proteins (Mullet, 1988). There is therefore a basal level of expression of genes coding for proplastid components in cells that contain proplastids, such as meristematic cells, pollen and egg cells (Mullet, 1988). Proplastids are involved in amino acid (Mullet, 1988, Wallsgrove et al., 1983) and lipid biosynthesis (Mullet, 1988; Browse et al., 1985) in meristematic cells. Some tissue cultured cells contain proplastids, especially in the presence of sucrose.

4.1.4 Effects of Light and Darkness on Gene Expression

It is well established that environmental factors such as light can regulate the expression of several nuclear genes encoding chloroplast proteins, which are involved in photosynthetic electron transport as well as carbon assimilation (for reviews, see Kuhlemeier et al., 1987; Link, 1988). Light can act to either induce or to suppress transcription of nuclear genes encoding chloroplast proteins (for a review see Link, 1988). Genes coding for chlorophyll a/b binding protein (cab) and small subunit ribulose bisphosphate carboxylase/oxygenase (ssu) are positively regulated by light (Link, 1988). However, both of these genes belong to multigene families whose members respond to light differently, in a tissue-specific and developmental stage-specific manner (Link, 1988; Fluhr, 1986a & b; Kulhemeier et al., 1987). There are two well documented examples of nuclear encoded genes that are negatively regulated by light. These are phytochrome and NADP-protochlorophyllide oxidoreductase, which upon illumination exhibit decreased levels of transcription (Colbert et al., 1983; Kay and Griffith, 1983). All the above work was carried out with intact plants, so care has to be exercised when tissue culture material is compared to intact plants. This is because there is evidence to suggest that gene regulation in calli is different to gene regulation in intact plants (Kuhlemeier et al., 1987). Further-more, blue-light control appears to be more important in controlling gene expression in photomixotropic or photoautotrophic suspension cultures of Chenopodium rubrum L (Richter et al., 1987) rather than the expected red light. Light can also affect processes such
as post-transcriptional RNA degradation, translation, and protein turnover (Slowin and Tobin, 1982; Bennett, 1981); although there is a lot of evidence to suggest that transcription is the main control point in photoregulated expression of nuclear genes for chloroplast proteins (Link, 1988).

4.1.5 Effects of Plastids on Nuclear Gene Transcription

Deng and Gruissem (1988) have observed that there is constitutive transcription in non-photosynthetic plastids though some of the mRNA's encoding photosynthetic proteins are not translated in proportion to their abundance. They have concluded that in non-photosynthetic plastids of spinach plants, gene expression is controlled primarily by post-transcriptional and translational mechanisms (Deng and Gruissem, 1988). There is evidence to suggest that mature chloroplasts are required, together with light treatments to obtain optimal rates of transcription from cab and ssu genes (Giuliano and Scolnik, 1988). It is thought that a chloroplast signal from an early stage of development is continuously required for optimal cab gene transcription (Burgess and Taylor, 1988). This signal is not synthesised when chloroplasts are exposed to photooxidative damage (Burgess and Taylor, 1988). Analysis of DNA extracted from plastids of tomato fruits and leaves have revealed that DNA fragments containing certain genes that are barely transcribed in chromoplasts are methylated and active genes are not methylated (Ngernprasirtsiri et al., 1988). There appears to be conflicting information on the role of transcription in non-photosynthetic plastids.

4.1.6 Photosynthetic Characteristics of Protoplasts and Plants Subjected to *Shock*

The biosynthesis of rubisco was examined in freshly isolated protoplasts by a radioactive labelling technique, followed by SDS PAGE (Fleck et al., 1979). It was discovered that the biosynthesis of rubisco stopped almost completely as soon as protoplasts were incubated in culture medium (Fleck et al., 1979). Further experiments based on mRNA extraction and *in vitro* translations showed that nuclear genes which encode chloroplast proteins are not highly transcribed in protoplasts (Fleck et al., 1980). Analysis of proteins synthesised in freshly isolated protoplasts showed that there are changes in protein profiles (Fleck et al., 1982).

In chapter 5 (section 5.1.3 & 5.1.14) it was suggested that subculturing induces a "stress"
response in cultured cells. However, it has also been established that there is an increased synthesis of both plastids and plastid DNA following media renewal in cultured tobacco cells (Yasuda et al., 1988).

Heat shock has been shown to decrease the synthesis of in vivo labelled proteins mainly by post-transcriptional means (Lindquist, 1986). For example, the synthesis of both SSU and LSU decreased under heat shock conditions (Vierling and Key, 1985); the cells potential to synthesise SSU and LSU returns after several hours depending on heat shock conditions used (Vierling and Key, 1985). Vierling and Key also observed that during and after heat shock, SSU synthesis corresponded with the amount of mRNA present but LSU synthesis showed little relationship to corresponding mRNA levels. There was no apparent coordination of LSU and SSU mRNA levels under heat shock conditions (Vierling and Key, 1985).

4.1.7 Effects of Carbohydrate and Senescence on Photosynthesis

When the sucrose concentration in culture medium of suspension cultured spinach cells is at, or above, 2.5 mg ml\(^{-1}\), chlorophyll synthesis is inhibited (Dalton and Street, 1977). This same effect was also observed with readily utilisable sugars such as glucose, fructose and maltose but not with slowly utilised sugars such as inulin and raffinose (Dalton and Street, 1977). It was observed that sucrose rather than its monosaccharide components directly inhibited photosynthesis or promoted photorespiration (Dalton and Street, 1977).

During senescence there is an increase in protease and hydrolase activity which leads to an increase in protein degradation (Veierskov and Thimann, 1988). There is an increasing permeability of the vacuolar membrane which eventually allows proteases to enter the cytosol and to attack cytosolic and organellar proteins (Veierskov and Thimann, 1988). The loss of chlorophyll is often used as a measure of senescence (Malik, 1987). Senescence of mustard cotyledons can be induced by continuous white light or darkness (Kasemiv et al., 1988). The loss of rubisco does occur during senescence induced by continuous white light, but rubisco loss is more pronounced in darkness (Kasemiv et al., 1988). In the dark-mediated senescence of mustard, rubisco loss is phytochrome controlled and senescence can be reversed by irradiation with red light (Kasemiv et al., 1988). Phytochrome is thought to decrease rubisco
loss by the retardation of enzyme degradation and to a lesser extent by an increase in gene expression (Kasemiv et al., 1988).

4.1.8 Photosynthetic Characteristics of Asparagus Cells

Mechanically isolated asparagus cells will retain their photosynthetic capacity for over 24 hr if maintained in the dark in aerated buffer (Hills, 1984). The photosynthetic activity of extracted cells is not affected by the presence of sorbitol up to concentrations of 1.5 M unlike other plant species cell preparations (Hills, 1986). Although the response of asparagus cells to light, temperature and carbon dioxide is similar to that obtained for other plant species, pH was observed to have little effect on photosynthesis (Hills, 1986). However, the photosynthetic capacity of isolated cells of asparagus was only 40-60 % of that of intact cladodes (Hills, 1986). This was thought to be due to an inhibition of the sucrose-synthesis pathway (Hills, 1986).

4.1.9 Dark-Induced Chloroplast Dedifferentiation

When *Euglena gracilis* cells are transferred to a medium containing organic carbon in the dark, the cells reduced their chloroplast efficiency both ultrastructurally and functionally (Scheer and Parthier, 1982). During this incubation period in darkness within glucose-containing medium, dark-dependent dedifferentiation of mature chloroplasts to form proplastids occurred (Scheer and Parthier, 1982). However, no parallel decreases were demonstrated to occur in chlorophyll content, thylakoid number and photosystem I and II activities during dark-induced chloroplast dedifferentiation (Ophir et al., 1975). This fact suggests that some of the chloroplast components are more stable than others (Scheer and Parthier, 1982). In *Euglena* cells grown under photoautotrophic conditions, each chloroplast contains 20-30 lamellae, whereas the heterotrophic cells have chloroplasts that contain 5-10 lamellae (Neumann and Parthier, 1973). Six generations (140 hr) in darkness results in the production of bleached heterotrophic cells containing proplastids (Ophir et al., 1975). There is a direct relationship between a dark-dependent, glucose-induced, decrease in the size of chloroplasts and the number of photosynthetic lamellae, with the loss of chlorophyll. Scheer and Parthier (1982)
observed that the loss in activity and abundance of rubisco strictly follows the cell division curve, which was interpreted as being consistent with a diluting-out effect of the enzyme amongst the progeny cells. A similar diluting out phenomenon was observed with aminoacyl-tRNA synthetases in *Euglena* (Scheer and Parthier, 1982). By using eukaryotic and prokaryotic protein synthesis inhibitors Scheer and Parthier (1982) concluded that dark-induced chloroplast dedifferentiation occurs independently of plastid gene expression. This is unlike light-induced biogenesis of chloroplasts, when gene expression programmes of both the nuclear and plastid genomes are turned on and in most cases coordinated. *Euglena* cells cultured in media lacking glucose in the light are able to synthesise new chloroplasts by re-utilizing carbon and nitrogen compounds resulting from the turnover of cytoplasmic proteins (Scheer and Parthier, 1982). There is some evidence to support the view that the blocked uptake of substrates is a primary cause for the arrested turnover of chloroplasts in dark-incubated cells cultured in media lacking organic carbon (Scheer and Parthier, 1982). This is however not true for cells cultured in media containing an organic carbon source in darkness.

4.1.10 Summary of Objectives

There were two major aims in this chapter and they were firstly to identify physiological markers for dedifferentiation and to examine the switch in general metabolism from photoautotrophic to heterotrophic in detail. Both of these aims can be realised in similar experiments utilising cytological and physiological techniques. There are several obvious factors that can contribute to a loss of photosynthetic potential. These are a loss of chloroplasts or a decrease in the efficiency of either or both of the dark reaction and light reaction of photosynthesis. The use of microscopic techniques in conjunction with nucleic acid stains will enable plastid replication to be followed together with an estimation of plastid numbers to be achieved on small quantities of tissue.

The switch in metabolism from photoautotrophic to heterotrophic can be followed by using an oxygen electrode. This type of analysis is both simple and requires very little tissue (Colman et al., 1979; Hills, 1986). Any reproducible changes observed could be used as a physiological marker for dedifferentiation. It is important that any method used to highlight
physiological markers of dedifferentiation, work on small quantities of tissue. The integrity of the light reaction components can be determined by the measurement of electron transport.

The efficiency of the electron transport can be monitored by measuring the oxygen consumption in the presence of the terminal electron acceptor methyl viologen with an oxygen electrode (Coombs et al., 1985; Walker, 1987). The other component of photosynthesis that can cause a loss of photosynthetic potential is the breakdown of the Calvin cycle. The abundance of some of the enzymes in this pathway can be easily determined by Western blotting. In particular the enzyme rubisco is highly abundant and both antibody probes and DNA probes are easily available to monitor both the transcript abundance and concentration of this enzyme.

The data from all of these experiments will give an idea of physiological markers for dedifferentiation and the mechanism which contributes to the loss of photosynthetic potential; and how this interacts with cellular dedifferentiation.

4.2 Results

4.2.1 Chloroplast Numbers

The morphology of chloroplasts in relation to cell age in culture was examined by light and ultra violet (U.V.) microscopy. The chloroplasts were large, disc shaped and prominent in the cytoplasm of freshly-isolated cells (see Fig. 4.1, A & C). The chloroplasts remained the same size (8.6 \( \mu m \)) until cell division had occurred, then there were signs after 10 days that the chloroplasts had divided; these divided chloroplasts are generally smaller (2.86 \( \mu m \)) than undivided chloroplasts (see Fig. 4.1, B & D). This division did not resemble normal "cellular" division but rather resembles a "fission" division as there was no increase in size of chloroplasts prior to division, and that the net result of division was a further reduction in plastid size. More of these "fission" divisions occurred in rapidly-dividing cells as opposed to cells with slower rates of cell division (see Fig. 4.1 B & D). The plastids in the non-dividing daughter cell (Fig. 4.1 D) are considerably larger (8.6 \( \mu m \)) than the smaller plastids (2.86 \( \mu m \)) found in the faster dividing daughter cells (Fig. 4.1 D). In general the plastids in the non-
**Fig 4.1 Chloroplast development during cell culture.** (A) Light micrograph of freshly isolated cells. (B) Light micrograph of day 10 cultured cells. (C) UV illuminated freshly isolated cells from the same field of view as A, chloroplasts are fluorescing brightly (8.6 μm). (D) UV illuminated 10 day old cultured cells. Chloroplasts in non-dividing cell (extreme left) are larger than chloroplasts in dividing cells (2.86 μm). Scale bars are 10 μm.
dividing cells appear not to undergo "fission" division during the first 15 days of culture, unlike plastids from fast dividing cells which show "fission" divisions after 8 days in culture. Division of the chloroplasts leads to an increase of chloroplast numbers per cell by day 10 in culture (see Fig. 4.2). This is a difficult phenomenon to follow accurately in U.V. illuminated cell microaggregates, as by this time multiple divisions have occurred and it is difficult to distinguish individual cells.

The chlorophyll content of a million starting cells was examined with time in culture as a measure of senescence and thylakoid membrane integrity. Chlorophyll is bound to cab in thylakoid membranes; if cab is not associated with chlorophyll then it is turned over rapidly (Apel and Kloppstech, 1980). The amount of chlorophyll per million starting cells remained at relatively constant levels during the first 7 days of culture (Fig. 4.3). Since cell division occurred at day 4 (see chapter 3) which increased cell numbers, the chlorophyll is shared amongst the daughter cells, and therefore chlorophyll content per cell was effectively reduced.

4.2.2 Oxygen Electrode Measurements

The photosynthetic capacity of cultured cells and dark incubated 6 week old asparagus plants was followed by using an oxygen electrode. An oxygen electrode measures oxygen concentration in a suspension of cells. The rate of oxygen evolution/uptake can be calculated if the cells are placed in a transparent, airtight chamber. Freshly isolated cells photosynthesize in the light and, as a result, release oxygen; the same cells incubated in the dark no longer photosynthesize but respire instead and so take up oxygen from the medium. By using such an oxygen electrode the switch in metabolism from photoautotrophic to heterotrophic could be followed. A fully heterotrophic culture would only consume oxygen, both in the light and the dark and therefore the oxygen electrode would measure the reduction in concentration of oxygen. A photosynthesizing culture would evolve oxygen and also show some oxygen consumption as all tissues respire, this is why there is a distinction between net and gross photosynthesis.

Gross photosynthesis rate is the oxygen evolution/sec without any adjustments to take into account the oxygen consumed by respiration. Net photosynthesis is when the dark incubated
**Fig 4.2** Chloroplast numbers in cultured cells. Chloroplast numbers per cell was determined by calculating the average of 10 separate fields of view for each day for 4 separate cultures. 0-10 represent day 0 to 10 of culture.
Chloroplast numbers in cultured cells

![Graph showing chloroplast numbers in cultured cells over days in culture.](image-url)
Fig 4.3 Chlorophyll content of cultured cells. Chlorophyll content of cultured cells was determined for a million viable starting cells, and was measured as in section 2.4.3. 0-7 represented day 0 to 7 of culture.
chlorophyll content of cultured cells

0 2 4 6 8

days in culture

ng chlorophyll/million starting cells
oxygen consumption measurements are taken into account by addition to the measured oxygen evolution rate. It was assumed with the cultured cell experiments, that no cell division occurred and so the oxygen evolution rates per million cells, should be taken as being oxygen evolution per million starting cells.

The gross photosynthesis of cultured cells (see Fig. 4.4) appeared to decrease a day prior to cell division. After cell division occurred the photosynthetic rate decreased more dramatically until eventually gross oxygen uptake in the light was recorded by day 5-6 (Fig. 4.4). This phenomenon was not observed in cells from dark-treated plants, where the gross photosynthetic capacity decreased with time in darkness but did not decrease to give oxygen uptake after 6 days (see Fig. 4.5). The net photosynthetic capacity of cultured cells (Fig. 4.6) also dropped at a similar rate to that observed for gross photosynthetic capacity (see Fig. 4.4). After day 4 when over 10% of the cells had divided, the net photosynthetic rate reversed to become oxygen uptake in the light (Fig 4.8).

The respiratory rate of cultured cells increased with time in culture (Fig. 4.7) and appeared to be a mirror image of gross photosynthesis; the light-driven electron transport rate had not decreased by day 10 of culture (Fig. 4.8). The electron transport data is the result of only experiments which showed considerable variation at each day of culture (this experiment was carried out in collaboration with D. Leader). This fluctuation of electron transport rate is thought not to be significant and appears to be the result of the experimental technique used. With this technique, problems were often encountered with permeability of the cells to substrates so only the trends from these results can be commented on. Overall trends suggest that the electron transport rate appears not to decrease noticeably during the duration of the experiment, indicating that there appears to be no lesion in the electron transport chain. It is therefore unlikely that the rapid loss of photosynthetic potential is caused by a decrease in the efficiency of the electron transport chain.
Fig 4.4 Gross photosynthetic rate of 3 separate cell cultures. 0-7 represent day 0 to 7 of culture. The arrow on each graph represents when cell division was first observed. Units are n mol oxygen evolved/sec/million viable starting cells.
Gross photosynthesis of cultured cells

n mol oxygen evolved/sec/million cells

days in culture

Gross photosynthesis of cultured cells

n mol oxygen evolved/sec/million cells

days in culture

Gross photosynthesis of cultured cells

n mol oxygen evolved/sec/million cells

days in culture
Fig 4.5 Gross photosynthesis of dark incubated asparagus plants. Six week old asparagus plants were incubated up to 7 days in the dark. Cells were mechanically isolated and incubated in asparagus medium before photosynthetic rates were measured. 0-7 represent day 0 to 7 of dark treatment.
gloss photosynthesis of dark incubated plants

![Graph showing the relationship between days incubated in darkness and nmol oxygen evolved/sec/million cells. The graph shows a decrease in oxygen evolution as days incubated increase.]
Fig 4.6  Net photosynthesis of cultured cells. Net photosynthesis was determined by measuring the rate of respiration from day 0 to 5 and adding it to the gross photosynthetic rate of the same cells. These rates were determined for a million viable starting cells. This graph is derived from data obtained from a single culture. Although the timing and absolute values were different for other cultures tested (variability of cultures), the same trend was followed.
Net photosynthesis in cultured cells

n mol oxygen evolved/sec/million cells

days in culture
Fig 4.7 Respiration rate of cultured cells. The respiration rate per million viable starting cells was determined by measuring dark incubated oxygen consumption between day 0 to 9.

This graph is derived from data obtained from a single culture. Although the timing and absolute values were different for other cultures tested (variability of cultures), the same trend was followed.
Respiration rate of cultured cells

Days in culture vs. nmol oxygen uptake/sec/million cells
**Fig 4.8** Electron transport rate of cultured cells. The electron transport rate per million starting viable cells was measured as oxygen consumption in the presence of methyl violigen (see section 2.4.2) under illumination between day 0 to 11. This graph is derived from data obtained from a single culture. Similar trends were observed in experiments on 2 other separate cultures.
Electron transport rate of cultured cells

mmol oxygen uptake/sec/million cells in the presence of methyl viologen

days in culture
4.2.3 Effects of Tissue Culture on Chloroplast Stromal Proteins.

Stromal proteins (such as rubisco) involved in carbon assimilation are extremely abundant and easy to monitor. The effect of tissue culture on the abundance of large and small subunits of rubisco was examined by means of Western blotting and total protein SDS PAGE analysis. Western blots were carried out initially with polyclonal antibodies raised to spinach rubisco and asparagus LSU. However, the spinach polyclonal antibody (a gift from Dr. G. Whitelam) did not cross react with asparagus SSU. The asparagus LSU polyclonal antibody was a gift from Miss. O. M. Fioroni. Identical results were obtained by Western blotting using both antibodies; the amount of LSU protein per µg of total protein decreased with time in culture (Fig. 4.9, A & B), so that by day 6 there was considerably less LSU than that present at day 1. The SSU protein (Fig. 4.9, C) followed the same reduction in amount per µg of total protein as observed for LSU. This 14 kD band was putatively identified as ssu as purified spinach rubisco ran to the same position on SDS polyacrylamide gels (Fig. 4.9, C).

4.2.4 Effects of Tissue Culture on mRNA Encoding LSU and SSU

A cDNA probe for barley ssu and a genomic probe for barley Isu were available at Leicester for use as heterologous probes. Since both of the above genes are evolutionally conserved it was decided to use them to examine message abundance by Northern blotting and RNA dot blots. However it was not known if the homology of both the probes would be sufficient for hybridisation with asparagus mRNA. Preliminary DNA dot blots were therefore performed to ensure that both of the probes would cross-hybridise with asparagus genomes, both the Isu and ssu probes were found to cross hybridise with reasonable efficiency.

The RNA dot blots probed with the Isu probe (Fig. 4.10, A) showed that message abundance of Isu decreased with time in culture. The Northern blot probed with Isu also showed a similar loss of message abundance with time in culture (Fig. 4.10, B & C). Poly (A)$^+$ RNA dot blots were probed with a barley ssu cDNA (Fig. 4.11, A) and showed an immediate reduction in ssu message with time in culture. Within a day of culture the reduction tailed off to a basal level which was maintained thereafter. Total RNA from different cultures were used in Northern blot experiments and probed with ssu. In this case ssu also showed
Fig 4.9 Abundance of LSU and SSU protein in cell cultures. (A) is a Western blot of LSU to asparagus LSU antibody. 1 μg of total protein loaded per lane. Lane 1 to 6 are 1 to 6 days in culture and lane 7 is partially purified spinach rubisco (0.25 μg). (B) Data derived from gel scan of Western blot A. (C) Is a total protein gel of day 0 to 6 in culture. Arrow marks SSU protein.
Gel scan of Western blot

A

56 kD (LSU)

B

Gel scan of Western blot

Mol.wt. 0 1 2 3 4 5 6

C

SSU
Fig 4.10 *Lsu* messenger RNA levels in dedifferentiating cultured cells. (A) Dot blot of total RNA from day 0 to 6 (0-6), 7, 8 & 9 are 1 to 3 day dark incubated plants and 10 is 7 day dark incubated plants, all probed with *lsu* gene probe. (a) is 5 μg total RNA. (b) is 0.5 μg total RNA. (c) is 5 μg tRNA (negative control). (B) Northern blot of 15 μg total RNA from day 0 to 6 cultured cells probed with *lsu* gene probe. (C) Data derived from gel scan of Northern blot B. The *lsu* gene probe is a *PstI, Hind III* fragment (Poulsen, 1983 b).
Gel scan of Northern blot probed with LSU
**Fig 4.11** ssu messenger RNA levels in dedifferentiating cultured cells. (A) Poly(A)$^+$ RNA dot blot probed with a ssu gene probe. The poly(A)$^+$ RNA samples 0 to 12 are as follows; 0 is cladode, 1 is decapitated cladodes, 2 is day 0, 3 is day 1, 4 is day 2, 5 is day 3, 6 is day 4, 7 is day 5, 8 is day 6, 9 is day 7, 10 is day 8, 11 is 2 months suspension culture and 12 is 6 months suspension culture. (a) 0.4 μg poly(A)$^+$ RNA from cell batch 1 (b) 0.04 μg poly(A)$^+$ RNA from cell batch 1. (c) 0.04 μg poly(A)$^+$ RNA from cell batch 2. (d) 0.4 μg poly(A)$^+$ RNA from cell batch 2. Samples from cladodes, decapitated cladodes, 2 to 4 days, 8 days, 2 months and 6 months consisted of only 1 batch of RNA. (B) Northern blot of 15 μg total RNA probed with ssu. 0 to 6 are 0 to 6 days of culture. (C) Data derived from gel scan of Northern blot B. The ssu gene is a full length cDNA from barley (Barkardottir et al., 1987).
Gel scan of Northern blot probed with SSU

Gel scan of Northern blot probed with SSU

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02

0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09

days in culture
a similar decrease in message abundance with time in culture (Fig. 4.11, B & C) though basal level expression was only observed after day 2. It is most likely that the detection limit of the assay causes this irregularity, as the lower amounts of poly(A)\(^{+}\) RNA (0.5\(\mu\)g) used in the dot blots compared to Northern blots (15\(\mu\)g/lane) cannot be detected.

4.2.5 Effects of Tissue Culture on Chloroplast Ribosomal RNA

Total RNA from cultured cells was fractionated on a 2% formaldehyde RNA denaturing gel. The amount of chloroplast rRNA decreased in proportion with an increase in cytoplasmic rRNA with time of culture (Fig. 4.12). Chloroplast rRNA from dark-treated asparagus plants (results not shown) did not display the same reduction of abundance. It should be noted that there are additional bands related to specific degradation products of the chloroplast 23S rRNA as observed in barley (Poulsen., 1983 a). Ribosomes are involved in protein synthesis and if their abundance is reduced then there will be a concomitant reduction of protein synthesis. In this case there appears to be a reduction of the protein synthesis required for chloroplast manufacture.

4.2.7 Discussion

There are no reliable visual markers for dedifferentiation prior to cell division (see chapter 3). The switch from a photoautotrophic metabolism to a heterotrophic metabolism was examined by monitoring oxygen evolution rates from cultured cells as a possible candidate for a physiological marker for dedifferentiation. A series of experiments were set up to try to attribute the loss of photosynthetic potential (see Fig. 4.4) to a single or several lesions within the photosynthetic apparatus. Since antibodies to LSU, a cDNA probe for the ssu gene and a probe for Isu was available, it was decided to extend the search for a physiological marker, to include a detailed analysis of the effect of this metabolic shift on rubisco.

The most obvious possibilities that could lead to a loss of photosynthetic potential might include a loss of chloroplast numbers per cell, degradation of chlorophyll, a lesion in the carbon fixation cycle, a lesion in the photosynthetic electron transport chain, or a combination of some or all of the above possibilities.
Fig 4.12 Ribosomal RNA composition during dedifferentiation. Fractionation of RNA in a denaturing gel (see section 2.7.2) of total RNA from 0 to 7 day old cultured cells, lanes 0-7 (2μg/lane). 23S,a and 23S,b are 23Sf1 and 23Sf2 as observed by Poulsen (1983 a).
Chloroplast numbers were examined microscopically using U.V. illumination, which causes chloroplasts to autofluoresce red (see Fig. 4.1). The numbers of chloroplastic units per cell actually appeared to increase with time in culture (Fig. 4.2). The chloroplasts however, appear to decrease significantly in size after day 8 when "fission" division has occurred (see Fig. 4.1), this results in a 33.3% reduction in size of plastids. The chlorophyll content per million starting cells remained relatively constant in the first week of culture. This effectively means that chlorophyll content per cell is reduced, by the observed cell divisions that occur from day 4 onwards (Fig. 3.2). Furthermore, the results suggest that little chlorophyll biosynthesis or degradation occurs during asparagus tissue culture. The relatively constant levels of chlorophyll also implies that photosynthetic membranes are relatively intact, and the membrane associated CAB levels do not decrease significantly with time culture. It is generally found that any CAB which was not bound to chlorophyll is degraded rapidly (Apel and Kloppstech, 1980). Chlorophyll degradation has been used as an indicator of senescence (Malik, 1987); the fact that chlorophyll levels remained constant with time in culture suggests that the process is dissimilar to senescence. However, the chlorophyll content per cell decreased with time in culture which seems to suggest a diluting out of associated chloroplast thylakoid membranes amongst newly divided cells.

The gross and net oxygen electrode measurements of photosynthesis with time in culture indicated that the photosynthetic rate decreased a day prior to cell division (Fig. 4.4 & 4.5). The decrease in photosynthetic rate after cell division was dramatic if either the gross or net rates were considered (Fig. 4.4 & 4.5). The same decrease in gross photosynthetic rate culminating in net oxygen uptake after 6 days of culture, was not observed in dark-incubated asparagus plants, although a decrease to very low levels of photosynthesis was noticed (Fig. 4.6). This implies that the mechanism of reduction of photosynthetic rate in the cultured cells is different from the dark-treated plants.

There are two simple, easily testable possibilities that may explain the loss of photosynthetic rate with time in culture. One possibility is a lesion in the electron transport chain and the other possibility is a lesion in carbon fixation. The first possibility was tested out by using methyl viologen, a terminal electron acceptor, to accept electrons from the electron transport
The oxygen uptake of asparagus cells in the presence of methyl viologen directly relates to the rate of electron flow. The light driven electron transport rates (Fig. 4.8) do not decrease significantly with time in culture and so cannot account for the rapid loss of photosynthetic potential. Therefore it is unlikely that a lesion in electron transport causes the reduction of net photosynthesis.

The second possibility was then examined by monitoring levels of the enzyme rubisco by total protein analysis and rubisco subunit message levels by Northern and Western analysis (Fig. 4.9). In the literature it has been suggested that dilution of the dark reaction components occurred during heterotrophic growth of *Euglena gracilis* cells (Scheer and Parthier, 1982). This also appeared to be the case with cultured asparagus cells, as the reduction in LSU and SSU protein (Fig. 4.9) paralleled the increase in cell numbers (see chapter 3, Fig. 3.2). This possibility also can explain the loss of photosynthetic capacity. With gross photosynthetic rates of cultured cells the loss of photosynthetic capacity occurred earlier than with the net rates. It was also observed that the respiratory rate of the cells increased with time in culture (Fig. 4.7). This rate appears to parallel the cell division rate and is a mirror image of the gross photosynthetic rate. Therefore, it appears that the dramatic increase in respiratory rate prior to cell division causes the rapid reduction of gross photosynthetic capacity. When the effects of this increase in respiratory rate is removed from photosynthesis as given by the net photosynthetic rates, it was observed that a decrease in abundance of both LSU and SSU occurred at the same time as the reduction in net photosynthetic rate. Therefore it appears that the rate of reduction of net photosynthetic capacity is correlated with the reduction in rubisco protein per mg total protein.

The chloroplast rRNA band intensities decrease with time in culture as a proportion of total rRNA (Fig. 4.12). This decrease in relation to nuclear-encoded rRNA bands is not found in dark-incubated plants, where the ratio of abundance of chloroplast rRNA to cytoplasmic rRNA is similar up to day 7. This can be interpreted in one of two ways; firstly chloroplast rRNA synthesis and chloroplast rRNA degradation may be reduced and increased respectively. Alternatively, the levels of chloroplast rRNA remain the same throughout culture and are reduced on a per μg total RNA basis as a result of an increase in cytoplasmic rRNA synthesis.
The first possibility may be more likely as from Fig. 3.6 in chapter 3 the cellular rRNA content does not increase until after day 2, by which time a reduction in chloroplast rRNA has already occurred.

The abundance of both \textit{lsu} and \textit{ssu} messenger RNA during culture was examined in order to compare with observations from chapter 5 (Fig 5.8 C), which showed a large number of down-regulated polypeptides soon after cells are incubated in culture. Both the abundance of \textit{lsu} and \textit{ssu} mRNA decreased rapidly to basal levels with increasing time in culture. I believe that this represents a genuine down-regulation as the rRNA content and therefore the total RNA content only increased rapidly after day 2 (Fig. 3.6). Therefore this does not represent a simple dilution of \textit{ssu} and \textit{lsu} mRNA. Since rubisco has been shown to possess a number of control levels i.e. both at the transcriptional, post-transcriptional and post-translational levels (see section 4.1.4) it is not possible to draw any further conclusions from this data. However, since the message coding for rubisco decreased rapidly with time in culture, while the protein abundance of rubisco did not show the same rapid decrease, then rubisco must be relatively stable in cultured cells. In general, chloroplast messenger RNA, in this case \textit{lsu}, is more stable than \textit{ssu} mRNA. It is not surprising to observe that \textit{ssu} mRNA reduces to lower basal levels than \textit{lsu}. It has previously been observed that a very small increase in Mg\textsuperscript{2+} concentration and an increase in pH could cause a large change in enzyme activity of fructose 1,6-bisphosphatase, phosphoribulokinase, sedoheptulose 1,7-bisphosphatase and rubisco (Buchanan, 1980). All these enzymes are members of the reductive pentose phosphate pathway and are stromal proteins. Light can induce an increase in Mg\textsuperscript{2+} concentration as well as cause an alkalization of the stroma of chloroplasts (reviewed by Buchanan, 1980). There is evidence to suggest that dark-induced deactivation of stromal enzymes is extremely complicated and involves the presence of a soluble oxidant (Buchanan, 1980). It is quite possible that such a mechanism may interact with transcription in asparagus.

Overall the observations made in this chapter are consistent with those made by Scheer and Parthier (1982), and together with a reduction of enzyme activity can explain the rapid loss of photosynthetic potential over the first week of culture. Since only rubisco protein and message levels were monitored it is quite possible that a decrease in either enzyme activity
or abundance of other stromal proteins involved in carbon assimilation could also contribute to the rapid loss of photosynthetic potential. It is thought that chloroplast dedifferentiation occurs during the switch to heterotrophic growth in *Euglena* leading to the formation of proplastids. There is circumstantial evidence to suggest that this is occurring in asparagus as well, as mRNA abundance for both *isu* and *ssu* are low, the chloroplast rRNA decreases as a proportion to cellular rRNA to basal levels and the size of chloroplasts decreases in culture.

The only sure method available to prove that the plastids formed in culture are proplastids is to use electron microscopy. It will be interesting to see if the reverse of the observations in this chapter occur during shoot regeneration in organogenic cultures. From the data presented in this chapter it can be concluded that the loss of gross photosynthetic potential and the increase in respiration rate are also useful markers for cellular dedifferentiation in mechanically isolated cultured asparagus mesophyll cells, especially when used in conjunction with cell division observations (see chapter 3).
Chapter 5 Gene Expression During Wound-Induced Cellular Dedifferentiation

5.1 Introduction

5.1.1 General Outline of Chapter

Land plants are generally sessile and are at the mercy of the environment and consequently have developed methods to cope with environmental changes. Plants therefore have had to develop mechanisms by which the switching between developmental pathways can be influenced by an interaction between the environment and the plants genotype. One such developmental pathway is dedifferentiation, and differs from most other developmental pathways in that it often represents a transition pathway between two differentiated cell types. However, it is important to note that dedifferentiation can only occur in undetermined tissue; determined cells such as lignified vascular elements and suberizised cells are incapable of dedifferentiation. One component of cellular development during this transition pathway may be changes in the amounts and activity of various enzymes and structural proteins. There is little known about differences in protein synthesis during dedifferentiation. Previous studies have concentrated on investigating the products of the dedifferentiation process i.e. callus cells, and have not attempted to study dedifferentiation itself at the molecular level. It is quite possible that there are no novel proteins synthesized during dedifferentiation, and that all the structural (see chapter 3) and physiological (see chapter 4) changes observed, result from differential processing of proteins, translatability of mRNA, mRNA stability or post-translational modifications such as phosphorylation, acetylation, methylation, adenylation, hydroxylation, carboxylation or glycosylation (Alberts, et al., 1983).

Any information gained from analyzing steady state mRNA populations or protein profiles would greatly contribute to our understanding of the process of dedifferentiation. In this chapter it is hoped to examine dedifferentiation at the molecular level. Initially the aim was to examine protein profiles, and if there appeared to be any novel proteins synthesized, to study their accumulation and finally to clone genes coding for such polypeptides. Consequently an approach which would allow the above strategy to be realised had to be adopted, or
developed. Resolution was an important consideration as Kamalay and Goldberg (1980) have demonstrated that rare mRNA species make up over 95% of nucleotide sequence diversity and it is this fraction that contains most of the differences in mRNA species between organ types. Therefore, the system chosen to study dedifferentiation has to be able to resolve the products of these rare messages.

The introduction will discuss phytohormone-induced gene expression in cultured cells, the control of the cell cycle, wounding, systems used to analyse wounding, wound-induced gene expression, light and other environmental induced gene expression. Expected changes during dedifferentiation will be highlighted and methods that have been used to examine gene expression during development will be discussed. The reasons for using the adopted experimental approach to examine dedifferentiation will be discussed.

5.1.2 Expected Changes in Gene Expression

From microscopical and physiological studies it is clear that detectable changes in structure and metabolism occur prior to cell division in asparagus cells (3-4 days in culture). However, it has not been possible to find any useful markers for dedifferentiation prior to day 3 in culture (see chapter 3). During mechanical isolation and tissue culture, plasmodesmatal links are disrupted, and a new hormonal and nutritional environment is artificially imposed on the cells in order to induce dedifferentiation. With the information obtained from other studies e.g. hormone-induced genes, wounding and light- or dark-induced genes, it is possible to postulate several changes that might be expected to be associated with growth in this new external cellular environment.

5.1.3 Auxin and Cytokinin Induced Gene Expression

It is well established that plant growth regulators can affect gene expression, growth rate, cellular morphology and cellular function. The plant growth regulator auxin has been implicated in promoting numerous phenomena in plants, and together with cytokinin is essential in promoting cell division and DNA synthesis in protoplasts (Meyer and Cooke, 1979; Cooke and Meyer, 1981). There is considerable variation in the effects of auxin on plant gene
expression between tissues of different organs.

Most callus culture media contain an auxin often together with a cytokinin. By manipulation
of the ratios of these growth regulators, different forms of growth can either be promoted or
suppressed. For example, low concentrations of auxins and high levels of cytokinins will
induce many types of callus to produce shoots (Alberts et al., 1983). Auxin has been
demonstrated to increase synthesis of rRNA and mRNA of ribosomal proteins 3-8 fold in
soybean hypocotyl (Gantt and Key, 1985).

Zurfluh and Guilfoyle (1980) observed that auxin induced an increase in RNA and protein
synthesis in mature hypocotyl segments of soybean but did not induce any cell elongation.
However, the auxin treatment of younger internal segments of soybean hypocotyl induced
cell elongation and large changes in the polypeptide pattern (Zurfluh and Guilfoyle, 1980).
In all these experiments proteins were in vivo labelled with ^S-methionine. The result of these
experiments indicate that a tissue specific pattern of protein synthesis is induced by auxin.
Further experiments which used cytokinin to prevent auxin-induced cell elongation were found
to change the polypeptide pattern, as well as the amount of incorporated radiolabel (Zurfluh
and Guilfoyle, 1980). Auxin, however, differentially effects gene expression in different plant
genera (Bevan and Northcote, 1981 a). For example, when auxin starved suspension cultured
cells of soybean are subcultured into auxin containing medium, there is a rapid increase in
3 polypeptides (35 kD, 45 kD, 65 kD) which is directly dependent on the presence of auxin
(Bevan and Northcote, 1981 b). When a Phaseolus vulgaris suspension culture was treated
in a similar manner, the observed changes in expression of some polypeptides were transient
and dependent on subculture rather than on the presence of auxin (Bevan and Northcote,
1981 b). Bevan and Northcote (1981 b) also found that the introduction of cytokinins did
not significantly affect polysome formation, and that most changes in polysome formation was
dependent on subculture. Suspension cultures of bean and soybean displayed an increase
in translatable mRNA of several polypeptides (Bevan and Northcote, 1981 a,b) following
subculture into auxin- or cytokinin-containing medium from a auxin-deficient medium.
5.1.4 Gene Expression and the Cell Cycle

The effects of auxin on the cell cycle has been investigated by several research groups (Yeoman and Mitchell, 1970; Meyer and Chartier, 1981). With protoplasts, both auxin and cytokinin are required for mitosis; the absence of one or other growth regulator causes the cells to be G1 arrested. Yeoman and Mitchell (1970) postulated that auxin may act at a specific G1 cell cycle control point in artichoke tubers. Cytokinin does not alter the protein synthesis profile in tobacco protoplasts (Meyer and Chartier, 1981) but was found to decrease the abundance of a 33 kD polypeptide in cultured pith and leaf tissue of tobacco (Eichholz et al., 1983).

There are considerable problems when using a protoplast system to study the effects of exogenous growth regulators, as freshly isolated protoplasts reform their cell walls quickly. Proteins involved in this cell wall reformation can be confused as being either hormone-induced proteins or osmotic shock-induced proteins (Fleck et al., 1982). Meyer et al. (1984) recognised this factor and attempted to separate proteins involved in cell wall synthesis from those responding to hormones by using cell wall synthesis inhibitors. They concluded that proteins $\alpha$ and $\beta$ recognised by Meyer et al. (1984) were involved in mitosis. Both of these proteins are affected by the presence of auxin; $\alpha$ is detected only in the presence of auxin and $\beta$ is increased by auxin (Meyer and Chartier, 1981). It is doubtful that these two proteins were involved in cell cycle regulation as cell cycle specific proteins have not been detected in synchronised mammalian cells in experiments using higher resolution methods (Bravo and Celis, 1980). Furthermore, recent cell cycle analysis of yeast mutants, showed that functional proteins involved in the cell cycle are present throughout the cell cycle and that the cell cycle is controlled by cell cycle phase dependent phosphorylation of these proteins (see section 5.1.5).

A callus specific polypeptide was identified in callus from epicotyl and embryo explants of sorghum. This polypeptide was glycosylated with a molecular mass of 27 kD and an isoelectric point of between pH 6.9-7 (Wozniak and Partridge, 1988). This polypeptide was not found in any mature organs except in crown tissues at very low abundance, but its abundance could be increased by the exogenous application of auxin (Wozniak and Partridge,
Wozniak and Partridge (1988) speculated that this protein has a role either in cellular differentiation or as an auxin binding protein.

Analysis of the complexity of poly(A)$^+$ RNA in auxin depleted and auxin treated soybean hypocotyl and suspension cultured cells, revealed that auxin decreased the complexity of poly(A)$^+$ RNA (Ulrich and Key, 1988). Saturation single copy analysis of auxin deficient and auxin treated suspension cultured cells revealed that auxin deficient cultures contained 57,000 diverse sequences and auxin treated cultures contained 41,000 diverse sequences (Ulrich and Key, 1988). Mixtures of poly(A)$^+$ RNA from both auxin depleted and auxin treated hypocotyl and suspension cultured cells showed that 20,000 sequences were shared between auxin depleted suspension cultures with auxin treated or depleted hypocotyls, but were not present in auxin treated cultured cells (Ulrich and Key, 1988). So auxin treatment can cause a dramatic change in complexity of poly(A)$^+$ RNA and subsequently in protein complexity (Ulrich and Key, 1988).

5.1.5 Cell Cycle Control Mechanisms

The eukaryotic cell division cycle is generally thought to have three control or transition points. These are, firstly the conversion of a quiescent cell to be competent to become a proliferating cell; secondly, the initiation of DNA synthesis, and finally the induction of mitosis in cells that contain newly-replicated genomes. The above key events are controlled by an interacting network of regulatory factors. Proteins implicated in the control of the cell cycle are highly conserved from yeasts to vertebrates and therefore the molecular regulation of mitosis in eukaryotes should be fundamentally similar (Dunphy and Newport, 1988).

A protein called maturation-promoting factor (MPF) was identified in M phase cells of frogs. MPF is stored in an inactive form in *Xenopus* oocytes, and the translation of an activator is required for its subsequent activation. It was observed by Dunphy and Newport (1988) that meiotic metaphase was induced by injected MPF extracted from metaphase arrested frog cells, in G2/prophase-blocked oocytes. Many cellular proteins are hyperphosphorylated during mitosis (Davies et al., 1983) consequently the proteins involved in phosphorylation reactions have been extensively investigated. Bravo and Celis (1980) found that there were no
polypeptides specific to the cell cycle, but they did detect changes in the relative abundance of certain polypeptides. Many of these proteins oscillate in abundance in relation to the cell cycle.

MPF is a phosphoprotein which has been recently purified and was found to contain two polypeptide subunits of 32 kD and 45 kD respectively (Lohka et al., 1988). The phosphorylase activity of MPF is selective and histone H1 is the best identified substrate. The activation of MPF causes interphase cells to enter mitosis, with concomitant chromosome condensation and nuclear envelope disassembly. Recently it was observed that the 32 kD component of MPF is identical with the 34 kD product of the cdc2 gene of fission yeast (Dunphy and Newport, 1988). This gene, cdc2, is central in controlling cell division, and is found to be essential for the initiation of DNA synthesis as well as for the proper execution of mitosis (Simanis and Nurse, 1986; Beach et al., 1982). The product of the cdc28 gene of budding yeasts is the homologue of cdc2 (Dunphy and Newport, 1988) and has kinase activity identical to all other cdc2 gene products. The cdc2 gene is highly conserved in evolution and is found in human cells as well. The product of cdc2 is a 34 kD protein; the human form of this protein can substitute functionally for its counterpart in yeasts (Lee and Nurse, 1987).

It is well documented that cdc2 interacts with a number of other genes for correct mitotic control. These genes include suc1, cdc25, wee1, and nim1. Apart from suc1, little is known regarding the biochemistry of these genes. The gene suc1 encodes a 13 kD protein (p13) which can exist in a complex with cdc2 kinase (Hindley et al., 1986; Brizuela et al., 1987). Over expression of suc1 slows down the rate of cell division in wild type yeasts but overexpression of suc1 rescues mutants with a temperature sensitive lesion in cdc2 (Dunphy and Newport, 1988). Dunphy and Newport (1988) suggests that the intracellular concentration of p13 might be critical for faithful division control. The gene cdc25 is an activator of cdc2 and wee1 is a negative regulator of cdc2 whilst nim1 counteracts the inhibitory effects of wee1 (Dunphy and Newport, 1988). Purified p13 can regulate the function of Xenopus MPF in a cell free system; micromolar concentrations of p13 block the ability of interphase arrested Xenopus egg extracts, to go into mitosis (Dunphy et al., 1988). By affinity chromatography to p13, two polypeptides of 34 kD and 42 kD were purified. These were subsequently shown to be the
frog homologue of $cdc_2$, as the 34 kD peptide cross reacts with antibodies specific to $cdc_2$ (Dunphy and Newport, 1988).

A M-phase-specific kinase which shows a preference for histone H1 (H1 kinase) was purified from starfish and was found to be identical to $cdc_2$ (Labbe et al., 1988). Histone H1 becomes hyperphosphorylated during mitosis but does not appear to be the sole target for MPF. In human cells $cdc_2$ kinase is inactive throughout interphase but is active upon entry into mitosis. The stimulation of $cdc_2$ kinase correlates with the formation of a complex with a 62 kD protein, that becomes phosphorylated in an in vitro kinase assay (Dunphy and Newport, 1988). In *Saccharomyces cerevisiae*, $CDC28$ kinase activation was observed to correlate with binding of a 40 kD protein that acts as a phosphate acceptor in vitro (Wittenberg and Reed, 1988). As previously mentioned, the active MPF contains a 34 kD protein and a 40-45 kD protein. At the end of the cell cycle the 62 kD protein (human cells) and the 40 kD (budding yeast) protein dissociates from the $cdc_2$ subunit. This results in the inactivation of MPF and the cessation of the cell cycle. Recently it was demonstrated that MPF inactivation results in the phosphorylation of $p34^{cd_2}$, in this form the protein has only weak protein kinase activity (Gautier et al., 1989). The $p34^{cd_2}$ H1 kinase activity appears to oscillate during the cell cycle of *Xenopus* as a result of phosphorylation/dephosphorylation (Gautier et al., 1989).

There is evidence that a protein displaying oscillating abundance was important in controlling the cell cycle, and that the translation of this protein is required for the progression of the cell cycle (Dunphy and Newport, 1988). This protein was later identified as being cyclin in sea urchin embryos. Cyclins have a molecular weight of between 50-60 kD and display cell cycle-specific accumulation. They accumulate through interphase and disappear at the end of mitosis (Dunphy and Newport, 1988). Mitosis is induced if the mRNA encoding cyclin is injected into frog oocytes (Pines and Hunt, 1987). The $cdc_13$ gene product of fission yeasts interacts with $cdc_2$ in an allele-specific manner and recently, has been identified as being strongly homologous to clam cyclin and sea urchin cyclin (Solomon et al., 1988). Dunphy and Newport (1988) postulate that the evidence favours the 62 kD protein associated with $cdc_2$ from humans, to be a cyclin. The 40 kD protein from frogs and budding yeast is smaller than the identified cyclin but Dunphy and Newport (1988) have postulated that it
represents cyclin homologue or unidentified regulators of cdc2.

A protein called proliferating cell nuclear antigen (PCNA) can increase the efficiency of chain elongation stage of DNA replication 5 fold (Prelich et al., 1987) and can also raise the activity of calf thymus DNA polymeraseΔ up to 1000 fold (Prelich et al., 1987; Bravo et al., 1987). However, PCNA is not required for the formation of the pre-synthesis complex on the template DNA but acts later in DNA replication when chain elongation is occurring (Prelich et al., 1987). PCNA is synthesised and accumulated in the nucleus only during DNA synthesis (Blow, 1987). It is likely that the presence of PCNA can control eukaryotic DNA replication in conjunction with MPF and the phosphorylation cascade system.

There is little detail known about the role of these proteins in the plant cell cycle. However, it has been demonstrated that, like the animal and yeast cell cycles, there are very little transcriptional changes in synchronised cell suspensions during the cell cycle; only a small number of transcripts displayed changes in abundance during the cell cycle (Kodama et al., 1989). With the same synchronised system it was demonstrated that there were some qualitative and quantitative differences of proteins observed during the cell cycle (Kodama et al., 1989). These differences were thought to be due to translational or post-translational regulation and not transcription (Kodama et al., 1989). It is assumed that a similar system of cell cycle regulation exists in plant cells although it has not been proven yet. Therefore an examination of transcriptional differences in asparagus cells would be unlikely to show cell cycle dependent changes.

5.1.6 General Effects of Wounding

As mentioned in chapter 1 wounding is an expression of the plants plastic response and is a complex phenomenon. This is partly due to wounding being a broad term and to the numerous changes that occur as a result of wounding. The synthesis of the hormone ethylene can be induced by wounding as well as by other stresses. It has been suggested that stress-induced ethylene may enable the plant to cope successfully with the trauma (Yu and Yang, 1980). For example wound-induced ethylene may play a role in stimulating the growth of a seedling as it emerges through the soil (Yu and Yang, 1980). Wounding has also
been demonstrated to result in the formation of a systemic signal in tomato plants which results in the weakening of cell membranes (Walker-Simmons et al., 1984). These two examples only represent a small part of the diversity of responses induced by wounding. This complexity in response together with different systems which have been used to examine various aspects of wounding, have made generalisations difficult to make. Therefore the observations made on these systems should only be used as a guide to what might be expected happen as a result of wounding in asparagus.

5.1.7 Wound Healing

Wound healing has mainly been analysed in anatomical and histological terms. There are however examples where analysis of cell sizes and cell number have been carried out during wound regeneration (Wilson and Grange, 1984; Yeoman and Aitchison, 1973). This type of analysis has made it possible to divide wound healing into three different phases. The initial phase is the lag phase which lasts 2 days in Capsicum annum and Lycopersicon esculentum, followed by a division phase which lasts 2-10 days and finally a differentiation phase (Wilson and Grange, 1984). The lag phase is characterised by an increase in cell metabolic activity (Yeoman and Aitchison, 1973) with no cell enlargement. The division phase is characterised by cell enlargement and cell division; cells in this phase are regarded as being fully dedifferentiated and meristematic (Yeoman and Aitchison, 1973) and the differentiation phase is characterised by the formation of differentiated structures such as vascular and cork cambium (Rittinger et al., 1987). This thesis only really covers the first two phases during which dedifferentiation occurs.

5.1.8 Biochemical Changes Induced by Wounding

Several biochemical and developmental changes take place during wounding, for instance there has to be cell cycle reactivation for cell proliferation to occur. Precursors for lignin and suberin production have to be synthesised and synthesis of proteins involved in wound healing has to be promoted. It has also been observed that there is an increase in free radicals by the activation of hydrolase activity (Thompson et al., 1987) as a result of wounding. This
activated oxygen species can damage lipid membranes. The plant decreases this threat by increasing the synthesis and activity of catalase (Esaka et al., 1983) and peroxidase (Thompson et al., 1987). Some of this activated oxygen activity is utilised in the formation of lignin, and so could be part of the plant's wound repair system.

5.19 Systems Used for Analysis of Wounding

There have been several basic types of plant systems used to study wounding at a molecular level; two examples include the wounding of storage organs (Kahl, 1978) and wounding of mature tissues (Davies and Schuster, 1981 a & b; Graham et al., 1986) and both are characterised by not containing any rapidly dividing cells. There are disadvantages associated with these systems used to examine wounding. With storage tissues only a tissue layer 2-3 cells thick below the wound site respond by cell proliferation. The majority of cells in the organ do not respond to wounding by dedifferentiation and, unless they are removed, a dilution of the wound-induced changes in gene expression will occur. Most analysis on wounding has not been carried out with a true control (Davies et al., 1986). This is especially true for experiments that have been based on radioactive in vivo labelling, where it is almost impossible to incorporate label into protein of a non-wounded control. This has lead to differences of opinion about the rate of protein synthesis during wounding. Wounding leads to the formation of polysomes in storage tissues or mature tissues (Kahl, 1978; Davies and Schuster, 1981 a & b), but wounding of actively growing tissue has either no effect (Davies and Schuster, 1981 b) or reduces the numbers of polysomes (Theillet et al., 1982). However, recently, Davies et al. (1986) found that the level of monosomes to polysomes did not affect the rate of protein synthesis and that wounding resulted in a massive transient fall in protein synthesis which eventually returned to unwounded translation levels.

5.1.10 Wound Induced mRNA's and their Associated Proteins

Recently numerous mRNA's and their associated proteins induced by wounding have been identified, and in some cases cloned. These proteins include phenylalanine ammonia lyase (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 (PR; Van Loon, 1985), hydroxyproline-rich glycoproteins (HRGP; Chen and Varner, 1985), chalcone synthase (Cramer et al., 1985), chalcone isomerase (Mehdy and Lamb, 1987), stilbene synthase (Vornam et al., 1988), peroxidases (Espelie et al., 1986) and chorismate mutase (Kuroki and Conn, 1988).

5.1.11 Phenylpropanoid Pathway Enzymes

Enzymes such as PAL, 4-coumarate CoA ligase, chalcone synthase and chalcone isomerase are active in the phenylpropanoid pathway which is stimulated by many environmental factors, one of which is wounding. This biosynthetic pathway leads to the formation of aromatic nine carbon-containing compounds, which are precursors for lignin synthesis, flavonoid pigment synthesis and isoflavonoid-type phytoalexins (Lamb et al., 1989). These products of the phenylpropanoid pathway are implicated directly with wound healing and in the plants defence towards pathogen infection.

5.1.12 Proteinase Inhibitors and Thionins

Proteinase inhibitors reduce the palatability of tissue to insects and are localised in vacuoles (Walker-Simmons and Ryan, 1977). Their expression is induced by wounding but can also be induced by pectic cell wall fragments called proteinase inhibitor inducing factors (Walker-Simmons et al., 1984). These proteinase inhibitors have been extensively analysed in solanaceous plants. Wounding any leaf on a tomato or potato plant for example leads to an induction in proteinase inhibitor synthesis in all their leaves. There is a rapid synthesis and accumulation of both proteinase inhibitor I and II mRNA after wounding, which begins at 2 to 4 hr post-wounding and peaks by 8 hr post-wounding. This protein synthesis is followed by a gradual decline in accumulation and by 20 hr accumulation had nearly ceased (Graham et al., 1986). This pattern of accumulation can be changed by continuous wounding of leaves over several hours and will lead to inhibitor 1 mRNA representing 0.5 % of total poly(A)^+ RNA (Graham et al., 1986).

Thionins are highly abundant polypeptides with antifungal activity that have been detected
in the cell walls of barley leaves (Bohlmann et al., 1988). Similar proteins (Thionins) are found in dicot plants as well, where they are found to be leaf specific. They are encoded by a multigene family that is confined to chromosome 6 in barley, and their synthesis can be triggered by wounding or by pathogen infection.

5.1.13 Pathogenesis Related Proteins

Pathogenesis related proteins are a group of proteins synthesised in response to a whole range of environmental stimuli. They are characterised by their low molecular weight (10-40 kD), large charge differences (pi 4-10.7), solubility at low pH, resistance to protease action, vacuolar and extracellular localisation (Van den Bulcke et al., 1989) and serological similarity of some isoforms (Van Loon, 1985). The PR proteins can be serologically separated into 3 groups of related proteins, the PR-1; PR-2, N and O; and the P and Q group. The biological function of these PR proteins has recently been identified, PR-O, -N and -2 all have 1,3-β-glucanase activity (Kauffmann et al., 1987). The group of PR proteins PR-P and PR-Q have been identified as endochitinases (Legrand et al., 1987). PR proteins have also been identified in monocots e.g. in maize, where four have been identified as 1,3-β-glucanases (Nasser et al., 1988). PR proteins can be induced by plasmolysis, polyacrylic acid derivatives, salicylic acid, senescence, ethylene, high concentrations of plant hormones, culture filtrates of pathogens, TMV infection, wounding and flower development. They have also been found in both habituated and non-habituated callus resulting from the combined action of cytokinins and auxins (Van Loon, 1985; Kombrink et al., 1988; Van den Bulcke et al., 1989).

5.1.14 Stilbene Synthase, Peroxidase and Chorismate Mutase

Stilbene synthase catalyses the formation of 3,4,5-trihydroxystilbene which functions as a phytoalexin in grape and peanuts. Vornam et al. (1988) observed that subculturing peanut suspension cultures induced synthesis of stilbene synthase. Wounding and fungal cell fragments can also induce synthesis of stilbene synthase. However, the extent and the time taken for induction was dependent on the growth phase of the suspension culture, e.g. induction of log phase cultures led to less selective induction but more pronounced synthesis.
of stilbene synthase, whilst induction of stationary phase led to a greater selectivity of induction but slower synthesis of stilbene synthase (Vorman et al., 1988).

Peroxidases exhibit altered isozyme patterns depending on the tissue type, developmental stage of an organ and in response to environmental stimuli (Cassab and Varner, 1988). In fact tissue samples from leaf, root, pith and callus of tobacco showed different peroxidase isozyme “fingerprints” (Cassab and Varner, 1988). The 12 isozymes of tobacco have been classified into 3 subgroups; the anionic (pI 3.4-4), the moderately anionic (pI 4.5-6.5) and the cationic (pI 8.1-11). The moderately anionic peroxidases are wound-induced and are localised in cell walls and have moderate activity towards lignin precursors (Lagrimini and Rothstein, 1987; Cassab and Varner, 1988). The anionic peroxidases accumulate in wound-healing potato tubers, are localised in the cell wall and have high activity towards the polymerisation of cinnamyl alcohols in vitro (Cassab and Varner, 1988; Espelie et al., 1986). The cationic peroxidases are localised in the central vacuole and catalyse the formation of hydrogen peroxide from NADH and water (Cassab and Varner, 1988). These enzymes are thought to be responsible for generating free radicals for polymerisation of lignin precursors. Peroxidase has also been implicated in cross-linking proteins, hemicellulose and ferulic acid (Cassab and Varner, 1988). Bochert (1978) observed a strong correlation between peroxidase activity and suberization during wound healing. Wound healing is defined as the physiological, cytological and biochemical changes involved in tissue regeneration post-wounding. Suberin is a polymer of aliphatic and aromatic domains and the aromatic domain is similar to lignin (Espelie et al., 1986). Peroxidase content of suberizing cells is ten times higher than that of adjacent dividing cells and such suberizing cells contain different isoperoxidases from adjacent dividing cells (Bochert, 1978). An anionic peroxidase has been implicated in suberization during wound healing (Espelie et al., 1986). This same anionic peroxidase has been observed during abscisic acid (ABA)-induced suberization in a potato suspension culture (Cottle and Kolattukudy, 1982). The anionic peroxidase showed its largest increase in concentration between 4 to 6 days post-wounding and was associated with the inner side of cell walls in the periderm layer (Espelie et al., 1986). There is a negative correlation between cell wall peroxidase activity and growth rate. For example with gibberellin-induced cell expansion in
suspension cultured spinach cells, there was a suppressed secretion of peroxidase, a reduction in PAL activity and an accumulation of wall-esterified ferulate (Fry, 1979; Fry, 1980). Therefore peroxidase has many functions, but it is most important in plant defence, in lignification and in the crosslinking of extensin monomers and feruloylated polysaccharides (Cassab and Varner, 1988).

Chorismate mutase activity increased during the wounding of potato tubers and was activated by tryptophan (Kuroki and Conn, 1988). Incubation with cyclohexamide inhibited the wound response and suggested de novo synthesis of chorismate mutase (Kuroki and Conn, 1988). Chorismic acid is located at a branch point between tryptophan biosynthesis and phenylalanine and tyrosine biosynthesis. Chorismate mutase is involved in the regulation of flux among the 3 aromatic amino acids and is reported to exist in multiple forms in a number of plant species (Kuroki and Conn, 1988).

5.1.15 Hydroxyproline-Rich Glycoproteins

Hydroxyproline-rich glycoproteins (HRGP) are a group of cell wall proteins, often called "extensins", which are found in most dicotyledonous and some monocotyledonous plants e.g. Zea mays (Kieliszewski and Lamport, 1987). HRGPs are characterised by a conserved pentapeptide Ser-Hyp-Hyp-Hyp-Hyp amino acid sequence and can be present as soluble or insoluble forms (Chen and Varner, 1985 a). These HRGP are induced by wounding, fungal elicitors or infection (Cassab and Varner, 1988; Corbin et al., 1987; Chrispeels et al., 1974; Esquerre-Tugaye and Lamport, 1979) and become insolubilised in cell walls with time. HRGPs are coded for by a large group of genes. Different transcripts and proteins are produced in response to different environmental stimuli. For example, there are three different transcripts for the apoprotein of HRGP induced by fungal elicitors, wounding or infection (Corbin et al., 1987). Each of these transcripts is encoded by a separate or low copy number gene and each transcript exhibits marked differences in accumulation under different stress conditions (Corbin et al., 1987). Corbin et al. (1987) used this observation to suggest the operation of several distinct intercellular stress signal systems in higher plants. The HRGPs have been postulated to be involved in controlling cell expansion (Wilson and Fry, 1986), in cell wall
strengthening (Stafstrom and Staehelin, 1988) and in defence against infection (Corbin et al., 1987).

5.1.16 Light, *Stress*, and Chemically-Induced Gene Expression

Changes in carbohydrate status has been demonstrated to affect protein profiles and gene expression in *Pennisetum americanum* (Baysdorfer and Van Der Wouder, 1988). One such enzyme effected by carbohydrate status is manganese superoxide dismutase (MnSOD). This protein can also be induced, to a lesser extent by wounding. Sucrose is the preferred carbohydrate that is responsible for the a massive induction of MnSOD (Bowler et al., 1989). This protein is a nuclear encoded mitochondrial enzyme and catalyses the dismutation of superoxide radicals to oxygen and hydrogen peroxide. These superoxide radicals are produced by enhanced respiratory oxidation of sugars in the mitochondrion. This increase in mitochondrial sugar respiration is often stress related, e.g. conditions of metabolic stress, and the pathogenesis response can induce this form of respiration (Bowler et al., 1989). Bowler et al. (1989) observed that MnSOD synthesis was increased by approximately 20-fold in suspension cultured *Nicotiana plumaginifolia* cells. Since MnSOD is widely distributed in eukaryotes and prokaryotes, it is therefore expected that the culture medium would affect gene expression and protein synthesis in cultured asparagus cells. Stress-induced translational alterations have also been observed to occur in maize (Wu et al., 1988). A 57 kD protein accumulated under stress in maize leaves and was associated with polysomes. This protein was found to inhibit polysome translational activity *in vitro* (Wu et al., 1988). Stress also contributes to the dissociation of polysomes and decreased protein synthesis (Wu et al., 1988).

Since the asparagus cell cultures are incubated in darkness, it is expected that genes which are light up-regulated would be down-regulated in such cells and likewise genes which are light down-regulated might be up-regulated. Light-induced genes, such as the nuclear encoded small subunit ribulose bisphosphate carboxylase (ssu), and light harvesting chlorophyll a/b binding protein (cab), are controlled by light and their expression is greatly reduced in the dark (Link, 1988; Kasemiv et al., 1988; Prioul and Reyss, 1987). There is evidence to suggest that the level of control for the induction of these light regulated genes
is transcriptional (Gallagher and Ellis, 1982; Gallagher et al., 1985). The well characterised examples of light down-regulated genes are phytochrome and NADPH-protochlorophyllide oxidoreductase (per). They represent only a small percentage of dark up-regulated genes (Hershey et al., 1984). Changes associated with light depravation should occur in the dark cultured asparagus cells. Scheer and Parthier (1982) have observed in *Euglena gracilis* cells, exposed to darkness and carbon-containing media, a functional decomposition of the photosynthetic apparatus. Since similar conditions occur in the asparagus cell cultures, it is expected that proteins involved in this degradation will be observed. Similarly nuclear genes involved in the maintenance of chloroplast photosynthetic components, should be down-regulated (Scheer and Parthier, 1982, see chapter 4).

5.1.17 Expected Changes During Dedifferentiation

Changes that are expected to occur during dedifferentiation can be separated into 3 groups; morphological changes, physiological changes and biochemical changes. The morphological changes expected during dedifferentiation are; redistribution of plastids (observed in chapter 3), increase in vacuolar size, cell expansion, cell division, changes in nucleolar morphology (see chapter 3) and an increase in vesicular fusion (Fransz and Schel, 1987). The major changes in physiology expected during dedifferentiation would be the switch in mode of metabolism from photoautotrophic to heterotrophic (see chapter 4) as well as a switch from quiescent to dividing cells. The biochemical changes expected to occur during dedifferentiation include; gene expression related to stress and medium constituents, synthesis of cell walls and changes in composition to contain less cellulose and an altered arabinose/xylose ratio (Blaschek et al., 1981), an increase in ornithine decarboxylase activity (Chiriqui et al., 1986) as well as changes in the type of lipids synthesized and isoperoxidase profiles (Goldberg et al., 1986). The above changes are expected to contribute to an altered protein synthesis profile during dedifferentiation.
5.1.18 Approaches Used to Examine Developmental Pathways

Several different approaches have been used to study gene expression and/or protein synthesis in plant developmental pathways. All these approaches have inherent advantages and disadvantages over each other.

5.1.18.1 Analysis of Total Protein.

The most simple approach is to study total protein extracts separated on 1 or 2-D polyacrylamide gels by direct staining with Coomassie blue or silver nitrate. This approach has been used successfully to compare protein profiles in different organs of Japanese morning glory (Bassett et al., 1988). The disadvantages of such an approach are that it has a poor detection limit as it requires more than 5 ng total protein per polypeptide spot in order to be detected; hence low abundance proteins which may be associated with regulation of development cannot be detected. Also, a further disadvantage is that it is impossible to distinguish single gene products from multiple gene products. In addition, post-translational modifications can result in alteration of isoelectric point (p.I) and molecular weight which in turn can cause a single gene product to appear to be different in different organs and thus makes interpretation difficult. These modifications also effectively increase the number of polypeptides associated with a single gene. Further disadvantages with this approach are concerned with technique, i.e. different extraction buffers can lead to different polypeptide patterns due to differential solubility and extractability of proteins. Salts in the extract have to be removed as they can affect resolution and therefore increase the sample volume and a reduce the protein concentration. To obtain good resolution there has to be a very fine balance struck between concentration and volume loaded onto an IEF gel. The methods available for the removal of nucleic acids do lead to artifacts which further complicate interpretation. Further artifacts can be introduced by proteolysis of proteins to yield lower molecular weight products. However, the ability to observe these different modification products can be an advantage, especially if this system is used in conjunction with systems utilising in vitro translation. In this way the extent of transcriptional and post-transcriptional control on dedifferentiation can be elucidated. A further advantage is that direct staining
techniques are simple and quick to perform, and require very little material.

5.1.18.2 In Vivo Labelling Approach

A similar approach is to use *in vivo* labelling with radioactively-labelled amino acids in conjunction with a high resolution gel electrophoresis system (O'Farrell, 1975). This approach offers advantages over the total protein system as only proteins undergoing synthesis are radioactively labelled. This effectively reduces the number of resolved polypeptides on gels and makes data interpretation easier. It also will enable different modified gene products of a single mRNA to be resolved (post-translational modification may be the process that controls differences between different developmental pathways). The disadvantage of this system is that very often plant cells have large endogenous pools of amino acids and so it is difficult to obtain efficient labelling without having to resort to extreme measures such as starvation of cells, which in themselves can induce changes in gene expression. A second disadvantage is that single gene products cannot be identified and with over 1000 polypeptides resolved it is difficult to identify differences.

5.1.18.3 In Vitro Translation Approach

Another well established technique is to run *in vitro* translations of purified poly (A)$^+$ RNA on polyacrylamide gels (Dayton Wilde *et al.*, 1988). With this method it is possible to identify non-processed single gene products with high resolution, also the mRNA populations would directly represent the transcript population in a cDNA library and these mRNA's also give some idea of protein synthesis at the time of harvesting (especially if polysomes are extracted and purified). However, as with other methods there are disadvantages such as the fact that the extraction of RNA is complex and difficult to carry out, mRNA populations may not be translated in proportion to their abundance and such translations may give an inaccurate impression of gene expression; i.e. during stress such as heat shock, certain mRNA's are translated very efficiently and other messages have depressed translations and are protected from degradation by RNase (Wu *et al.*, 1988).
5.1.18.4 Immunological Approach

Another technique which can detect very low concentrations of proteins is the immunological approach where either monoclonal or polyclonal antibodies are generated to total protein extracts of the developmental stage of interest. Both these methods have been used successfully to study somatic embryogenesis in carrot (Choi et al., 1987; and Smith et al., 1988). The advantage of this system is that it can detect nanomole concentrations of protein and therefore is likely to identify low abundance proteins that may modulate development. The disadvantages are that this approach does not offer any information concerning gross protein changes, it is a complicated and time-consuming procedure and many monoclonal antibodies raised may not cross-react with denatured proteins (Smith et al., 1988). Another major problem with such an approach is the number of monoclonal cell lines that can be handled by a single person is small. This will reduce the probability of identifying low abundance proteins that are developmental stage specific.

5.1.18.5 Approaches Involving the Use of Nucleic Acid Hybridisation

There are two high resolution approaches that involve the principle of nucleic acid hybridisation. One approach that has enabled differences in transcript abundance and diversity to be quantified, is sensitive RNA-excess/single-copy DNA hybridisation (Kamalay and Goldberg, 1980). This method uses two $^3$H-single copy DNA fractions isolated by hybridisation with polysomal RNA. One of these fractions is termed $^3$H-mDNA and represents a population enriched for single-copy sequences complementary to leaf mRNA, in the case of Kamalay and Goldberg (1980). The other fraction termed $^3$H-null mDNA was depleted of the above leaf sequences. Therefore the hybridisation of non-leaf polysomal RNAs with $^3$H-mDNA will represent the fraction of leaf mRNAs that are present in the cytoplasm of other organs, while hybridisation with $^3$H-null mDNA identifies mRNAs absent from leaf polysomes. This method will therefore allow the determination of the extent of difference between differentially-expressed genes at different developmental stages. However, this approach will not give any information about any of the proteins encoded by differentially-expressed mRNA's or information about the expression of these mRNA species. Further disadvantages of this method are that
hybridisation conditions such as temperature and concentration of nucleic acids are critical.

Another method that uses nucleic acid hybridisation, is in differential screening of cDNA libraries to obtain differentially-expressed genes. For example if differentially-expressed genes specific to dedifferentiation are required then a cDNA library of RNA extracted from dedifferrentiating cells is first constructed. To identify dedifferentiation-specific genes the library would be screened with 1st strand cDNA from cladodes and dedifferrentiating cultured cells. Any colonies or plaques hybridising with one sample of cDNA but not the other is differentially expressed. The genes that are dedifferentiation specific are colonies or plaques that do not hybridise with the cladode cDNA but with the dedifferrentiating cell cDNA. Additional information can be deduced from the construction of such libraries, for example the relative abundance of differentially expressed transcripts can be calculated. The expression of such transcripts can be determined by Northern blots. The disadvantage of this approach is that it requires high quality RNA for the construction of cDNA libraries, it is often difficult to obtain large numbers of recombinants and does not directly give any information concerning the gross protein changes. For these reasons, this approach is commonly used in conjunction with other approaches, such as in vitro translations to enable a more complete picture of gene expression to be built up (Logemann et al., 1988; Davison et al., 1987).

5.1.18.6 An Approach Based on DNA Copy Number

A method based on cloning digested Bam HI DNA fragments which changed in intensity between callus and leaves, has been used to examine the copy number changes induced by dedifferentiation in rice (Kikuchi et al., 1987). This approach was used to study rice dedifferentiation because anther cultured rice tissue was found to have increased chromosome numbers (Kikuchi et al., 1987). This approach is very time consuming, does not give any idea of changes in protein and mRNA complexity and may be applicable to some systems only. It was observed that dedifferentiation of asparagus did not result in polyploidy (Paul et al., 1989).
5.1.19 The Adopted Approach

Early pulse chase experiments using $^{35}$S methionine to in vivo label newly synthesised proteins did not work efficiently, so this approach could not be used with much utility in Asparagus. The approach that I decided to use for this thesis was in vitro translation of mRNA followed by one or two dimensional gel electrophoresis of translated products. One dimensional sodium dodecyl sulphate (SDS) polyacrylamide gels did not give the required resolution (see Fig 5.5), though a newly-synthesised polypeptide band (16 kD) could be identified. The two dimensional gel electrophoresis system chosen included a broad pH range urea equilibrium isoelectric focusing gel (IEF) first dimension and a denaturing SDS polyacrylamide gel second dimension. The rational of this approach is that proteins are separated on the basis of their charge in the first dimension and on the basis of their size in the second dimension. This method has been used to obtain high levels of resolution and can resolve down to 0.001 to 0.00001 % of total radioactivity applied (O'Farrell, 1975; Garrels, 1979). Since an average rare mRNA constitutes between 0.001 % to 0.005 % of total eukaryotic RNA (Sargent, 1987) and, if these mRNA’s are translated in proportion to their abundance, then their gene products should be resolved, as it is well within the detection limits of the two dimensional gel system (Celis and Bravo, 1984). The broad pH range equilibrium isoelectric focusing gels were chosen in preference to non-equilibrium isoelectric focusing gels (NEPHAGE) because better acidic resolution could be obtained. Since the majority of proteins have pI’s that fall between the pH 4-6 range, an increased resolution of the acid range is preferable to increased resolution of the basic range (obtainable by NEPHAGE). As mentioned earlier, the ultimate aim of the study was to construct a cDNA library and to identify any differentially-expressed clones, if they existed. The use of in vitro translations was the most logical approach to study mRNA profiles in order to decide at which stage during dedifferentiation the cDNA library should be constructed, which should contain differentially-expressed transcripts.

The experiments carried out in chapter 3 optimised the cell culture system but did not identify any cytological markers prior to cell expansion and cell division. But experiments carried out in chapter 4 has shown that a loss of photosynthetic potential occurred just prior
to cell division. This physiological marker was the only identified marker for dedifferentiation prior to cell division. However, the dedifferentiating cells appeared to go through 3 phases during dedifferentiation; cell cycle reactivation, DNA synthesis and the first cell division and the continuation of the cell cycle. It would be interesting to examine transcripts from each of these phases to identify associated proteins.

5.1.20 Expected Gene Expression can be Divided into Three Distinct Phases

Since the initial isolation of the cells involved some stress it was expected that gene expression could be roughly divided into three main phases; an initial wounding and stress phase, a dedifferentiation phase and finally a division phase. By analysis of in vitro translated protein profiles it should be possible to identify protein changes associated with these 3 major phases. It is expected that a proportion of translated mRNA are involved in "house keeping" activities as postulated by Kamalay and Goldberg (1980) and since these represent 55-75 % of polysomal RNA in plant organs, it should be possible to identify such proteins. Treatments such as wounding or exposure to auxin often reduce the numbers of polypeptides translated, with increasing abundance of certain polypeptides and decreasing abundance of others (Meyer et al., 1984 a; Ulrich and Key, 1988; Zurfluh and Guilfoyle, 1980; Logemann et al., 1988; Theillet et al., 1982). Consequently it was expected that this form of gene expression would be encountered during cell culture and that it should be possible to identify the associated gene products. The final class of gene expression expected during cellular dedifferentiation was the synthesis of up-regulated proteins i.e. those which are either not detectable, or are detectable at very low abundance, in cladodes but are greatly up-regulated in cultured cells. It was hoped that such proteins may play a role in the dedifferentiation process and would be good targets for cloning following the differential screening of a c-DNA library.
5.1.21 Summary

Gene expression can be controlled at different levels; at the level of transcription, post-transcription, translation and post-translation. One or all of these processes may be implicated in the control of development. It is not clear the role and extent each of the different controlling points play during dedifferentiation. However, it is clear that post-translational modifications such as phosphorylation, can control fundamental pathways such as the cell cycle. Transcription plays only a minor role in cell cycle control. Therefore, it is quite possible that dedifferentiation can be controlled by post-translational modifications with transcription not playing a significant role. If this is so then the examination of transcripts, by in vitro translation will not give a great deal of information about the controlling mechanisms of dedifferentiation. This method however, will show the transcript population complexity and directly represents the complexity of cDNA libraries constructed from this material. It is therefore an ideal method to examine mRNA populations to identify stages that contained differentially expressed transcripts, if they occur. It is possible to make an assessment of the changes in transcription expected during dedifferentiation. There are however, numerous environmental factors that can cause gross changes in gene expression. Some of these factors are components of the asparagus cell culture medium such as growth regulators, sucrose and salts. Therefore it is expected that wound-induced dedifferentiation involves some transcriptional changes that are a result of the medium but may not be involved in the control of dedifferentiation.

This chapter describes the examination of the mRNA populations in dedifferentiating cultured asparagus cells. The mRNA profiles were examined by using in vitro translation followed by 2-D gel electrophoresis. The most complex population of mRNA containing many up-regulated transcripts as determined from 2-D gel electrophoresis was used to construct cDNA libraries.
5.2 RESULTS

5.2.1 Optimisation of RNA Extraction

High quality mRNA had to be extracted and purified in large quantities from cultured asparagus cells and cladodes both for \textit{in vitro} translations and cDNA synthesis for library construction. It was estimated that 1 mg of total RNA was required to make sufficient poly (A)$^{++}$ RNA for several attempts at constructing a cDNA library. Initially, to test the RNA extraction procedure, RNA was extracted from 6 month old asparagus suspension cultured cells, as large quantities of material were available and the material was mitotically active. The modified Covey and Hull (1981) protocol was very successful and yielded milligram quantities of translatable RNA. However, when this method was used to extract RNA from asparagus cladodes it was considerably less successful. The extracted RNA was found to be contaminated by carbohydrates which proved almost impossible to remove. A further complication was that, what appeared to be pure undegraded RNA did not translate efficiently. This was attributed to very small amounts of spectrophotometrically undetectable tannins and other low molecular weight contaminants which inhibit translation (Roberts and Ranu, 1986; Gordon and Payne, 1976). Eventually, it was determined that the only method available to reduce the effects of these contaminants was to extract RNA from cladodes younger than 5-6 weeks after sowing (when they are used for tissue culture). However, using tissue younger than three weeks after sowing led to considerable problems with carbohydrate contamination. There was therefore a window of approximately two weeks, when good quality RNA could be extracted from cladodes (3-4 weeks after sowing). Carbohydrate contamination was further reduced by watering plants with Hoagland’s solution (Epstein, 1972) once weekly and increasing the intensity of illumination. The effect of these two treatments was to increase growth rate and to reduce the amount of stored starch. Further modifications were made to the RNA extraction protocol (Covey and Hull, 1981) to remove any insoluble contaminants (see chapter 2 & 5.2.12).
5.2.2 Development and Optimisation of pH Gradient and Attained Resolution

The equilibrium isoelectric focusing (I.E.F) gel system had to be set up using equipment built in the faculty workshop. The initial protein extraction and running buffer used was O'Farrell's (1975) lysis buffer. This buffer proved to be unsatisfactory for total protein extractions in asparagus, due to its poor protein solubilising properties. Therefore a series of I.E.F compatible buffers were tested before 2 DMH (see chapter 2) was chosen as the ideal buffer. It proved possible to obtain undegraded protein extracts quickly and efficiently with this buffer.

Initially it was difficult to generate linear pH gradients using O'Farrell's (1975) protocol with ampholytes obtained from Pharmacia. Numerous parameters were changed with no net improvement in the linearity of the pH gradient. A new broad range ampholine obtained from B.D.H. was used in conjunction with cooling (10°C) and different focusing conditions (focus for 4900 V hr at 400 V and then for a further 1500 V hr at 1500 V) enabled reproducible linear gradients to be obtained (see Fig. 5.1). This pH gradient extended from pH 3.6 to pH 8.0, which should allow most proteins to be resolved. When these gels were combined with a second dimension SDS polyacrylamide gel a very reproducible protein profile could be generated (see Fig. 5.2 & 5.3). The polypeptide patterns produced on Fig. 5.2 are the result of different in vitro translations from a single batch of RNA separated on 2-D gels. The ideal test would be to compare polypeptide patterns from the same in vitro translations on several 2-D gels. However, this was not possible, as each in vitro translation does not yield sufficient incorporated counts to run on more than two, 2-D gels. Fig. 5.2 presents a magnified comparison of the most complicated region of the 2-D gels; it can be seen clearly that they display highly reproducible polypeptide patterns. There were noticeable differences in intensity of certain polypeptides, which were probably due to the different efficiency of translation within a single batch of mRNA. The numbers of polypeptides resolved ranged from 100-300, which was equivalent to that obtained in other analyses (Meyer and Chartier, 1981; Bassett et al., 1988; Schuster and Davies, 1983; Theillet et al., 1982). When a rough estimate of the number of counts resolved for polypeptides 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 represented between 0.04-0.09% (+/- 0.0037-0.0058%) of
Fig. 5.1 The pH gradient of isoelectric focusing rod gels.
pH gradient of I.E.F gels

pH

distance in cm
Fig. 5.2 Reproducibility of separate *in vitro* translations of the same batch of RNA run on 2-D gels. Separate *in vitro* translations of mRNA from the same sample of cladodes (A-C). D is a magnification of the most complex parts of gel A-C (140,000 cpm/gel).
Fig. 5.3 Reproducibility of *in vitro* translations of separate batches of RNA run on 2-D gels. A & B are separate translations of the same sample of mRNA from 7 days (140,000 cpm/gel). C & D are separate translations of a second sample of mRNA from 7 days (140,000 cpm/gel).
the total radioactivity applied.

5.2.3 Reproducibility of Translation Profiles from Different Samples of RNA Representing a Specific Stage in Dedifferentiation

There are no good cytological markers for early dedifferentiation (i.e. prior to cell division at 4 days; see chapter 3) and the asparagus system was not totally synchronous. Thus, it was imperative to determine if the rough sampling times, which were only accurate to plus or minus 2 hr (except for 0 days, which is accurate to within 15 min), would contribute to unreproducible translation patterns. The process of sterilisation, mechanical isolation, filtration, washing of cells and plating out normally takes approximately 3 hr to perform (see chapter 2). Therefore, after mechanical isolation day 0 cells were incubated in media before harvesting 3 hr after mechanical isolation. Even though asparagus tissue cultures display very high levels of reproducibility in the frequency and timing of cell division, some cultures were found either to divide earlier (often mistakes in plating density) or to divide considerably later with very low division frequencies (see chapter 3). These cultures were not representative so it was important to determine that cells harvested for RNA extraction came only from cultures which conform to the expected patterns of behaviour in dedifferentiating cells (i.e. that they lose photosynthetic capacity and that they divide at the right time with the right frequency). From chapter 4 it was determined that the loss of photosynthetic capacity determined in an oxygen electrode was a reproducible marker for dedifferentiation that preceded cell division. Therefore as a rule, oxygen electrode measurements were made every day (see chapter 4) and several culture dishes were kept over after harvesting to ensure that the cytological and physiological markers for dedifferentiation were achieved as normal. In order to test the validity of this approach the translation products of two individual samples of mRNA from two separate cultures, which had fulfilled the above criteria were compared on 2-D gels (see Fig. 5.3). Any identified polypeptides which are referred to in this thesis were all checked for the presence or absence in RNA extracted from at least two individual samples of cultured cells.
5.2.4 Numbers of Polypeptides With Time in Culture

It is possible to resolve between approximately 140-300 different polypeptides on these two dimensional gels (see Fig. 5.4). Fig. 5.4 shows the maximum number of polypeptides resolved per day of tissue culture; the data represents the optimal separation achieved from the most efficient translations on the best 2-D gels. The patterns are consistent although there are differences in the numbers of maximum resolved polypeptides. It is thought that these differences were due mainly to an interaction between different efficiencies of translation, as separate translations of the same sample of mRNA often resulted in totally different incorporated counts (data not shown), rather than a real change in mRNA abundance and complexity.

The first time point in sampling of extracted cells was called 0 days as it represented gene expression of the cells when they are initially plated out. The polypeptide numbers were low at 0 days and then increased to approximately 277 resolved polypeptides by day 2 of culture. The maximum numbers of detectable polypeptides (Fig. 5.4) increased gradually with time in culture to a maximum at day 5. The numbers of resolved polypeptides decreased after six days, to a stable number between 7 days and 2 months of culture. It was also observed that cladode samples which included apical tips (WC, Fig. 5.4) contained more resolved polypeptides than non-meristematic cladodes.

5.2.5 Are There Any Resolvable Protein Changes Between Different Aged Cultures?

As mentioned in the introduction (section 5.1) it is not known if the major mechanism of control of gene expression during dedifferentiation is post-translational or transcriptional. Although a single "novel" polypeptide band (DD-1) was identified on 1-D SDS polyacrylamide gels (Fig. 5.5), no idea of the extent of transcriptional or post-translational control on gene expression could be determined. The intensity of several bands fell at day 0 (see Fig. 5.5), and then the abundance of several of these translation products increased to cladode levels by day 1 (see Fig. 5.5). Furthermore it was observed that a new polypeptide band (16 kD, DD-1) appeared by day 1 and was maintained thereafter (see Fig. 5.5). Therefore, if a comparison of the translational profiles of mRNA from different developmental stages was
Fig. 5.4 Polypeptide numbers with time in culture. Maximum number of polypeptides from *in vitro* translated mRNA on 2-D gels with time in culture (140,000 cpm/gel). nmc) non-meristematic cladodes (decapitated cladodes), wc) whole cladodes, 0) 0 day, 1) 1 day, 2) 2 days, 3) 3 days, 4) 4 days, 5) 5 days, 6) 6 days, 7) 7 days, 2 mths) 2 months in culture.
Polypeptide numbers with time in culture

days in culture

resolved polypeptide numbers
**Fig. 5.5** *In vitro* translated polypeptide profiles on a 1-D gel. One dimensional SDS gel (12 %) of *in vitro* translated mRNA from 1) cladodes, 2) 0 day, 3) 1 day, 4) 2 days, 5) 3 days, 6) 4 days, 7) 5 days, 8) 6 days, 9) $^{14}$C methylated molecular weight markers, 10) water control (50,000 cpm/lane).

A "novel" band is arrowed (16 kD).
made, with a high resolution system, an idea could be obtained of the level of transcriptional control over gene expression.

As can be seen quite clearly in Fig. 5.6, there are considerable differences in the polypeptide patterns between the developmental stages. Polypeptides displaying different forms of expression can be easily identified. There are polypeptides which are shared by all the different stages; these may be products of the expression of "house keeping" genes. There are polypeptides that are down-regulated and there are those which are up-regulated based on polypeptide intensity (see Fig. 5.6). Two different types of up-regulated polypeptides were observed in cultured cell samples. The first type were designated type I up-regulated gene products and represented polypeptides present at low or medium abundance in the cladodes which were up-regulated by at least 10 fold in tissue culture. The second type were designated type II up-regulated gene products and represented polypeptides present at either very low abundance or undetectable levels in the cladodes which were then greatly up-regulated (more than 100 fold) in tissue cultured cells. Both types of up-regulated gene expression can be observed in Fig. 5.6 B-D.

The benefits of increased resolution offered by 2-D gel electrophoresis was strikingly demonstrated in the low molecular weight region of these gels (see Fig. 5.6). The 16 kD band identified on 1-D gels (see Fig. 5.5, DD-1) was resolved into 3-5 separate polypeptides depending on mRNA sample. Many polypeptides found in cladodes were down-regulated, to the extent that some were undetectable by 1 day of culture (polypeptides enclosed within circle, Fig. 5.6). Type I up-regulated polypeptides were present at all the different developmental stages (example polypeptides enclosed within triangles, Fig. 5.6). There were also numerous type II up-regulated polypeptides that were either not present in cladodes or at very low abundance but is found in each of the developmental stages shown in Fig. 5.6 enclosed within squares. The 16 kD band in Fig. 5.5 (DD-1) was resolved by 2-D gels into initially 3 major polypeptides at day 1 of culture; by day 5 of culture the 16 kD band was resolved into 6 major polypeptides. This region of the 2-D gels suggested that there were considerable transcriptional changes occurring during dedifferentiation. When this region of the 2-D gel was considered, together with overall translated polypeptides profiles, it provided
Fig 5.6 A comparison of 2-D gel profiles of non-meristematic cladode, 1 day, 5 days and 6 months of culture. A) 2-D gel of \textit{in vitro} translated mRNA from non-meristematic cladodes; (140,000 cpm) displaying the cladode pattern of protein synthesis. 
B) 2-D gel of \textit{in vitro} translated mRNA from 1 day old cultured cells; (140,000 cpm) displaying a pattern influenced by wounding and dedifferentiation. 
C) 2-D gel of \textit{in vitro} translated mRNA from 5 day cultured cells; (140,000 cpm) displaying a pattern influenced by cell division (50%).
D) 2-D gel of \textit{in vitro} translated mRNA from 6 month suspension cultured cells; (140,000 cpm) displaying a pattern of a stable suspension culture.

\( \triangle \) = Type I up-regulated polypeptide.
\( \square \) = Type II up-regulated polypeptide.
\( \bigcirc \) = Down regulated polypeptide.
strong evidence that transcriptional control was critical in this developmental pathway. Hence, the conclusion was that the present approach used to study this pathway was adequate and the initial observations were worthy of further detailed analysis. It is interesting to note that there appears to be no major polypeptides of between 17-20 kD on both 1-D and 2-D gels that corresponds to ssu.

5.2.6 Differences in 2-D Gel Profiles of Whole and Decapitated Cladodes

Since no cells from the apical meristem survived mechanical isolation, it was decided to determine whether the 2-D gel pattern of in vitro translated poly(A)^+ RNA from cladodes was identical to that isolated from decapitated cladodes. The cladode RNA sample was extracted from cladodes stripped off 4 week old plants possessing apical tips. The decapitated cladode RNA sample was extracted from cladodes stripped off decapitated 3 week old plants and was designated non-meristematic cladodes. It was necessary to use young plant material, as samples without apical tips yielded considerably less RNA and consequently it was more difficult to remove contaminating carbohydrates. Younger plant material was growing rapidly and contained less starch and other contaminating carbohydrates.

It was discovered that several polypeptides differed between cladodes and decapitated cladodes (boxed polypeptide spots. Fig. 5.7). These polypeptides were either of very low abundance or were undetectable on 2-D gels. Fig. 5.7 gives a distorted view of polypeptide abundance and numbers due to different photographic exposures used during printing. Autoradiographs used in Fig. 5.7 show clearly more polypeptides in cladodes than in non-meristematic cladodes.

5.2.7 Detailed Analysis of 2-D gels and Reconfirmation of Differentially-Expressed Polypeptides

Since the in vitro translation profiles of dedifferentiating cells appeared to be so different from that of cladodes, a series of experiments were designed to simplify, confirm and highlight these apparent differences. Three different approaches were employed to identify and confirm differences in translation profiles. The first of these approaches was to run "mirror images" of the acid ends from IEF gels on the same second dimension gel (see Fig. 5.8C). This enabled the analysis of differences in polypeptide intensity and allowed the identification of "novel" polypeptides. A further approach was to run mixtures of samples from cladode and cultured cells on the same IEF gel (Fig. 5.9C). This type of 2-D gel analysis made it possible to identify "novel" polypeptides on the basis of small differences in pl. If analysis of this more complex gel was used in conjunction with gels of cladode and cultured cells it was possible
Fig 5.7 A comparison of 2-D gel profiles of non-meristematic (decapitated) cladodes and whole cladodes. A) 2-D gel of in vitro translated mRNA from non-meristematic cladodes (decapitated cladode, 140,000 cpm).
B) 2-D gel of in vitro translated mRNA from cladodes (140,000 cpm).
□ = Type II up-regulated polypeptide.
**Fig 5.8 Mirror image method of analysis.**  
A) 2-D gel of *in vitro* translated mRNA from non-meristematic cladodes (140,000 cpm).  
B) 2-D gel of *in vitro* translated mRNA from 5 day cultured cells (140,000 cpm).  
C) 2-D mirror image gel of acid ends of *in vitro* translated mRNA from non-meristematic cladodes (X, 140,000 cpm) and 5 day cultured cells (Y, 140,000 cpm).

□ = Identified type II up-regulated polypeptides (DD-1 group).  
○ = Identified down-regulated polypeptides.  

The acid ends of the IEF gels were flipped over and run side by side on the same SDS PAGE to produce a mirror image.
Fig. 5.9 The mixture method of analysis. A) 2-D gel of *in vitro* translated mRNA from non-meristematic cladodes (140,000 cpm).

B) 2-D gel of *in vitro* translated mRNA from 2 day cultured cells (140,000 cpm).

C) 2-D gel of a mixture of *in vitro* translated mRNA from non-meristematic cladodes (70,000 cpm) and 2 day cultured cells (70,000 cpm).
to identify more polypeptide differences (see Fig. 5.6). In order to reconfirm all observations and to minimise human error, other members of the lab independently reconfirmed the observations; this was the third approach.

Detailed analysis carried out on the gels containing mixtures of mRNA populations (Fig. 5.9C) enabled considerably more type II up-regulated polypeptides to be identified. Consequently a nomenclature had to be devised for naming these type II polypeptides. It was finally decided to name these polypeptides by virtue of their position on 2-D gels; i.e. to name them using an approximate pI and molecular weight grid reference. For example on Fig. 5.10, M44.7 P5.32 was identified, where the approximate molecular weight was 44.7 kD and the approximate pI was 5.32. The pI value was obtained by measuring the distance in mm, from the acid end of the IEF gel and reading the pH value of the graph on Fig. 5.1. The standard error bars represent the range of pH at that point on the gel. So Fig. 5.1 had to be consulted to obtain an idea of the pI range a polypeptide fell in. The "real" pH however, could be several pH units lower than the values on the graph as urea affects pH measurement (Uni, 1971).

The use of "mirror image" gels made intensity comparisons of different polypeptides relatively simple (see Fig. 5.8). Furthermore, type II polypeptides induced in cultured cells could also be quite clearly visualised (see M16 P5.6 a & b, enclosed in square on Fig. 5.8). Only those polypeptides that were observed in all samples of translated mRNA, and which were clearly present, were considered fit for further detailed analysis. Examples of type II polypeptides which were identified at 2 days in culture are shown in Fig. 5.10.

5.2.8 Wounding, Stress and Early Culture Induced Polypeptides

It was quite remarkable that the profiles obtained from in vitro translated mRNA from day 0 (3 hr) showed considerable differences from cladode profiles. There were several cladode polypeptides that were down-regulated within 3 hr (see Fig. 5.11). It was not surprising to observe that there are type I up-regulated polypeptides within 3 hr. It is a well known phenomenon that up-regulation of certain mRNA species occurs during wounding (Thiellet et al., 1982; Stanford et al., 1989; Logemann et al., 1988; Logemann et al., 1989). An example
Fig. 5.10 Identified type I and type II polypeptides discussed in this chapter.

2-D gel of *in vitro* translated mRNA from 2 day cultured cells (140,000 cpm). The type I and type II up-regulated polypeptides referred to in this thesis are indicated above.
Fig. 5.11  Wound, stress and early culture induced polypeptides. Examples of stress-induced gene expression (within square).

A) Type I up-regulated polypeptide M89 P3.7 a + b on 2-D gels of in vitro translated mRNA from 1) cladodes 2) day 0 3) 1 day in culture 4) 2 days in culture.

B) Type II up-regulated polypeptide M41.5 P6.3 on 2-D gels of in vitro translated mRNA from 1) cladodes 2) day 0 3) 1 day in culture 4) 2 days in culture. 140,000 cpm loaded on each 2-D gel.
of such a set of up-regulated polypeptides is provided by M89 P3.7 a & b (see Fig. 5.11A). This set of polypeptides displayed classical "wound gene" expression; they were up-regulated to a large extent by day 0, followed by a drop in intensity to a basal level by day 1 when the lower spot (M89 P3.7 b) was more prominent. This characteristic was continued up to day 7 in culture when analysis was discontinued.

There are type II up-regulated polypeptides that were expressed by day 0, an example of these includes M41.5 P6.3 (see Fig. 5.11B). They displayed different patterns of expression with time in culture. The polypeptide M41.5 P6.3 was first observed at day 0, thereafter the intensity of the polypeptide spot decreased to barely detectable levels by day 1. This polypeptide was not observed after this age in culture and therefore appeared to be a good candidate for a "stress response" protein. Stress responses were expected to arise (refer to section 5.1.15), and this result confirms that there are new mRNA species transcribed as soon as the cells are plated out. However, the duration of this early stress response seems to last just over a day, since the polypeptide changes observed are barely detectable after 1 day in culture.

5.2.9 Type II Polypeptides that Display Intermediate Expression

The period between early stress-induced gene expression and cell division was defined as the intermediate phase. The polypeptides expressed during this period were unlikely to be involved in stress-induced effects or cell division. Cell division is an extremely complicated process that involves DNA synthesis as well as phragmoplast formation. In G1 arrested asparagus cells DNA synthesis occurs prior to nuclear division and cytokinesis (see chapter 3, section 3.2.5). There were polypeptides identified in Fig. 5.12 that displayed type II differential expression within this phase. The polypeptides M38 P7 (Fig. 5.12A) and M60.5 P6.53 (Fig. 5.12B) are examples of polypeptides that display this type of expression. The polypeptide M38 P7 was initially observed at 1 day when its intensity was very weak, its intensity increased to a maximum by 2 days and thereafter it was barely detectable. This polypeptide was undetectable at 7 days in culture. The polypeptide M60.5 P6.53 first appeared at 2 days when it was at its most intense, its intensity fell off to very low levels by
Fig. 5.12 Intermediate gene expression. Examples of intermediate gene expression (enclosed within square, 140,000 cpm/gel).
A) Type II up-regulated polypeptide M38 P7 on *in vitro* translated 2-D gels of mRNA from 1) cladode 2) 1 day cultured 3) 2 day cultured 4) 5 day cultured cells.
B) Type II up-regulated polypeptide M60.5 P6.53 on *in vitro* translated 2-D gels of mRNA from 1) cladode 2) 1 day cultured 3) 2 day cultured 4) 5 day cultured cells.
day 5 and was undetectable thereafter.

5.2.10 Type II Polypeptides Expressed During Intermediate and Late Phase of Culture

The late phase is when nuclear division and cytokinesis occur and in this context is taken to mean between 4-7 days of culture, by which time one to four cell divisions from each starting cell have occurred (see chapter 3). Polypeptides that were expressed in intermediate and late phase cultures should be detectable after 1 day of culture and remain detectable until 7 days of culture. Examples of polypeptides that displayed this type of expression include M47.6 P8.3 (see Fig. 5.13A) and M37.2 P5.32 (see Fig. 5.13B). The polypeptide M47.6 P8.3 was detectable after 1 day of culture but its intensity changed with time of culture. The polypeptide M37.2 P5.32 was detectable after 1 day of culture and remained detectable until 7 days of culture.

5.2.11 Early Phase-Induced Type II Polypeptides that are Expressed until the Late Phase of Cell Culture

These polypeptides were expressed during the early stress phase of gene expression, through the intermediate phase, to the late phase of gene expression, and may be involved in the production and maintenance of a callus phase. There are likely to be polypeptides involved in the maintenance of high metabolic rates encountered during wounding and cell division. It was therefore expected that the different sets of polypeptides found expressed during the above phase have different functions, and consequently display variation in expression profile. The polypeptides that were visible in all three phases were M44.7 P5.32, M95.5 P3.82, M16 P5.6 a & b, M16 P5.32 and M16 P6.34. Polypeptide M16 P5.6 a & b, M16 P5.32 and M16 P6.34 make up the 16 kD band in Fig. 5.1 called DD-1 (see Fig 5.15). The polypeptide M44.7 P5.32 was a type I polypeptide which was expressed at low abundance in cladodes and non-meristematic cladodes (see Fig. 5.14A) and showed a slight up-regulation by 3 hr (day 0). Its intensity increased to a maximum by day 2 and was maintained at high levels thereafter up to day 7, when analysis was discontinued. The polypeptide M95.5 P3.82 (type II) (see Fig. 5.14B) displayed a slightly different expression pattern. This polypeptide was
**Fig. 5.13 Intermediate to late phase gene expression.** Examples of type II polypeptides expressed during intermediate phase to late phase (enclosed within square, 140,000 cpm/gel).

A) Type II up-regulated polypeptide M47.6 P8.3 on *in vitro* translated 2-D gels of mRNA from 1) cladode 2) 1 day cultured 3) 2 day cultured 4) 5 day cultured 5) 7 day cultured cells.

B) Type II up-regulated polypeptide M37.2 P5.32 on *in vitro* translated 2-D gels of mRNA from 1) cladodes 2) 1 day cultured 3) 2 day cultured 4) 5 day cultured 5) 7 day cultured cells.
Fig. 5.14 Stress or wound induced polypeptides that are expressed through intermediate phase to late phase. Examples of stress-induced polypeptides that are expressed through to late phase of culture (enclosed within square, 140,000 cpm/gel).

A) Type II up-regulated polypeptide M95.5 P3.82 on in vitro translated 2-D gels of mRNA from 1) cladode 2) 1 day cultured 3) 2 day cultured 4) 5 day cultured 5) 7 day cultured cells.

B) Type I up-regulated polypeptide M44.7 P5.32 on in vitro translated 2-D gels of mRNA from 1) cladode 2) day 0 3) 2 day cultured 4) 5 day cultured 5) 7 day cultured cells.
**Fig. 5.15 DD-1 gene expression.** Examples of polypeptides (DD-1) that are stress-induced and are expressed throughout culture (the polypeptides are arrowed, 140,000 cpm/gel).

Type II up-regulated polypeptides M16 P5.6 a & b (second arrow from left) and M16 P6.34 (third arrow from left) on *in vitro* translated 2-D gels of mRNA from 1) cladodes 2) day 0 3) 1 day cultured 4) 2 day cultured 5) 5 day cultured 6) 7 day cultured 7) 2 month cultured cells 8) 6 month cultured cells.
undetectable in cladodes and was first identified at a very low intensity at day 1; the intensity increased thereafter and it was detectable until day 6. This polypeptide was not resolvable after 7 days of culture; this would preclude this polypeptide being involved in maintenance of the callus state or an involvement with cell division. A diagram summarising all the types of expression encountered during dedifferentiation is displayed in Fig. 5.16. The data in Fig. 5.17 tabulates the presence or absence of 12 up-regulated polypeptides during dedifferentiation; the majority of these proteins have been used previously as examples in this chapter. The other two polypeptides in the table (Fig. 5.17) were present within the cladodes at higher abundance and are examples of products of type I up-regulated genes.

5.2.12 The Choice of Type II Up-Regulated Polypeptides for cDNA Cloning

The analysis of the different stages of dedifferentiation by 2-D gel electrophoresis made possible the identification of novel, differentially-expressed products of mRNA's. It was also possible to identify stages when the mRNA of these polypeptides were at their most abundant and therefore most readily cloned. It is generally rather difficult to synthesise and select a cDNA clone of an identified polypeptide. The ideal method would be to use several different approaches to isolate and check the clone. This was the reason why M16 P5.6 a & b (see Fig. 5.15) were chosen as candidates for cloning.

These polypeptides were possibly related and may be isoforms of an enzyme. If this was true, then provided one of these polypeptides (M16 P5.6 a & b or M16 P6.34) was cloned it should be possible to clone the other members of this group (DD-1) easily. The nucleotide sequence should be quite similar, so any cDNA of this group of polypeptides should hybridise with other members of the DD-1 group. Polypeptides of this group were visible on both total protein (see chapter 3) and in vitro translated protein 1-D gels. Members of this DD-1 group of polypeptides also appeared to be present throughout dedifferentiation and early suspension culture (see Fig. 5.15).

The information that DD-1 was visible on Coomassie blue stained 1-D gels meant that this group of polypeptides were relatively abundant and easily resolved. Therefore it should be possible to raise antibodies to the DD-1 group of polypeptides. However, before this was
Fig. 5.16 A diagramatic representation of gene expression during dedifferentiation. M41.5 P6.3 is an example of type II upregulated wound/ stress, early gene expression. M38 P7 is an example of intermediate type II upregulated gene expression. M37.2 P5.32 is an example of a type II upregulated intermediate to late phase gene. M16 P6.34 is an example of a type II upregulated gene expressed through all phases.
Fig. 5.17 A summary of gene expression during dedifferentiation. A summary of examples of early phase, intermediate phase and late phase expressed polypeptides. ++ is highly abundant, + polypeptide present, - polypeptide absent and na is not analysed.
done it was imperative to determine the extent of post-translational modifications of these polypeptides. This was important, as if there had been a great deal of post-translational modification, the raised antibodies might not recognise the unprocessed protein produced by the expressing cDNA clone. Also, it was quite possible that the processed protein might have different p.I's and molecular mass, and so identification of the corresponding in vitro translated mRNA may be difficult.

Mixture 2-D gels containing in vitro translated proteins and total proteins from 5 day cultured cells were run to determine the extent of post-translational modifications and to identify the products of translated mRNA. It was found that the polypeptides from in vitro translated mRNA of the DD-1 group co-migrated with polypeptides from total protein extracts (Fioroni et al., 1989).

From Fig. 5.15 it was determined that M16 P5.6a was present at very low abundance in cladodes but not non-meristematic cladodes and displayed type II up-regulation by day 0 and hence was possibly stress-regulated. The expression increased and was maintained at high levels up until day 7; the intensity of this polypeptide was reduced by 2 months of culture and by 6 months of culture it had a very low intensity. The polypeptide M16 P5.6b displayed a different expression profile. This polypeptide M16 P5.6a was present at very low abundance in cladodes and not found in non-meristematic cladodes. M16 P5.6a was a type II up-regulated protein and by day 1 it was present at low intensity, whereupon its intensity increased and was maintained at high levels until 2 months in culture after which it was no longer detectable. The polypeptide M16 P6.34 was detectable in cladodes but not non-meristematic cladodes and was type II up-regulated by day 0 and maintained high levels until day 7. After day 7 the intensity of this polypeptide fell to average abundance levels by 2 months and remained so at 6 months. The polypeptide M16 P5.32 appeared to be present at very low abundance in cladode and non-meristematic cladodes, it displayed type II up-regulation within a day of culture and remained at high abundance levels until 2 months, then fell to low levels by 6 months.

The DD-1 group was identified, separated and purified on total protein 2-D gels which were then blotted onto PVDF membrane. After visualization with Coomassie Blue staining the
polypeptide spot was cut out and then used for N-terminal peptide sequence analysis as well as to raise antibodies. From the amino acid sequence an oligomer was synthesized and used as a primer for first strand cDNA synthesis, this effectively enriches the probe population by ensuring that only mRNA with sequence homology to the oligomer is synthesised in the second strand reaction. This enriched probe was used to probe a cDNA library (see section 5.2.14). The antibodies could also be used to select clones in an expression library.

In summary, the results from 2-D gel analysis allowed the identification of target proteins which displayed two different types of gene expression. There were polypeptides that displayed type I up-regulated gene expression where these polypeptides were present at low or medium levels before being up-regulated by at least 10 fold. There were other polypeptides that displayed type II up-regulated gene expression where polypeptides present at low or undetectable levels were greatly up-regulated by over 100 fold. Both types of gene expression are found during all stages of dedifferentiation. The transcription of type II up-regulated polypeptides can be associated with 3 overlapping phases; wounding, stress and early gene expression, intermediate gene expression, intermediate and late phase expression as well as genes expressed through all 3 phases. This separation of cultures into different phases of gene expression indicated at what stage cultures should be harvested for mRNA isolation and cDNA cloning (see 5.2.12). In some cases these target polypeptides could be isolated easily and used to prepare antibody and oligomers in order to recognise genes coding for such proteins.

5.2.13 Analysis of mRNA Populations by Screening cDNA Libraries

An approach based on in vitro translation and 2-D gel electrophoresis has been used to examine dedifferentiation. In order to obtain corroboratory evidence on the mRNA complexity during dedifferentiation a strategy based on nucleic acid hybridisation was considered worthwhile. A cDNA library represents the steady state mRNA population at a point in time when the RNA was extracted. Therefore if the library of cultured cells was probed with 1st strand cDNA from cladodes and cultured cells, the amount of difference in mRNA species can be evaluated (Fioroni et al., 1989). From the data amassed in the preceding experiments in
this chapter it was decided to attempt to make a cDNA library representing 3 hr post-isolation (day 0) to examine the number of stress-induced changes in mRNA profile. In collaboration, O. Fioroni constructed two further libraries, one at the most complex stage at day 5 and another at 6 months.

5.2.14 Extraction and Purification of RNA

Pelham and Jackson (1976) demonstrated that it was possible to obtain high efficiency in vitro translation of mRNA with a mammalian cell-free system. This approach, when used in conjunction with radiolabelled amino acids and high resolution electrophoresis systems, has made the identification of the products of unique mRNA species possible. For this method to work efficiently uncontaminated RNA has to be extracted and purified.

It might be appropriate to mention in detail the development of the mRNA isolation procedure used to provide substrate for cDNA synthesis was a modification of the method developed originally by Covey and Hull (1981). Messenger RNA produced by this method proved undegraded and pure and appeared to give reproducible in vitro translation data (see preceding sections). The Covey and Hull (1981) method used the detergent TNS (see chapter 2) to dissolve nucleoprotein complexes, facilitate protein denaturation, improve RNA yields and to inhibit ribonuclease activity (Taylor, 1979).

Contaminating protein was removed by alkaline phenol:chloroform:isoamyl alcohol extraction. Phenol extraction also has the effect of inducing aggregation of mRNA with other RNA species especially rRNA. A pH of 8.4 was used for the RNA extraction buffer as it had been observed that in the cold at neutral pH, RNA was found in the denatured protein interface during phenol extractions. This was thought to be due to an interaction between RNA and proteins mediated by monovalent cations such as Na\(^+\) and K\(^+\) (Taylor, 1979). Therefore the use of an alkaline buffer during phenol extraction will allow most of the RNA to partition into the aqueous phase. Phenolic compounds were also removed by phenol:chloroform from the aqueous phase (RNA containing) which reduced complexes between RNA and hydrophobic impurities. 8-Hydroxyquinoline, which is an antioxidant and a chelating agent, as well as isoamyl alcohol, which is an anti-foaming agent, were added to phenol:chloroform mixtures to improve their
Properties.

Problems with carbohydrate contamination were encountered when RNA was extracted from cladodes and day 0 cells. This problem was reduced by watering asparagus plants with Hoaglands solution (Epstein, 1972) once a week (see section 5.2.1). This resulted in a reduction of stored starch in the plants as a rapid growth rate and respiration was maintained by high temperatures and the availability of nutrients. However, even though carbohydrate contamination of RNA was reduced, it still remained a problem. These carbohydrate impurities absorb at 230 nm and sometimes result in a shift of the peak in the O.D. scan between 200-300 nm (Fig. 5.18B). These impurities co-migrated with poly (A)⁺ RNA in oligo-dT cellulose columns, and inhibited in vitro translations and reverse transcriptase-mediated cDNA synthesis. Other unidentified impurities cause a shift in the O.D. scan between 200-300 nm (Fig. 5.18C). By reducing the volume of phenol:chloroform extractions by between 5-10 fold it was observed that a large proportion of these impurities flocculated. This was achieved by ethanol precipitating the nucleic acid in the aqueous phase after two phenol:chloroform extractions. The RNA pellet was then resuspended in 1/5 volume 100 mM Tris-HCl pH 8.4 prior to centrifugation at low speed (5 K, SS34 rotor, in a Sorval 5 B). The resultant pellet consisted mainly of impurities and some RNA. Further phenol:chloroform extractions were performed on the extracts until the protein layer at the interface was almost absent.

The DNA and any other contaminants were removed by washing the salt/ethanol nucleic acid precipitate with 3M sodium acetate by resuspending and resedimentation. This had the effect of solubilizing DNA, tRNA, 5S rRNA... These substances inhibit translations and if removed give rise to good quality RNA. The RNA extracted with the modified extraction protocol contains no protein impurities (280 nm absorbance) and was very low in carbohydrate contamination (see Fig. 5.18A). The RNA extract could be purified to yield poly (A)⁺ RNA by the use of oligo-dT cellulose columns (see chapter 2). Since after a single column step the poly (A)⁺ RNA still contained approximately 50 % rRNA; two column steps were required to produce poly (A)⁺⁺ RNA which was sufficiently pure for cDNA synthesis. The poly (A)⁺ RNA was sufficiently pure to be used in in vitro translations.
Fig 5.18 Optical density scans of RNA preparations. (A) High quality pure RNA. (B) Carbohydrate impurities. (C) Unidentified impurities. All samples were extracted from asparagus cladodes. $A^\circ$ is relative absorption values.
5.2.15 Construction of an Asparagus cDNA Library

Extracted poly (A)$^+$ RNA from day 0 cells was used in reverse transcriptase mediated cDNA synthesis reactions (see chapter 2). The reverse transcriptase reaction was checked by running 1$^{st}$ strand cDNA on an polyacrylamide gel (Fig. 5.19A). The RNA was found to be pure and undegraded as the 1$^{st}$ strand cDNA had an average length of over 1353 base pairs. The synthesized double stranded cDNA was ligated into dephosphorylated Eco RI cut Lambda ZAP vector (see chapter 2). The packaged recombinants were titrated, and the library was found to contain $2.75 \times 10^4$ pfu at a concentration of $5.5 \times 10^4$ pfu ml$^{-1}$. A ratio between the number of recombinants within the library and the amount of cDNA used in the ligation will give the cloning efficiency. The cloning efficiency of the day 0 library was found to be $1 \times 10^5$ pfu $\mu$g$^{-1}$ cDNA and the library size was found to be 27,500 recombinants.

It has been reported that rRNA secondary structure can prime first strand cDNA synthesis (Meeks-Wagner, et al., 1989). Therefore a plate of the day 0 library was probed with a rDNA probe from Petunia in order to estimate the percentage of rDNA contamination in the cDNA library (Fig. 5.19B). There were 26 positive plaques for rRNA out of 350 pfu, which is 7.4%. Therefore the library contained 7.4% rRNA clones which is below the maximum acceptable figure of 10%. To check on the insert size, a number of random plaques were picked, grown for 4 hr in the presence of BB4 plating cells and R408 helper phage (see chapter 2). The plasmid was extracted from recombinant cells and the DNA digested with Eco RI to excise the cDNA insert and ran on a 2 % agarose gel (Fig. 5.19C). The average size of the inserts was 700 bp.

Due to a lack of time the day 0 library was not screened to identify differentially expressed clones. However, O. Fioroni used the above strategy to isolate several putative DD-1 cDNA clones from a day 5 library (Fioroni et al., 1989). It would be possible to use this approach for any of the other 12 differentially expressed polypeptides, provided the identification of the processed product of differentially expressed mRNA was possible.
Fig. 5.19 Quality of cDNA library. (A) 1st strand cDNA synthesis. (B) A portion of the cDNA library probed with a *Petunia* rDNA probe. (C) Eco RI digests of some random recombinants run on a 2% agarose gel. Lanes 6, 12 and 17 are DNA size markers.
5.3 Discussion

This chapter has been concerned with the study of changes in mRNA pools during dedifferentiation. Dedifferentiation in cultured asparagus cells is extremely complex and has many contributing factors which may alter gene expression, including wound responses, hormonal effects, possible effects of other media constituents and cell division. In order for any molecular analysis of this process to be carried out, efficient RNA extractions and purifications from small amounts of tissue had to be perfected. This proved possible to do with minor modifications to the Covey and Hull protocol (1981) (see chapter 6). The system adopted to study dedifferentiation was in vitro translation of mRNA followed by two dimensional gel electrophoresis and cDNA cloning.

The system used for the in vitro translations was the commercial rabbit reticulocyte lysate system (RRL) (see chapter 2) depleted of methionine. This system was chosen in preference to the wheat germ cell-free translation system because higher molecular weight products can be obtained with RRL. It is important to bear in mind that recent evidence suggests that plant and animal RNA’s are perceived differently within animals or plants. For example, plant and animal initiation codons can differ (Lutcke et al., 1987). So plant mRNA translated by an animal cell-free translation system may have introduced artifacts.

Since in vivo labelling shows up processed gene products, it is expected to obtain larger numbers of resolved polypeptides with in vivo labelling than with in vitro translations. Traditionally the numbers of resolvable polypeptides within plant systems used to be less than in bacterial or other eukaryotic systems. For example over 700 (Bravo and Celis, 1980) polypeptides are resolvable in animal or bacterial extracts but only approximately 250-500 polypeptides were detected in plant extracts (Meyer and Chartier, 1981; Thiellet et al., 1982; Basset et al., 1988; Schuster and Davies, 1983). This was thought to be due to the low protein content of plant cells together with less efficient labelling and the presence of substances that detrimentally affect IEF. However recently in some plant systems this problem has been overcome and it has been possible to obtain equivalent numbers of resolved polypeptides as in animal systems (Choi and Sung, 1984). The figures obtained in this thesis for maximum numbers of resolved polypeptides (see Fig. 5.5) are between 138-311
polypeptides. This figure is equivalent and in some cases higher than that obtained in some in vivo labelling studies (Meyer and Chartier, 1981; Thiellet et al., 1982; Schuster and Davies, 1983) where the numbers were expected to be higher. If high activity $^{35}$S is used to label polypeptides then, the 2-D gel system should be capable of resolving polypeptide spots of between 0.0001-0.00001 % of the total radioactivity applied (O'Farrell, 1975). With the crude method used to determine the approximate resolvable radioactive counts, the figure was 0.018 % for a low abundance spot. This figure is however for incorporated counts, since approximately 1 % of radioactivity is incorporated a low abundance spot will therefore represent 0.00018 % of total radioactivity loaded. This would be a slightly lower value than that theoretically possible to resolve, but since a polypeptide spot that is just detectable on an autoradiograph would have a considerably smaller figure than 0.018 % of incorporated radioactivity (not measurable by this method) the actual resolution of the system is comparable to the theoretical optimum. The total number of resolved polypeptides could be improved by leaving the gels down on film for longer than a period of two weeks. However, only very few, low abundance new polypeptides were resolved after a month. These spots representing low abundance polypeptides appeared to be clearer, and so easier to detect. There is however a concomitant loss of resolution of adjacent highly abundant polypeptides, these polypeptides are distinguishable when placed on film for two weeks but merge after longer exposures.

The decapitated cladodes (removal of apical tip) designated non-meristematic cladodes had lower numbers of resolvable polypeptides than intact cladodes. This was not surprising as the apical tip houses an apical meristem which should contain novel polypeptides (Stafstrom and Sussex, 1988) and hence should possess more resolvable polypeptides than the decapitated cladodes. The in vitro translated 2-D patterns from cladodes and non-meristematic cladodes do have several differences (Fig. 5.7). However, it is impossible to be sure if the observed differences are due to the presence or absence of the apical meristem or due to an age difference between the samples. Cells from the apical tips are damaged during the mechanical isolation process and do not contribute to the viable cell population in tissue cultures. Wound-induced gene expression is negligible as decapitated cladodes were immersed immediately into liquid nitrogen.
The polypeptide numbers decrease at day 0 (see Fig. 5.5); this is most probably a general suppression of normal transcription/translation by wounding. This was expected as mechanical isolation disrupts the plasmodesmatal connections between the mesophyll cells. However, at day 0 several upregulated 'stress' related transcripts appeared in the message population. It is well established that the presence of pectic fragments can elicit defence (Templeton and Lamb, 1988; Darvill and Albersheim, 1984) and wound (Ryan, 1987, Ryan and An, 1988) regulated gene expression. The asparagus cells are exposed to cell wall fragments for a period of up to 40 min during isolation. Wounding often causes marked changes in the level of translation both in the terms of proportion of ribosomes existing as polysomes and in the rate of protein synthesis (Theillet et al., 1982; Davies et al., 1986). However, the effects of wounding on different tissue types vary considerably. For example wounding (see section 4.1.6-14) has either no effect on actively growing tissues (Davies and Schuster, 1981) or will lead to a decline in polysome formation (Theillet et al., 1982) but in mature tissue wounding leads to an increase in polysome formation (Davies and Schuster, 1981). Davies et al. (1986) concluded that wounding inhibits translation in mature tissues by inhibiting the ribosome translocation/release process. But work by Logemann et al. (1988) has suggested that wounding suppresses certain gene transcriptional units, leaves some genes unaffected and induces other genes. It is expected that the maximum resolved polypeptide number would decrease under the influence of wounding. However, this was not observed as polypeptide numbers were higher than that for non-meristematic cladodes. The reason for this was that in vitro translation was used instead of in vivo labelling and therefore any translation inhibition mechanisms would not show up. Thiellet et al. (1982) observed that polypeptide numbers fell from 250 in non-wounded root tips to 80 after wounding.

Since the asparagus cell cultures were incubated in the dark in the presence of sucrose, the cultures switch from autotrophic to heterotrophic growth. It has been well documented that light can directly control the relative abundance of proteins involved in photosynthesis. For example, the mRNA from genes such as ssu and cab are reduced by darkness (see section 5.1.15; Link, 1988). Both genes belong to gene families and have members that respond to light differently. Therefore there could be several transcripts associated with each
proteins that are reduced. Vierling and Key (1985) observed that ssu mRNA decreased in abundance in response to heat shock. In chapter 4 (section 4.2.4), Northern blotting analysis with ssu and Isu confirmed the fact that dark treatment can reduce the polypeptide numbers and so in conjunction with wounding, it can contribute to the loss of polypeptides. This factor might contribute to the down-regulation of numerous polypeptide spots observed to occur within 3 hr of mechanical isolation. Together with this effect there is also a concomitant increase in transcripts of several genes that are up-regulated in the dark (see section 5.1.15).

During culture the polypeptide numbers increase and are maintained at high levels between 2-5 days (see Fig. 5.5). The polypeptide numbers fall thereafter to a constant level that is maintained between 7 days- 2 months of culture. The increase in polypeptide numbers could be due to the appearance of type II polypeptides involved in dedifferentiation and in the maintenance of highly active growth. The subsequent fall in polypeptide numbers could be due to the disappearance of some polypeptides involved in maintenance of cladode characteristics and those involved in dedifferentiation.

The polypeptide patterns are highly reproducible between the separate mRNA batches (see Fig. 5.4) of each sample time point. It was extremely important to determine this as there are no early visual markers for dedifferentiation. The results (see Fig. 5.4) show that the criteria used to check the cultures prior to harvesting or RNA extraction are adequate. There were however differences noted in the intensity of certain polypeptides between different translations of the same and separate batches of mRNA. The radioactive amino acid used as a label for in vitro translations was commercial high activity $^{35}$S-methionine (see chapter 2). This amino acid is a rare amino acid and is coded for by the initiation codon. This terminal end of a polypeptide is sometimes cleaved post-translationally. Since no post-translational processing should occur within the cell-free RRL, all newly synthesised polypeptides should contain incorporated $^{35}$S-methionine after in vitro translation of mRNA. However, some polypeptides contain several methionines and so could appear to be highly abundant even though they may represent a low abundance mRNA.

It appears that each stage of the tissue culture process has its associated type II polypeptides, as well as down-regulated and up-regulated type I polypeptides (see Fig. 5.6).
By various means (see Fig 8 & 9) over 20 type II polypeptides were identified in cultured cells, though a number of these are on the borderline of detection. These polypeptides are not mentioned further in this thesis, as they proved difficult to analyse and therefore it was impossible to ensure that some of them were not artifacts. However, 10 polypeptides were quite clearly type II and were easily resolved on 2-D gels.

How important is transcriptional control in developmental pathways? This seems to vary with the system in question (see section 5.1.1). Post-transcriptional control seems to perform a central role in the control of the cell cycle (see section 5.1.5). Analysis by *in vivo* labelling and by *in vitro* translations of mRNA extracted during carrot somatic embryogenesis (Choi and Sung, 1984) have revealed that there are very few "novel" proteins associated with this process. It is likely that other "novel" proteins involved are very low abundance and therefore below the detection limits of the system, or that the control is post-transcriptional/post-translational. There are however great differences in the mRNA composition of different plant organs even though in some cases the cells are morphologically identical (Goldberg, 1988). Evidence in the literature suggests that there are callus-specific proteins which cannot be detected elsewhere in the plant (Hahne *et al.*, 1988). This was attributed to the nature of callus which is apparently a tissue type without a direct equivalent in the whole plant (Hahne *et al.*, 1988). Other workers have tried to attribute functions to callus-specific polypeptides and have circumstantial evidence to suggest that the presence of some of these proteins is directly related to the presence of exogenous auxin (Wozniak and Partridge, 1988). Bevan and Northcote (1981) described that growth-arrested suspension cultures had an increased abundance of several proteins, which was directly correlated with subculturing (see section 5.1.3). However, they observed that different genera of plants responded to auxin and subculture treatment differently (Bevan and Northcote, 1981). *Glycine max* produced a rapid increase in 3 polypeptides after subculture which was dependent on the presence of auxin. *Phaseolus vulgaris* produced an increase in 3 polypeptides after subculture in the presence of auxin which was dependent on subculturing (Bevan and Northcote, 1981). They suggested that auxin effects in bean act within the primary stimulus of subculturing but, with soybean subculture it is not required for the induction of auxin-induced proteins. This system using cell
division arrested suspension cultures has similarities with asparagus dedifferentiation. For example the cell cycle is arrested and has to be reinitiated by tissue culture in the presence of auxin. However, it is important to bear in mind that the already dedifferentiated bean cells were dividing prior to subculture into auxin depleted media. In comparison the asparagus mesophyll cells have arrested cell cycles and have not divided since they were produced by the meristem. So the bean cells are competent to divide, given the addition of auxin, but the asparagus cells have to be made to be competent to divide by dedifferentiation; a process which may involve the de novo synthesis of some of the type II proteins. Also the polypeptides induced by subculture are more likely to be related to "stress" responses rather than with the reinitiation of the cell cycle. Subculture was observed to induce stilbene synthase synthesis in suspension cultured peanut cells (Vornam et al., 1988). This enzyme is involved in the synthesis of a phytoalexin, and as a consequence can also be induced by pathogen culture filtrates and wounding. This would suggest that the process of subculture is "stressful" to plant cells and is frequently thought of as being analogous to wounding (Bolwell, G., personal communication).

The changes in in vitro translated mRNA products on two dimensional gels suggested that transcriptional control was at least one controlling point during dedifferentiation. Further evidence was obtained from differential screening of a 5 day cultured asparagus cDNA library; out of 560 clones, 326 were culture specific (Fioroni et al., 1989). This suggests that over 50 % of the mRNA found at day 5 is either culture specific or is expressed at considerably higher levels than in the cladodes. This differential screening method will only detect mRNA species more abundant than 0.1 %. Though the real test to work out if transcription is a prerequisite for dedifferentiation is to prevent transcription by antibiotics such as α-amanitin (binds to RNA polymerase II & blocks mRNA synthesis) and study the effects.

As mentioned in the introduction (section 5.1) dedifferentiation is a transition pathway between two divergent cell types. The acquisition of embryogenic potential is also such a pathway. de Vries et al. (1988) have examined this pathway in carrots and have identified several novel polypeptides which appeared during the acquisition of embryogenic potential. Choi and Sung (1984) using the same system, but comparing embryogenic and non-
embryogenic callus, could not find any large differences. This suggests that the proteins involved in establishing the embryogenic pathway are either not being transcribed during embryogenesis or are below detection limits. This group (de Vries et al., 1988) also found that there were no gross protein changes during dedifferentiation of carrot hypocotyls. However, in asparagus it is quite clear that there are numerous differences observed in mRNA composition during dedifferentiation. It is highly likely that no differences in mRNA composition was noticed in carrots because of the masking effect of a large number of non-dedifferentiating cells. It is also highly likely that the different observations in the literature are due in some part to this effect of masking by non-dedifferentiating cells.

It is quite clear that mechanical isolation will induce wound responses (see Fig. 5.11) which in the case of asparagus cells appears to last the duration of a day. It is also clear there are initially some wound-induced polypeptides that continue to accumulate and proliferate throughout culture (see Fig. 5.15). There has been a great deal of debate about whether tissue culture is "stressful" to plants. Recently it has emerged that a wound-induced promoter element also functioned at high efficiency in suspension cultures (Logemann et al., 1989). It is quite possible that some of the type II polypeptides identified in the asparagus system are involved in plant defence and stress responses and are regulated in a similar fashion to that observed for PR proteins and peroxidases (see section 5.1.12-13). If this assumption is true then these polypeptides should be expressed in suspension cultures as well. In this way some tentative functions might be attributed to these novel polypeptides.

The constructed day 0 cDNA library was not differentially screened as there was insufficient time. However, the average insert size, library size and the percentage contamination by rRNA was determined. Even though the library was not large it contained a low rRNA contamination and a large average insert size (700 bp). This suggests that there is a good chance of obtaining full length cDNAs to abundant transcripts. If time had permitted differential screening would have been attempted to clone any wound-induced transcripts. However, a cDNA encoding one of the DD-1 group of proteins was isolated from the day 5 library by O. Fioroni. Ultimately using dedifferentiation specific cDNAs from either library dedifferentiation could be studied in the intact plant. Data from this type of experiment will show if wound-induced
dedifferentiation of isolated asparagus cells is the same as in the intact plant. And therefore also answer questions concerning hormonal gradients controlling development at wound sites.

The observations made in this chapter makes it possible to conclude that dedifferentiation involves de novo synthesis of type II polypeptides and that it is a very complex process. It is also quite clear that it is difficult to separate each phase from the other as there is considerable overlap. With any of the identified polypeptides in this chapter there is a real risk that some of them may be induced by phytohormones or other medium constituents and have nothing directly to do with dedifferentiation at all. Such hormone-induced proteins for example should be transcribed quite quickly i.e within a day in contact with the phytohormones. So intermediate and late expression polypeptides would not be likely candidates for such hormone-induced proteins. The DD-1 group however is expressed at day 0 and thereafter throughout culture (up to 6 months) and is continually exposed to the hormones in the medium. Therefore this group of proteins would seem to be possible candidates for hormone-induced proteins. This possibility was tested out by Paul (personal communication), by using antibodies to DD-1 to screen Western blots of proteins extracted from cells grown in auxin-free medium, cytokinin-free medium and hormone-free medium. The protein DD-1 was found in all extracts, including cells incubated in hormone free medium for a week. This effectively precludes the possibility that DD-1 is a hormone-induced protein, as well as it being involved in cell division, as cells incubated in hormone-free medium do not divide. Further experiments have to be carried out to preclude this group of proteins involvement in dedifferentiation. It is possible that the DD-1 group could be a "stress" related group of proteins.

In conclusion it has to be realised that the in vitro translated mRNA profiles represent a distorted view of gene expression during dedifferentiation. For example, eukaryotic mRNA’s are translated with unequal efficiencies in vivo and in vitro (Jobling and Gehrke, 1987). This effect has been attributed to the 5’ untranslated leader sequence of the mRNA (Jobling and Gehrke, 1987). Furthermore it has been documented that stress can reduce the rate of protein synthesis, and polysomal translation (Wu et al., 1988). Wu et al. (1988) suggested that
a 57 kD protein may be involved in altering translation efficiency in maize leaves by binding to polysomes and preventing translation during the imposition of stress responses, such as heat shock, herbicide treatment and pathogen infection. If such a system also functions in asparagus then it is quite possible that the translated mRNA profiles are artifactual and do not represent the population of newly synthesised proteins. Each mRNA is assumed to be products of a single gene, but this is not a valid assumption in all cases as there examples of single genes that produce different sized transcripts (Chen and Varner, 1985b). The extensin gene (pDC 5 A1) has two initiation codons and produces two different sized transcripts from a single gene.

Evidence from yeast and animal research is strongly in favour of post-translational control being involved in controlling the cell cycle and mitosis (see section 5.1.5). So cell cycle mediated changes are unlikely to be observed on the translated asparagus mRNA profiles. There are examples of cells, such as in seeds and pollen grains where mRNA is stored stably and is only translated at a particular stage of development (Gordon and Payne, 1976; Mascarenhas et al., 1984). In which case any mRNA extracted from these tissues may not represent any protein synthesis. Again such stable messages could give the wrong impression about gene expression at that point in time.

All the above factors, combined with the differences in the nucleotide consensus sequences surrounding plant and animal initiation codons may lead to artifacts caused by using in vitro translations in the interpretation of gene expression during dedifferentiation. However, in this thesis artifactual interpretations were minimised since the translated mRNA profiles were compared against each other and only clear differences were highlighted.

After consideration of the results in this thesis as well as results obtained by other workers, the following conclusions can be made; dedifferentiation is an extremely complex process which requires the interaction of several factors i.e. wounding, hormones and culture media which on their own can also affect gene expression. During dedifferentiation of asparagus cells there are a large number of mRNAs that display decreased abundance (down-regulated). Transcription of type I and type II mRNA's occurs during dedifferentiation. During dedifferentiation gene expression of the type II polypeptides vary and can be sub-divided into
five kinds according to when expression occurs; This may be either during one of 4 overlapping phases, i.e. wounding, stress and early gene expression, intermediate gene expression, intermediate and late phase expression, or throughout all phases.

There is a change in complexity of the mRNA population during dedifferentiation which is reflected by the polypeptide numbers. However, post-transcriptional control cannot be discounted as being important during dedifferentiation. A cDNA library directly represents the mRNA population complexity used to construct the library. In this way the 2-D profiles provide a guide to when cDNA libraries should be constructed as these profiles represent the mRNA population complexity. The 2-D profiles also have shown that changes in gene expression occur prior to any visual signs of dedifferentiation. The complexity of the 2-D profiles are mainly associated with wounding and dedifferentiation and not with cell division as the polypeptide numbers decrease to a constant number between 7 days and 2 months. Established (8 year old) suspension cultured Petunia hybrida Blue Lace cells showed virtually identical 2-D profiles to in vitro translations of mRNA extracted from whole seedlings, even though the cells were dividing rapidly (Fioroni, 1989). If in this extreme comparison no culture-specific polypeptides were found it is not surprising that cell division related polypeptides were not found in asparagus. Some cell cycle phase specific polypeptides were identified from synchronised cultures of Catharanthus roseus cells (Kodama et al., 1989). This study examined 2-D gel profiles of total protein, in vivo labelled protein and in vitro translated proteins and observed that these differences appear to be mainly due to translational or post-translational processing (Kodama et al., 1989). Therefore transcription does not appear to determine the abundance of some cell cycle phase associated polypeptides (Kodama et al., 1989). However, like the animal and yeast cell cycle proteins only a small number of polypeptides appeared to show cell cycle phase associated qualitative or quantitative change (Kodama et al., 1989). It is therefore not surprising that an examination of in vitro translated 2-D gels of dedifferentiating asparagus cells has not shown the presence of cell cycle phase associated polypeptides.

The major disadvantage of the approach used in this chapter is that it is difficult to work out the identity of target polypeptides. It may be possible to identify any of the type II
polypeptides by microsequencing to obtain probes to isolate it from a cDNA library or to compare with the protein sequence data bank. Perhaps in the future an unknown polypeptide function can be worked out by using reverse genetics to disrupt gene expression of the particular cDNA of interest (see chapter 6).
Chapter 6  General Discussion and Conclusions

6.1 General Discussion

The aim of this thesis was to use a mechanically isolated asparagus cell culture system to examine the developmental switch resulting in the formation of dividing callus cells from quiescent mesophyll cells. The factors that induce dedifferentiation in intact plants result from a combination of hormonal gradients, tissue age and type in response to wounding. This thesis is only concerned with the first two phases of wound healing, the lag and dedifferentiation phase (section 5.1.7). A cytological, physiological and molecular characterisation of the dedifferentiation developmental pathway was undertaken.

Dedifferentiation is defined in chapter 1, section 1.2. Molecular analysis requires a great deal of physiologically and biochemically similar tissue for analysis. As discussed in Chapter 1, section 1.4 there are considerable problems examining wound-induced cellular dedifferentiation in intact plants. These problems are mainly due to the inability to obtain large quantities of wound-induced, biochemically active tissue easily. For this reason an alternative in vitro system was chosen, as large amounts of wound-induced, biochemically and physiologically similar tissue can be easily obtained. However, the use of in vitro systems while removing some technical problems introduce others. For example, the wounded cultured cells are not exposed to hormonal and nutritional gradients which occur in the whole plant. So any observations made on in vitro systems cannot be directly compared to the intact plant. However, some idea of changes that occur as a result of wound-induced dedifferentiation can be obtained from an examination of an in vitro system. Mechanically isolated asparagus cells were chosen as model system to study wound-induced dedifferentiation as it best satisfied criteria for the ideal in vitro system (see section 1.7).

The cytological study was concerned with the optimisation of the culture system as well as the search for cytological markers for dedifferentiation. As large quantities of tissue is required for molecular analysis, such markers would enable dedifferentiating cells of the same stage to be harvested. To determine the feasibility of the system for molecular analysis, yields of macromolecules from these cultured cells were determined. In chapter 3
3.2.3), modification of culture conditions and plating density made molecular analysis feasible. During wound-induced dedifferentiation there is a change in the photosynthetic biology of the cells as the cells switch from photoautotrophic development to heterotrophic development. This physiological switch was monitored to identify further markers as well as to give further information on dedifferentiation (see Chapter 4).

The most obvious markers for dedifferentiation were cell expansion and cell division. Both of these events occurred quite late in the process of dedifferentiation. There is an increase in respiration rate (see section 4.2.2) which occurs prior to day 3 followed by a decrease of net photosynthesis after day 5 of culture. This proved to be a simple, useful marker for dedifferentiation. Cell division and changes in the photosynthetic rate were used routinely as markers to identify cultures at the same stage of dedifferentiation. It was also noted that the RNA content and protein content of the cultured cells increased after day 2 of culture. However, this is not suitable as a marker for dedifferentiation as RNA analysis is destructive and requires at least $4 \times 10^6$ viable cells. The mesophyll cells are in the G1 phase of the cell cycle and on mechanical isolation and subsequent cell culture, S phase is induced in some cells by day 3 of culture. This means that DNA synthesis has to be initiated in asparagus cells before cell division can occur.

After the first cell division the dividing cells do not change their morphology until after over 2 months of culture. Thus, this appears to suggest that dedifferentiation is completed by the first cell division. The amounts of protein, RNA and DNA increase dramatically after a lag of 3 days and appear to parallel the onset of cell division in the cultured cells. Therefore gross changes in these macromolecule levels also supports the idea that dedifferentiation is completed by the first cell division. A loss of photosynthetic potential is paralleled by a decrease in the amounts of rubisco per cell. The messenger RNA for both $ssu$ and $lsu$ decrease within a day of culture, which is similar to that observed in protoplasts (Fleck et al., 1980); the loss of the rubisco protein however appears to take longer possibly as the result of slow protein turn over. During the period of cellular dedifferentiation a loss of photosynthetic capacity and an increase in respiration rate occurs. However, it appears that chloroplast dedifferentiation is incomplete until after day 10 of culture. "Fission" division was
observed in chloroplasts of 10 day old cells. The resultant plastids appear to be similar to proplastids as their chlorophyll, protein and nucleic acid content is significantly reduced.

The molecular analysis of dedifferentiation enabled gene expression during dedifferentiation to be separated into 3 overlapping phases (see Chapter 5). Two types of up-regulated genes were observed and characterised. It appears that a large number of transcriptional changes occurs during dedifferentiation. This implies that transcriptional control of gene expression may be more important in this developmental pathway than in other developmental pathways such as embryogenesis (Sung and Okimoto, 1981) or cell division of undifferentiated suspension cultured cells. The 4 types of gene expression are wound, stress and early culture induced polypeptides; intermediate expression polypeptides; intermediate and late expression polypeptides and polypeptides expressed throughout all phases. By implication polypeptides that are expressed in the different phases may possibly have different biological functions. In this way some tentative functions might be attributed to some of these proteins.

Mechanically isolated cells appear to be an excellent system for the examination of the wound response. This is because all the cells are wounded (plasmodesmatal links severed), there are no complicating hormonal gradients to take into account (all cells are exposed to the same external environment) and there is little masking of wounded gene expression by non-wounded tissue. In chapter 5 it was observed that there were a large number of changes in transcript levels and profiles during early dedifferentiation (3 hr-1 day). This is unlike that observed during wounding of storage organs and leaves. In potato tubers, carrots and tomato leaves little change in gene expression is observed except for the appearance of one or two highly upregulated transcripts; proteinase inhibitor I & II in tomatoes (Ryan and An, 1988), induction of extensin in carrots (Varner) and wun1 & wun2 in potatoes (Logemann et al., 1988). However, in vivo labelling experiments carried out by Theillet et al. (1982) on wounded Vicia faba meristematic root cells showed that 6-7 novel polypeptides were resolved 15 hr after wounding. In this system there is no cell cycle reactivation and so it cannot be used to examine wound-induced dedifferentiation which includes cell cycle reactivation. However, it is clear that the mechanically isolated asparagus mesophyll cell system is ideal for the examination of wound-induced dedifferentiation. I believe the reason why little change in gene
expression was observed in most intact plant systems is due to the masking effect of non-wounded tissue. Messenger RNA from non-wounded tissue would swamp some of the lower abundance wound-induced mRNAs and so such messages will either be undetectable or be difficult to identify. The DD-1 gene expression profile is unlike expression profiles of other wound-induced genes such as chitinase, \textit{wun1} or PAL which show rapid accumulation of transcripts with short half lives. Chitinase transcripts peak at 1.5-2 hr post-wounding with basal levels reached by 4 hr (Hendrick \textit{et al.}, 1988). PAL has a peak in transcript abundance 3-4 hr post-elicitation or wounding after which levels rapidly decline (Cramer \textit{et al.}, 1985). The transcript for the gene \textit{wun1} in potato tubers is detectable 30 min post-wounding, reaches a peak after 10 hr and is maintained at constant high levels for the next 14 hr. However, an expression cassette with the \textit{wun1} promoter driving a CAT gene maintains high levels of expression in suspension cultured cells (Logemann \textit{et al.}, 1989). This suggests that tissue culture is in itself a stressful process. Therefore it is possible that DD-1 expression profiles are the direct result of stress inherent in cell culture.

What do the results obtained in this thesis tell us about dedifferentiation? Firstly, dedifferentiation appears to be a highly ordered, reproducible process that results in the formation of dividing callus cells. Secondly, it is an active process that results in an increase in respiration rate as well as changes in protein composition. Thirdly, a series of transcriptional changes occurs during dedifferentiation. Fourthly, a change in cell function results from dedifferentiation. Fifthly, dedifferentiation can be separated into 2 parts, cellular and plastid dedifferentiation. Finally, dedifferentiation can be further separated into 3 different phases on the basis of morphological and molecular analysis. It is interesting to note that dedifferentiation has a morphological lag phase (but not a molecular lag phase) that lasts 2 days during which gross transcriptional changes occur. An experiment carried out by Elizabeth Paul at Leicester (personal communication) where different aged dedifferentiating asparagus cells were transferred from normal temperature (20°C) growth conditions to 4°C showed that cultures more than 2 days old prior to transfer underwent cell division. However, cells transferred to cold conditions prior to 2 days did not undergo cell division. It appears that correct gene expression during this "lag" phase is a prerequisite for dedifferentiation.
A scenario for dedifferentiation might be as follows. Wounding induces the transcription of genes involved in plant defence, wound healing and in cell cycle reactivation. An increase in metabolic activity and therefore respiration rate occurs during cell expansion, DNA synthesis, protein synthesis and the synthesis of other cellular components required for cell division. At the same time there is an increase in rRNA concentration per cell, movement of nuclei and organelles as well as a reduction in the activity of chloroplasts. The loss of chloroplast activity could be partly attributed to the presence of sucrose in the medium and the lack of light. As functional chloroplasts are no longer required the cell conserves precursors by converting plastids to proplastids that demand less precursors to maintain structure. This change was evident by after day 10 of cell culture. Both nuclear division and cell division occurs, perhaps with phosphorylation/dephosphorylation of certain proteins being important as in yeast and animal cell cycles. The thus formed cells are cellularly fully dedifferentiated, but dedifferentiation of the whole cell is only final when plastid interconversions are fully complete. It is unlikely that the change in cell function is due to a general breakdown of cell structure. It is more likely that this change is due to a modification of cell structure caused by newly synthesised components. A summary of observations made during wound-induced asparagus dedifferentiation is given in Fig 6.1.

During the dedifferentiation of carrot hypocotyl explants no great changes in transcript complexity was observed (de Vries et al., 1988). However, de Vries et al. (1988) concluded the above from a comparison between suspension cultured cells and the original hypocotyl explants. Therefore the process of dedifferentiation was already complete when the comparison was made. Fioroni (1989) observed that there are no major differences in transcript complexity between suspension cultured cells and the whole plant in Petunia hybrida. This also appears to occur with carrot embryogenesis, where there are hardly any differences observed between non-embryogenic callus and embryogenic callus (Sung and Okimoto, 1981). However, de Vries et al. (1988) observed differences in transcript complexity when newly formed callus tissue acquiring embryogenic potential and hypocotyl explants were compared. In asparagus, transcriptional differences between the cladodes and 2 month suspension cultured cells was small when compared to cultured cells under a week of age. This seems
Fig. 6.1  An overall summary of asparagus dedifferentiation.
Summary of asparagus dedifferentiation

Differentiation Mechanical Isolation Dedifferentiation
Meristematic cell → Mesophyll cell (quiescent) → Cultured cell (dividing) → Chloroplast dedifferentiation (up to 14 days)

Cell and nuclear size

Cell cycle reactivation

Phases of dedifferentiation

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<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0-2</td>
<td>Day 3-4</td>
<td>Day 4-6</td>
</tr>
<tr>
<td>Little change in 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>
</tr>
<tr>
<td>Little change in respiratory rate or photosynthetic rate</td>
<td>Increase in respiration</td>
<td>Loss of photosynthetic capacity</td>
</tr>
<tr>
<td>Decrease in LSU &amp; SSU mRNA</td>
<td>Decrease in LSU &amp; SSU protein</td>
<td>Intermediate to late phase gene expression (M95.5 P3.82)</td>
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<tr>
<td>Wound, stress &amp; early culture-induced polypeptides (M41.5 P6.3)</td>
<td>Reduction in chloroplast rRNA</td>
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</table>
to suggest that during the switch between developmental pathways a large transient change in transcription has to occur before complexity decreases to approach that expected for such pathways. Given the resolution of the systems (in vitro translation followed by 2-D gel electrophoresis) used to examine these switches in development, only relatively large transcript steady state differences will be clearly seen. Therefore the examination of a switch in development will have to have sufficiently large transcriptional differences to be clearly identified. Once the switch in development has occurred it is very likely that the differences in transcript abundance are more subtle and less easily identified. Post-translational control would be the more likely controlling mechanism once the cells development is firmly directed down a particular developmental pathway. This is further evidence that the mechanically isolated asparagus cell culture system is ideal for the study of the developmental switch, dedifferentiation.

However, there are disadvantages with the asparagus system; for instance the lack of high synchrony would preclude the identification of very low abundance transcripts as well as transcripts involved in the cell cycle.

6.2 Future work

There are numerous questions that have not been answered in this thesis; is in vitro dedifferentiation identical to dedifferentiation in the intact plant? Why is the plant genome unstable during dedifferentiation? What leads to increased levels of transposition observed by Peschke et al. (1987) during dedifferentiation? These are some of the questions that may be answered by a continuation of this study. It would be relatively simple to compare in vitro dedifferentiation with dedifferentiation in the intact plant. The antibodies or differentially expressed cDNA clones identified in this thesis can be used to probe Western or Northern blots from macromolecules extracted from tissue undergoing dedifferentiation on the intact plant. If expression of such transcripts or proteins is similar then it is highly likely that in vitro dedifferentiation is similar to whole plant dedifferentiation. A similar comparison can be made with dedifferentiating tissues from other dicotyledonous and monocotyledonous species of plants. This will enable an estimation of the similarity of gene expression during
dedifferentiation to be made.

It will be more difficult to examine why the genome is unstable. For such a study DNA probes for hypervariable regions or DNA probes homologous with transposable elements have to be available. By examining DNA banding patterns during dedifferentiation some idea of DNA rearrangements might possibly be made. However, since no chromosome abnormalities were identified during within first wound-induced cell division of asparagus, it might be that analysis has to be performed on older dedifferentiated tissue or in a different species of plant. Such a study should also provide an idea of control over transposition during dedifferentiation.

A problem with the molecular approach used in this thesis is that the identification and the biological function of polypeptides is difficult to determine. This in part is due to very few plant proteins having been sequenced and so N-terminal peptide sequences very rarely show a match in data bases. One simple method available to work out biological functions of unknown cDNAs (polypeptides) is for reverse genetics to be used. For this to work, a cDNA clone specific to the polypeptide in question is required. A genomic clone has to be obtained, then expression cassettes can be constructed from sequences up stream of 5' end, driving an antisense cDNA sequence of the cDNA. This will effectively disrupt the expression of the gene in question, and by studying the effects of disrupted gene activity, valuable information can be obtained about the function of the protein of interest.

Though difficult it is not impossible to isolate cDNA clones for low abundance transcripts. Such random clones could be isolated from colonies that show little or no hybridisation with any probe population used during differential screening. These colonies show little or no hybridisation as the transcripts they represent are not found in sufficient abundance in the probe population. After purification the cDNA clone can be used to probe Northern blots, and the expression profiles worked out. Such transcripts may be involved in the of control cell cycle reactivation or of the cell cycle itself.

In conclusion dedifferentiation is a highly ordered process that involves transcriptional changes. The cloning of such differentially expressed transcripts has provided the opportunity for a more detailed examination of dedifferentiation to be undertaken. The promoter regions of such genes could be used to drive toxin genes with insecticidal properties or proteins with
anti-fungal or anti-bacterial properties during wound-induced dedifferentiation. This will conserve metabolites in the plant as the anti-insect proteins will only be synthesised when the plant is damaged and requires it.

This thesis provides an idea of the complex series of events that occurs during dedifferentiation and it is hoped that an even better understanding of plant plastic responses will result from the continuation of this work. It is also hoped that some aspects of dedifferentiation will be of some use to plant biotechnology and lead eventually to novel methods for controlling infection of crop plants at wound sites.
References


deposition in boundary layers formed after wounding in various plant species and organs. Canadian Journal of Botany., 65, 1886-1892.


measurements of photosynthesis. *Research Institute for Photosynthesis, University of Sheffield*, pub Oxygraphics Ltd, UK.


ABSTRACT

A Molecular Study of Dedifferentiation and Cell Cycle Reactivation
In Mechanically Isolated Asparagus Cells.

By Harikrishna Kulaveerasingam.

Mechanically isolated cell cultures were chosen as a model system to examine wound-induced dedifferentiation at the molecular level as large quantities of physiologically and morphologically similar G1-arrested mesophyll cells could be obtained. Within 5 days of culture such non-dividing, photosynthetic cells become heterotrophic, and have completed a first nuclear division and cytokinesis.

There are few changes in cell morphology during the first 2-3 days in culture. However, during this period there is a massive increase in respiration rate and total RNA synthesis. Following DNA synthesis there is a rapid cell expansion, mitosis and cytokinesis. Steady state transcript populations were monitored through the first 8 days of culture by analysis of the products of in vitro translations on 2-D gels. Large changes in gene expression were evident during the first 3 days in culture with several genes highly up-regulated and others down-regulated.

Dedifferentiation can be separated into 3 different phases. Firstly, reactivation of the cell cycle during which there are few cytological or physiological changes but gross changes in the expression of genes possibly associated with wounding or stress. Secondly, DNA synthesis, first mitosis event and phragmoplast formation during which there are minor changes in transcript abundance. Finally a continuation of the cell cycle with little alteration in transcript abundance.

Changes in plastid morphology are only apparent after 10-14 days resulting in the formation of proplastid like structures. However, mRNA for both large subunit ribulose bisphosphate carboxylase and small subunit ribulose bisphosphate carboxylase decrease to basal levels within a day of culture and photosynthetic capacity diminishes when the first cell division is evident. Plastid dedifferentiation can therefore be considered separately and proceeds slowly being more or less complete after 2-3 cell divisions.

Dedifferentiation is therefore seen to be a complex process which involves the interaction of several factors i.e wounding and hormones and results in temporal changes in transcript abundance, changes in the mode of respiration, morphology and cell proliferation.