Gene expression during regeneration of shoots in tobacco by tissue culture

Fernando, Kumudu

Award date:
1992

Awarding institution:
University of Bath

GENE EXPRESSION DURING REGENERATION OF SHOOTS IN TOBACCO BY TISSUE CULTURE

Submitted by Kumudu Fernando

for the degree of Ph.D

of the

University of Bath

1992

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my husband Nimal

and our children, Nishan and Milan

ABSTRACT

Leaf explants cultured on MS medium supplemented with 0.5 mg l^{-1} 2,4-D (CIM) for 4 days (first-stage) when transferred to a medium containing 1.25 mg l^{-1} BAP (SIM) produced shoots with very little callus within 12 days. When explants were subcultured onto second-stage CIM, a mass of callus was produced. The formation of endogenous root primordia was evident in some second-stage CIM cultures. Small cells with dense cytoplasm and prominent nuclei were more abundant and appeared earlier in second-stage SIM cultures as compared to second-stage CIM cultures. A tunica-like structure was evident in explants after 6 days on second-stage SIM. Total RNA and poly $(A)^+$ RNA increased when explants were subcultured onto second-stage SIM cultures whereas there was no significant increase in RNA levels in explants subcultured onto second-stage CIM cultures. Pulse labelling of proteins in *in vitro* cultures showed qualitative and quantitative differences between day 0 (before culturing) samples and the explants that had been on NHM, CIM or SIM for 2 days. Qualitative and quantitative differences were also seen between the second-stage SIM and second-stage CIM cultures. *In vitro* translations studies did not show any differences in translation profile between 1-8 day second-stage SIM and 2 day second-stage CIM (used as the control), except a band (Mr ~ 39 kDa) in 2 day second-stage CIM cultures. Differential hybridisation carried out with a cDNA library to poly $(A)^+$ RNA from SIM cultures identified five clones which appeared to be SIM specific. Slot blot and Northern analysis showed that there was an increase in the mRNA corresponding to one of these clones, SIM9, during the first three days on second-stage SIM. Similar increase was seen on day 5 on second-stage CIM, indicating that SIM9 was not SIM specific. Therefore SIM9 may be related to some protein that is commonly induced by both hormonal regimes, i.e., 2,4-D in CIM and BAP in SIM, but the protein appears more quickly and reaches a higher level in SIM cultures than in CIM cultures.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. J.R.Beeching and Professor G.G.Henshaw for supervising the project. My special thanks to Professor G.G.Henshaw for the concern during my illness and for the moral support given during the writing up of the thesis. I am deeply indebted to Dr. Jane Cavell for her helpful discussions and suggestions, and for proof reading this thesis. I would like to thank Pete Clark for his photography. I greatly appreciate the financial support of the Dept. of Agriculture, Sri Lanka, without which I could not have obtained this degree. I extend my eternal gratitude to my husband Nimal and to our two sons Nishan and Milan for their encouragement, patience and understanding.

ABBREVIATIONS

ABA	abscisic acid
APS	ammonium per sulphate
BAP	6-benzyl aminopurine
b	bases
bp	base pairs of DNA
cDNA	complementary DNA
Ci	Curie
CIM	callus induction medium
^{o}C	degrees centigrade
Da	Dalton
2,4-D	2,4-dichlorophenoxyacetic acid
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidine triphosphate
dGTP	deoxy guanosine triphosphate
dTTP	deoxy thymidine triphosphate
DC	damaged cells
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis (beta amino ethyl ether)-N,N,N',N'-tetraacetic acid
g	gram
GA	gibberellin
GA_3	gibberellic acid
h	hours
IAA	indole acetic acid
l	litre
LB	Luria broth
m	metre
M	molar
mAP	messenger affinity paper
min	minutes
MS	Murashige and Skoog medium
Mr	molecular weight
mRNA	messenger ribonucleic acid

NHM	no hormone medium
OD	optical density
PAL	phenylalanine ammonia lyase
pfu	plaque forming units
poly (A)	polyadenylic acid
poly $(A)^+$ RNA	polyadenylated ribonucleic acid
poly (U)	polyuridylic acid
RCP	root cap
RNA	ribonucleic acid
RNase	ribonuclease
RP	root primordium
rpm	revolutions per minutes
rRNA	ribosomal RNA
RRLS	rabbit reticulocyte lysate system
RUBISCO	ribulose-1,5-bisphosphate carboxylase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	seconds
SIM	shoot induction medium
TAL	tyrosine ammonia lyase
TBA	tertiary butyl alcohol
TCA	trichloroacetic acid
TEMED	N,N,N',N' tetramethyl ethylene diamine
tRNA	transfer ribonucleic acid
UV-	ultraviolet

CONTENTS

		Page
ABSTRACT		ii
ACKNOWLEDGEMENTS		iii
ABBREVIATIONS		iv
CONTENTS		vi
1	INTRODUCTION	1
1.1	Organogenesis	2
1.1.1	Developmental biology in organogenesis	2
1.1.2	Factors influencing organogenesis	4
1.1.3	Initiation of organogenesis	6
1.1.4	Histology during organogenesis	6
1.2	Plant hormones	8
1.2.1	Auxin and gene expression	11
1.2.2	Cytokinins and gene expression	14
1.2.3	Gibberellins and gene expression	19
1.2.4	Abscisic acid and gene expression	19
1.2.5	Ethylene and gene expression	20
1.3	Hormone-binding molecules	21
1.4	Gene expression during somatic embryogenesis	22
1.5	Gene expression during *in vitro* organogenesis	23
1.6	OBJECTIVE	27
2	*IN VITRO* CULTURES	28
2.1	INTRODUCTION	28
2.2	MATERIALS AND METHODS	28
2.2.1	Selection of *in vitro* systems	28
2.2.1.1	Species and source of explants	28
2.2.1.2	Preparation of explants for tissue cultures	28
2.2.1.3	Nutrient media and their preparation	29
2.2.2	Histological work	30
2.2.2.1	Fixing of plant material	30
2.2.2.2	Dehydration	30
2.2.2.3	Embedding the specimens in wax	30
2.2.2.4	Sectioning	31
2.2.2.5	Mounting	31

2.2.2.6 Staining 31
2.3 RESULTS 32
2.3.1 Selection of first-stage medium 32
2.3.2 Developmental measurements 34
2.3.3 Histological studies 36
2.4 DISCUSSION 40

3 CHANGES IN AMOUNTS OF TOTAL AND POLYADENYLATED RIBONUCLEIC ACID IN *IN VITRO* CULTURES 46
3.1 INTRODUCTION 46
3.2 MATERIALS AND METHODS 46
3.2.1 General methods 46
3.2.1.1 Removal of ribonucleases 46
3.2.1.2 Precipitation of RNA 47
3.2.1.3 Estimation of purity of RNA 47
3.2.1.4 Preparation of molecular weight markers 48
3.2.1.5 Denaturing gel 48
3.2.1.6 Quantification of RNA 49
3.2.2 Preparation of total RNA 50
3.2.2.1 Extraction method D 50
3.2.2.2 Removal of polysaccharides 51
3.2.3 Purification of polyadenylated RNA 51
3.2.3.1 Using messenger affinity paper 52
3.2.3.2 Affinity chromatography on oligo (dT) cellulose 52
3.2.4 Quantification of poly $(A)^+$ RNA 53
3.2.4.1 Determination of the amount of poly (A) in poly $(A)^+$ RNA 53
3.2.4.2 Determination of the ratio of poly (A) tail to poly $(A)^+$ RNA 54
3.3 RESULTS 55
3.3.1 Total RNA accumulation in *in vitro* cultures 55
3.3.2 Isolation of poly $(A)^+$ RNA from *in vitro* cultures 57
3.3.2.1 Determination of the ratio of poly (A) tail to poly $(A)^+$ RNA 57
3.3.2.2 Accumulation of poly $(A)^+$ RNA in *in vitro* cultures 57
3.4 DISCUSSION 58

4 *DE NOVO* SYNTHESIS OF PROTEINS IN *IN VITRO* CULTURES 64
4.1 INTRODUCTION 64
4.2 MATERIALS AND METHODS 65
4.2.1 Preparation of samples 65

4.2.2 Determination of incorporation of radioactivity 65
4.2.3 Preparation of molecular weight markers 66
4.2.4 Analysis of polypeptides by SDS-PAGE 66
4.2.5 Autoradiography and fluorography 67
4.3 RESULTS 67
4.3.1 Protein synthesis in first-stage cultures 67
4.3.2 Protein synthesis in second-stage cultures 70
4.3.3 Protein synthesis in second-stage SIM and CIM cultures 73
4.3.3.1 During the first four hours after transfer 73
4.3.3.2 During 1-8 days 73
4.3.3.3 During 12-24 days 78
4.4 DISCUSSION 78

5 ANALYSIS OF mRNA POPULATIONS UNDER DIFFERENT HORMONE REGIMES AND DURING SHOOT ORGANOGENESIS 84
5.1 INTRODUCTION 84
5.2 MATERIALS AND METHODS 84
5.2.1 *in vitro* translation of poly $(A)^+$ RNA 84
5.2.1.1 Rabbit reticulocyte lysate system N. 150 85
5.2.1.2 Wheat germ system 86
5.2.1.3 Rabbit reticulocyte lysate system N. 90 86
5.2.1.4 Two dimensional SDS-PAGE 87
5.3 Optimisation of translation systems 87
5.3.1 Rabbit reticulocyte lysate system-N. 150 87
5.3.2 Wheat germ system 88
5.3.3 Rabbit reticulocyte lysate system-N. 90 91
5.3.4 Comparison of wheat germ and RRLS-N. 90 92
5.4 RESULTS 92
5.4.1 *In vitro* translations of poly $(A)^+$ RNA isolated from tissue cultures 92
5.4.1.1 Poly $(A)^+$ RNA from SIM cultures 92
5.4.1.2 Poly $(A)^+$ RNA from shoot and callus cultures 94
5.5 DISCUSSION 98

6 CONSTRUCTION OF A COMPLEMENTARY DNA (cDNA) LIBRARY 103
6.1 INTRODUCTION 103
6.2 MATERIALS AND METHODS 103
6.2.1 Synthesis of double stranded cDNA 103
6.2.1.1 Synthesis of the first strand 104

6.2.1.2 Synthesis of the second strand 105
6.2.1.3 Calculation of percentage incorporation into cDNA 105
6.2.1.4 Alkaline gel electrophoresis of cDNA 106
6.2.1.5 Purification of double stranded (ds) cDNA 106
6.2.2 cDNA cloning 106
6.2.2.1 Methylation of *Eco*RI restriction sites 107
6.2.2.2 Addition of *Eco*RI linkers to cDNA 108
6.2.2.3 *Eco*RI digestion of linkered cDNA 108
6.2.2.4 Separation of cDNA from excess linker molecules 109
6.2.2.5 Ligation of cDNA to λ gt10 arms 109
6.2.2.6 *In vitro* packaging of ligation mixtures 110
6.2.2.7 Transfection of *E. coli* 110
6.2.2.8 Isolation of DNA from lambda clones 111
6.2.2.9 Determination of presence and size of insert 112
6.2.2.10 Amplification of the library 113
6.3 RESULTS 113
6.3.1 cDNA synthesis 113
6.3.1.1 Percentage incorporation during first strand synthesis 113
6.3.1.2 Amount of first strand cDNA synthesised 115
6.3.1.3 Percentage yield of first strand cDNA 115
6.3.1.4 Percentage incorporation during second strand synthesis 115
6.3.1.5 Amount of second strand synthesised 115
6.3.1.6 Percentage yield of second strand cDNA 115
6.3.1.7 Alkaline agarose gel electrophoresis 115
6.3.2 cDNA cloning 117
6.3.2.1 Separation of *Eco*RI linkers 117
6.3.2.2 Transfection of *E.coli* 118
6.3.2.3 Determination of presence and size of inserts 119
6.4 DISCUSSION 121

7 IDENTIFICATION OF SIM SPECIFIC CLONES 124
7.1 INTRODUCTION 124
7.2 MATERIALS AND METHODS 125
7.2.1 Preparation of plaque lifts 125
7.2.2 Differential hybridisation of the filters 125
7.2.2.1 Synthesis of single stranded cDNA probe 125
7.2.2.2 Chromatography through Sephadex G50 126
7.2.2.3 Hybridisation of plaque lifts 127
7.2.3 Subcloning of inserts into a plasmid vector 128

7.2.3.1 Purification of lambda recombinant DNA 128
7.2.3.2 Subcloning procedure 129
7.2.4 Probing RNA with insert DNA 130
7.2.4.1 Preparation of DNA to be labelled 130
7.2.4.2 Oligolabelling 131
7.2.4.3 Preparation of RNA slot blots 131
7.2.4.4 RNA for Northern analysis 132
7.2.4.5 Hybridisation of RNA with insert DNA 133
7.2.4.6 Removal of probe and re-use of blots 134
7.3 RESULTS 134
7.3.1 Differential hybridisation 134
7.3.2 Subcloning of inserts into plasmid vector 138
7.3.3 Purification of insert DNA from plasmids 138
7.3.4 Levels of SIM specific mRNA during culture 138
7.3.4.1 Probing RNA on a slot blot 138
7.3.4.2 RNA on a Northern blot 141
7.4 DISCUSSION 141

8 CONCLUSION 153

REFERENCES 159

APPENDIX A 174

APPENDIX B 176

APPENDIX C 178

APPENDIX D 183

1 INTRODUCTION

The plant cell is said to be totipotent in that it is supposed to possess the ability to regenerate into plants. Haberlandt (1902) was one of the first to predict that isolated cells could be cultured *in vitro* and subsequent studies brought this prediction into reality (White, 1939; Skoog and Miller, 1957; Steward *et al.*, 1958). It is now possible with many species to culture organs, cells and protoplasts and to induce them to regenerate into plants, although there are cell types and species in which totipotency has not been demonstrated.

In tissue culture, totipotency manifests itself through somatic embryogenesis or organogenesis. Somatic embryogenesis follows the same sequence of morphogenetic events that is found in zygotic embryo development, that is globular stage, heart shape stage, torpedo stage and finally the embryo. These somatic embryos may arise from a single cell or a group of cells (Maheshwaran and Williams, 1985). Organogenesis is the development of organs such as shoots, roots, flowers etc. from meristematic foci in *in vitro* grown tissues. In both organogenesis and somatic embryogenesis, the parental tissues do not possess initials for organ or embryo development, but cells become meristematic and subsequently develop either into an organ or an embryo during *in vitro* culture. These changes in development involve changes in gene expression.

Recent advances in the field of biotechnology such as protoplast fusion and gene transfer techniques have, respectively, made possible the production of somatic hybrids of distantly related plants and the introduction of genes with desirable characters into other plant cells. However, one of the constraints on these systems is the difficulty in regenerating plants from the hybrid and transformed cells. The precise understanding of organised development in tissue cultures at the molecular level would be of importance, if the regeneration of plants is to be achieved routinely in genetic manipulation. Therefore, it would be

very useful to increase our knowledge of the genes involved in regeneration of plants by tissue culture and the regulation of their expression. As an introduction to this project on gene expression during regeneration of shoots from tobacco, I propose to review the literature on important aspects of organogenesis, gene expression in response to plant hormones and also the present status of research on gene expression during organogenesis and somatic embryogenesis.

1.1 ORGANOGENESIS

Under natural conditions, if a shoot is separated from the parent plant and planted, roots will often be formed giving rise to a plant. Adventitious buds may be formed from a cut stump. These are examples of organogenesis in nature. These processes are controlled by hormones within the plant and one of the main factors controlling whether buds or roots develop *in vivo* is the cytokinin/auxin ratio. Auxin is produced near the shoot apex and cytokinin is produced in the roots. The loss of roots increases the auxin/cytokinin ratio in the shoot and enhances root development; loss of the shoot apex increases the cytokinin/auxin ratio and stimulates shoot production (Goldsworthy, 1988). Similarly, a variety of organs such as shoots, roots, leaves and flowers can be produced in cell and tissue cultures by manipulating *in vitro* conditions. The initiation of cell proliferation occurs due to wounding at the edges of the explant. The cell division normally stops after some time, but it can be induced to continue in the presence of an auxin in the medium to form an unorganised mass of tissue called callus. If the conditions in the medium are favourable for organogenesis, groups of cells in callus gradually form organ primordia. These will finally grow longitudinally with well defined growing points at the apex (e.g.: shoot or root apex) to give rise to a particular type of organ.

1.1.1 Developmental biology in organogenesis

The removal of a part from the entire plant disturbs the organisational constraints imposed by the plant and the immediate response by the explant will

be physiological changes at the wounded surface. In potato storage tissue, an increase in the size of the nucleus and nucleolus was noticeable within 24 h of injury and in Jerusalem artichoke the nucleus reaches maximal size within 24 h after wounding (Street, 1977). In addition to cell proliferation, increased ribosomal ribonucleic acid synthesis, increases in the amount of endoplasmic reticulum and synthesis of novel proteins have been observed in association with the wound response (Barckhausen, 1978). In the presence of an auxin, the process of cell division initiated due to wounding is triggered at the cut edges of the explant and produces an unorganised mass of tissue (callus). This loss of specialised function is sometimes referred to as dedifferentiation, that is reversion to the lowest level of tissue organisation. Transfer of these explants to a medium containing cytokinin may favour shoot organogenesis.

Henshaw *et al.* (1982) described three phenomena occurring in relation to organogenesis: competence, determination and differentiation. Competence is confined to a group of cells that can respond to an appropriate signal to undergo organogenesis. When these cells are committed to a specific developmental fate, e.g.: shoot development, they are said to be determined. Once determined the cells may undergo differentiation if exposed to permissive conditions for organ formation.

Christianson and Warnick (1988) studied the *in vitro* organogenesis of *Convolvulus arvensis*, using leaf material as explants. They observed that culturing explants on callus induction medium for 3 days prior to transfer onto shoot induction medium shortened the time required by the explant to acquire 'determination' for shoot production on shoot induction medium. They separated the process of organogenesis into 'dedifferentiation' (the process of converting differentiated cells to a state of callus formation), 'induction' (change in the destiny of a cell or a group of cells) and 'determination' (the process of obtaining a fixed destiny for a specific developmental state).

It is not yet known, however, whether each of these steps is regulated by the differential expression of genes, and it is not known how hormones establish the pattern of organogenesis at the cell and molecular levels. Meins (1986) stated that there are two hypotheses: (1) hormones act primarily to induce cells that are competent but not committed to a particular developmental fate; (2) the cells are already determined to follow a certain developmental pathway and hormones evoke the expression of the committed state. He suggested that hormones act in both ways depending on the experimental system. The most critical event in organogenesis may be the acquisition of competence but the number of cells involved in this event may be very small (Meins *et al*,1982). Hence one of the problems in investigating the molecular mechanisms underlying organogenesis using cell and tissues is likely to be the difficulty of detecting genes that are rare.

1.1.2 Factors influencing organogenesis

The literature on plant tissue culture reveals that many factors can affect organogenesis. These may broadly be divided into (i) physical effects such as light, pH, temperature, culture environment, (ii) biochemical effects such as growth substances, metabolites and mineral salts, and (iii) the genetic or epigenetic state of the explant, affecting the ability to alter patterns of gene expression in culture.

Skoog and Miller (1957) discovered an important principle controlling organogenesis in tissue cultures of tobacco. They found that a high cytokinin/auxin ratio encourages shoot formation whereas a high auxin/cytokinin ratio enhances root formation. This concept of the overall regulatory effect of the auxin/cytokinin balance has been confirmed by many workers (for example: Vasil and Vasil, 1972; Street, 1977). Recent confirmation of this idea has come from experiments which altered the hormone ratio *in vivo*. Inze *et al.* (1984) and Schröder *et al.*(1984) transformed plant cells with the T-DNA from the Ti

plasmid of *Agrobacterium tumefaciens* in which the T-DNA genes responsible for auxin biosynthesis had been inactivated. This increased the cytokinin/auxin ratio resulting in shoot formation. The converse experiment involving inactivation of the genes that encode cytokinin biosynthesis resulted in root formation (Akiyoshi *et al.*, 1984).

Although the most important factor for the regulation of organogenesis is the auxin/cytokinin ratio, other hormones may also affect the process. There are reports of GA having inhibitory effects on shoot formation in *in vitro* grown tobacco tissues (Murashige, 1961). Kochba *et al.* (1978) observed suppression of *in vitro* shoot formation and somatic embryo formation when GA was applied to citrus ovular callus. Webb *et al*. (1983) employed GA in combination with auxin and cytokinin for enhancement of shoot proliferation from *in vitro* cultured potato leaf discs. Abscisic acid was also found to promote shoot growth in cultures of sweet potato (Shepard, 1980; Yamaguchi and Nakajima, 1984). Huxter *et al.* (1981) observed the effect of ethylene on *in vitro* shoot formation in tobacco, showing inhibition, especially during shoot initiation; and stimulation of shoot growth during later stages of development.

There are also many examples in the literature where organogenesis was brought about by changing levels of macro and micro elements, by adding diverse plant extracts such as coconut milk to the medium, by changing culture conditions (e.g: liquid medium as opposed to solid medium) and by providing amino acid supplements (see review by Tran Thanh Van, 1981). Trewavas (1983) argued that nitrate should be considered as a growth regulator rather than as a nutrient, as it dramatically modifies plant development. Myo-inositol, a sugar alcohol, was found to promote bud formation (Wolter and Skoog, 1966).

The multiplicity of hormone combinations that stimulate organogenesis and the factors other than exogenously applied hormones that affect organogenesis, raise the question whether organogenic expression is controlled by endogenous hormones and other critical metabolite levels within the tissue,

which determine the response to exogenously applied hormones and other factors that will bring about organogenesis. Considering the above information about the variables influencing organogenesis an important question emerges: are the different agents/conditions directly or indirectly affecting the gene expression responsible for organ initiation?

1.1.3 Initiation of organogenesis

The initiation of organogenesis involves the formation of meristemoids in the explant. Meristemoids are cells capable of producing the whole plant, and consist of small spherical cells with dense cytoplasm and large nuclei (Thorpe and Murashige, 1970). Meristemoids are rare, with only one meristemoid per 1000-10000 cells (Meins *et al.*, 1982) and it has been suggested that they are initially capable of giving rise to either roots or shoots (Thorpe, 1978). These meristemoids undergo further modifications cytologically, ultrastructurally and biochemically to bring about organ formation.

1.1.4 Histology during organogenesis

When the explant is transferred to a suitable nutrient medium, the initial cell division results in callus formation at the periphery of the explant. During organogenesis this normal cell proliferation at the cut edges is slowed down and sometimes stops. Cell division in the deeper tissues begins, giving rise to smaller closely packed cells, resembling vascular tissues. It was suggested by Thorpe (1978) that the formation of these localised meristematic tissues is important in morphogenesis and a new morphological state has been reached. Thorpe and Murashige (1968) detected starch accumulation in cells at loci which eventually formed shoot primordia. They suggested that starch served as an energy reserve to be used during organogenesis. Meada and Thorpe (1979) studied the histological events during shoot organogenesis in tobacco from callus cultures. According to them histological events related to shoot organogenesis were (1) appearance of patches of tracheids somewhat interior in

the callus, (2) formation of meristemoids with prominent nuclei in close proximity to the tracheids, (3) formation of broad protrusions composed of elongating parenchymatous cells with starch grains, (4) appearance of cell strands connecting groups of meristemoids and (5) extension of cell strands towards the surface from which the shoot apex was formed. They observed that not all the meristematic nodules gave rise to shoot primordia. The meristematic nodules in the upper part of the callus remained without forming shoots and the ones in the lower part of the callus (closer to the medium) with less lignified elements developed shoot primordia. They stated that this difference in shoot formation within the callus tissue was due to the differences in the physiological gradient within the tissue.

Christianson and Warnick (1988) observed histological changes in the development of shoots from *Convolvulus arvensis* leaf explants. After 7 days on shoot induction medium they observed cell proliferation at the cut margins with more extensive proliferation at cut veins. They observed meristemoids similar to those described by Maeda and Thorpe (1979) by day 10 on shoot induction medium and well-formed shoot axes were seen after 12 days on the same medium. Formation of epidermis was observed as new shoot epidermis which was contiguous with the outermost cell layer of the callus. These authors suggested that the shoot apex formation was a superficial phenomenon. Maeda and Thorpe (1979) also observed cells at the surface that stained dark with safranin with thick granular deposits, suggesting regeneration of epidermal-like cells at the shoot apex.

It is seen that most experiments in tissue culture have been largely empirical, being limited to the identification of the substances which will induce development of organised structures. Despite extensive work on morphogenesis during regeneration of shoots *in vitro*, the basic molecular events of the process have not been studied in detail. Specific genes essential for the regeneration process, if they exist, have not been identified, and neither have the precise roles

and mechanisms of endogenous hormones in modulating the process of organogenesis been elucidated.

1.2 PLANT HORMONES

Plant hormones or plant growth regulators are defined as organic compounds which in small amounts promote, inhibit or otherwise modify any physiological process in plants (Tukey *et al.*, 1954). The five major groups of plant hormones; i.e. auxins, cytokinins, gibberellins, abscisic acid and ethylene, have all been shown to influence plant growth and development *in vivo* and *in vitro*.

Auxins

The naturally occurring auxin indole acetic acid (IAA) has been shown to influence various physiological processes including cell expansion during shoot elongation, apical dominance and root initiation (Finkelstein *et al.*, 1988). It is believed that the elongation of cell is at least partly due to hydrogen efflux from the cytoplasm to the cell wall. This acidification of the wall is thought to enhance wall loosening bringing about cell elongation (Cleland, 1973). The auxin requirement in plant tissue culture suggests that auxin plays a fundamental role in plant development. The synthetic auxin 2,4-D has been used widely in tissue culture. Depending on the tissue involved, and the culture conditions, auxins are known to stimulate cell division, cell elongation and cell differentiation.

Cytokinins

Cytokinins are a group of plant hormones basically resembling adenine (6-aminopurine). They include the synthetic cytokinins kinetin (6-furfurylamino-purine), benzyl adenine and the naturally occurring zeatin. The group of cytokinins generally regulate cell division and in combination with auxin bring about cell division and cytodifferentiation (Fosket, 1980). Fukuda and Komamine (1985) found that *Zinnia* cells grown in a high concentration of

cytokinin and low concentration of auxin differentiated into tracheary elements without undergoing cell division and the transformation from mesophyll cells to tracheary elements was observed after 48 h and found to be completed by 96 h. In Jerusalem artichoke, a high concentration of BAP (5.0 mg l^{-1}) in combination with a low concentration of 2,4-D (0.2 mg l^{-1}) induced mitosis after 20 h, and tracheary elements appeared after 50-52 h (Phillips, 1987). The nature of proteins formed during tracheary element formation has been speculated as phenylalanine ammonia lyase (PAL) and peroxidases, which are associated with lignification or enzymes that are involved in biosynthesis of wall components (Roberts, 1988). There is also evidence that cytokinins affect chloroplast development. Cytokinins were found to exert a stronger and much more specific effect on chloroplast development than on cell proliferation in tobacco (Feierabend, 1981; Parthier, 1979).

Gibberellins

Gibberellins (GA) are naturally occurring hormones and are thought to be ubiquitous in higher plants. Chemical characterisation of gibberellins has identified at least 72 different forms (Sponsel, 1987). Physiological studies have shown that GA is involved in cell division at shoot apices, stem elongation in seedlings and the production of hydrolytic enzymes during germination of seeds (Finkelstein *et al.*, 1988). Gibberellic acid (GA_3) is often used in combination with auxin and cytokinin in plant tissue culture. Physiological studies have suggested that gibberellin (GA) is involved in stimulating cell division at shoot apices (Sachs, 1978), and during periods of rapid growth during embryogenesis in seeds (Chory *et al.*, 1987).

Abscisic acid

Abscisic acid (ABA) is generally considered as a growth inhibitor because it is associated with seed and bud dormancy (Wareing and Phillips, 1983). In addition it has an influence on seed germination and embryogenesis.

It is rarely used in *in vitro* cultures because of its known inhibitory effects such as prevention of cytodifferentiation in callus cultures, especially in root organogenesis (Roberts, 1988). It is less widely used in tissue culture than are auxins and cytokinins. Shepard (1980) reported that ABA induced shoot initiation in callus, derived from potato protoplasts. ABA was also found to reverse the inhibitory effect by GA on tobacco *in vitro* shoot formation (Thorpe and Meier, 1973).

Ethylene

Ethylene is an olefin which exists as a gas. Physiological studies have shown that ethylene influences various developmental processes including germination, fruit ripening and senescence. Ethylene is not usually used as an exogenous hormone in tissue cultures. However, it is produced as an immediate response to wounding during excision of the explant from the parent tissue. The synergistic effects of ethylene and auxin lead to cell division (Roberts, 1988). Sauerbrey *et al.* (1987) reported the accumulation of ethylene in sunflower cell suspension cultures grown in a medium containing 2,4-D as the growth hormone. These cultures produced a large amount of ethylene (2 nmoles/10^6 cells/h) during the exponential growth phase; the authors concluded that ethylene evolution as a byproduct of actively dividing cells in suspension cultures was stimulated by 2,4-D. Ethylene is known to affect seed germination, seedling growth, leaf and root growth, plant senescence and fruit development (Abeles, 1973; Liebermann, 1979). Biosynthesis of ethylene has been conclusively linked with fruit ripening; fruit exposed to exogenous ethylene begin ripening and inhibitors of ethylene biosynthesis prevent this ripening (Yang, 1985). Trace amounts of ethylene have been shown to initiate fruit ripening and once ripening starts there is an autocatalytic burst of ethylene production (Yang, 1985; McGlasson, 1985). A stimulatory effect on somatic embryogenesis in citrus was demonstrated by Kochba *et al.* (1978). However, Tisserat and Murashige

(1977) observed an inhibitory effect of ethylene on somatic embryogenesis in citrus. Production of ethylene by plant material and its influence on morphogenesis is somewhat contradictory because of the positive and negative effects reported by various workers.

It is therefore evident that each plant hormone can be involved in more than one event, and more than one plant hormone appears to be involved in any given event. However, in most of the *in vivo* and *in vitro* experiments on the effects of hormones on gene expression, only the role of applied, exogenous hormones has been investigated. The results have been interpreted as effects of a specific plant hormone on a specific event. The roles of endogenous hormones and their interaction with exogenous ones are difficult to assess.

1.2.1 Auxin and gene expression

Auxins are a class of hormones that control both cell division and cell elongation (Thimann, 1969). Generally a positive correlation was found between the ability of auxin to enhance RNA synthesis at concentrations that increase cell growth, and to inhibit RNA synthesis at concentrations that would decrease cell growth (Trewavas, 1968; Key, 1969). Addition of actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) inhibited auxin-induced cell elongation (Key and Ingle, 1969; Hagen, 1987; Walker and Key, 1982; Walker *et al.*, 1985, Theologis, 1986). These results indicate that the action of auxins is mediated by alteration in gene expression.

Baulcombe and Key (1980) and Baulcombe *et al.* (1981) reported the expression of a few highly abundant poly $(A)^+$ RNAs in soybean hypocotyl tissues treated with auxins. The authors identified 20 polypeptides which were enhanced and another 20 reduced in auxin treated tissue. They isolated 3 cDNA sequences that were decreased in response to 2,4-D. Time course experiments indicated that mRNAs hybridised to clones, p3. p9 and p11 decreased by 66, 9

and 20 fold respectively, after 4 h and became undetectable after 20 h of auxin application. Zurfluh and Guilfoyle (1980) showed the alteration of proteins in soybean hypocotyl due to 2,4-D treatment; analysis of *in vivo* labelled proteins from auxin treated elongating hypocotyl sections and non-elongating basal sections showed that induction of protein synthesis by 2,4-D was tissue specific. These authors also reported that auxin induced polypeptides could be masked by those induced by wounding. Subsequently, Zurfluh and Guilfoyle (1982 a, b, c) demonstrated the appearance of specific polypeptides by carrying out *in vitro* translations with poly $(A)^+$ RNA isolated from auxin treated and untreated tissues and separating the resultant polypeptides by 2D gel electrophoresis. They also found that some of these polypeptides were identical to those induced by IAA in pea epicotyl sections, suggesting that both hormones increased the levels of identical sets of translatable mRNA.

Similar observations were reported by Theologis and Ray (1982a, b) when excised epicotyl tissues were treated with auxins. They reported about five mRNAs responsive to IAA in the pea epicotyl, based on their work on *in vitro* translations. Three of these mRNAs increased their levels within 10-20 min of IAA application indicating an early response to the auxin. It was earlier thought that these may be involved in cell elongation by activating the proton extrusion pump. However, Theologis and Ray (1982b) showed that although high levels of these mRNAs were found in cut epicotyl sections treated with auxin, cell elongation was lower in these sections than in the untreated intact plant where these mRNAs were much less abundant. They proposed that these mRNAs may not be related to cell elongation by proton efflux, but may be involved in cell enlargement and the steady state of cell elongation. They categorised those mRNAs which were found to be abundant after 1 h of auxin application as late responding mRNAs. It was suggested that these might be involved in events such as cell wall biosynthesis, ethlylene production, cell division and root initiation. Subsequently Theologis *et al.* (1985) constructed a

cDNA library from RNA isolated from IAA treated pea epicotyl tissues and identified auxin induced clones. They found that induction of some of these specific mRNAs occurred, rather than the alteration of pre-existing translatable mRNAs. The induced mRNAs increased rapidly (2-50 fold) 10-30 min after auxin application. McClure *et al.*, (1989) characterised a gene cluster in soybean hypocotyl that encodes a group of auxin-regulated RNAs. They detected an increase in the rate of transcription of this cluster 10 min after application of 2,4-D to sections of soybean hypocotyl. They revealed that there were five genes in the portion of DNA they sequenced, at least three of which were transcriptionally regulated by auxins. Reddy *et al.* (1990) identified two auxin induced genes in auxin treated strawberry receptacles. They observed a high level of auxin-induced mRNAs in auxin treated pollinated fruits as compared to unpollinated fruits. This demonstrates that the developmental state of a tissue can affect its response to hormone treatment.

Change in gene expression due to auxin in cell cultures has also been reported. For example, Bevan and Northcote (1981b), using suspension cultures of bean and soybean, observed increased levels of mRNAs within 2 h of subculturing to a medium containing 2,4-D. They identified two 2,4-D responsive polypeptides of 15 and 40 kDa, the levels of which were reduced 10 h after subculture.

Van der Zaal *et al.* (1987) applied 2,4-D to tobacco cell suspension cultures at the stationary phase of the growth curve and observed that cell division was restored within 10-12 h of 2,4-D application. They identified two 2,4-D induced mRNAs by carrying out cell free translations of mRNAs and determined that the lowest 2,4-D concentration required to induce these mRNAs varied from less than 2.2×10^{-8} M to 2.2×10^{-6} M. By carrying out differential hybridisation of a cDNA library made with poly $(A)^+$ RNA isolated from auxin treated cells, they identified seven clones which were responsive to 2,4-D.

Auxin-induced mRNA production was also observed by Meyer *et al.*

(1984). They worked with tobacco protoplasts and found that auxin specific mRNAs appeared within 30 min of application and reached a constant level after 2-4 h of auxin treatment. The level of synthesis of some other proteins was reduced after 6 h of auxin application. They concluded that the early induced mRNAs occurred as a direct effect of auxin and the late responsive ones as direct or indirect effects of auxin. In addition, they concluded that the early responsive mRNAs were involved in cell elongation and the late responsive ones in mitotic cell division. Similar effects were observed in the experiments mentioned earlier with pea epicotyl and soybean hypocotyl. Meyer *et al.* (1984) also concluded that auxin induced changes in protein synthesis are not a result of proton extrusion; this was also confirmed by Theologis (1986).

In summary, it is therefore evident that changes of some mRNAs and induction of others are caused by application of auxins to plant tissues; the earliest observed mRNAs are induced after 10-20 min auxin application. The effect of auxin on mRNA levels occurs in two stages: first within 5-60 min of application which may be involved in cell elongation and cell enlargement, and the second where changes in mRNA occur after 2 h of application. The latter may be involved in longer term effects such as cell division, vascular differentiation, and adventitious meristem initiation.

1.2.2 Cytokinins and gene expression

Cytokinins are well known to activate the synthesis of total RNA and proteins in higher plant tissues. It has been demonstrated that cytokinin activated the production of all RNA types; rRNA, tRNA and some mRNAs (Kuleava, 1981). Ananiev *et al.* (1987) investigated the effect of cytokinin on ribosomal RNA gene expression in excised cotyledons of *Cucurbita pepo* L. They used ^{6}N benzyl adenine and ^{1}N-(2 chloro-4 pyridyl) ^{2}N phenyl urea on isolated nuclei. They observed a rapid increase in RNA polymerase-I activity with both cytokinins. The maximum stimulation was seen after 4-6 h of cytokinin

application and stimulation did not occur when cotyledons were treated with cytokinins in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that cytokinins induce synthesis of RNA polymerase-I. The benzyl adenine and the derivative of phenyl urea both appeared to have the same effect on ribosomal gene expression, although they have quite different structures.

Several authors have reported cytokinin-induced changes in mRNA and polypeptide populations. For example, Fosket *et al.* (1981) carried out *in vivo* labelling experiments in soybean cell cultures grown in a medium containing cytokinin, using [^{35}S] methionine, and observed qualitative and quantitative differences in proteins which occurred prior to cytokinin induced cell division. They detected a protein Mr 55 kDa, which is approximately the molecular weight of tubulin, the major component of microtubules necessary for spindle formation. Crowell *et al.* (1990) constructed a cDNA library to mRNA isolated from soybean suspension cultures grown in a medium supplemented with cytokinin. They carried out differential hybridisation and identified two cytokinin induced cDNA clones for ribosomal protein genes.

Chen *et al*. (1987) studied the response of pumpkin cotyledons to exogenous BAP and reported that cytokinins enhanced, reduced, induced or inhibited synthesis of some polypeptides. They observed that the earliest response was within 60 min of application, when BAP was applied to excised cotyledons, and after 7.5 h, when BAP was applied to cotyledons cultured on filter papers. Hybridisation of a cytokinin induced cDNA clone to slot blots of RNA from BAP treated cotyledons showed a three fold increase in mRNA levels after 60 min and a 12 fold increase after 5 days. The mRNA corresponding to this cDNA clone was detectable in every organ of 20 day old pumpkin plants with the highest concentration being in the roots.

One of the cytokinin-responsive proteins is the light harvesting chlorophyll a/b-binding protein (LHCP). Teyssendier De La Serve *et al.* (1985) used a LHCP cDNA as a probe to measure the levels of homologous mRNAs in

kinetin treated tobacco suspension cultures, incubated for 9 days under continuous light. They found that the regulation of LHCP gene expression by kinetin and light was at the transcriptional level. However, Flores and Tobin (1986) examined the effects of BAP and light on mRNA levels in duck weed plants and concluded that regulation of LHCP gene expression by BAP and light was not at transcriptional level but post-transcriptional.

Memelink *et al.* (1988) transformed tobacco cells with the T-DNA from the Ti plasmid of *Agrobacterium tumefaciens*. These cells had an altered developmental potential due to changes in their endogenous phytohormone level and significantly they also exhibited changes in levels of specific mRNAs. Transgenic cells with T-cyt (cytokinin synthesis gene) produced numerous stunted shoots but no roots. They also found differences in gene expression between transformed and untransformed cells.

Incorporation of cytokinins into transfer RNA (tRNA) has been reported by many workers (Fox, 1966; Armstrong *et al.* 1976; Burrows *et al.* 1971). Burrows *et al.* (1971) identified the cytokinin bases that occur in tRNA of a cytokinin requiring tobacco callus grown on benzyl adenine (BAP). Assays carried out with tRNA hydrolysates showed the presence of benzyl adenosine, 2-iso-pentenyl adenine, zeatin riboside and the methyl derivative of zeatin riboside which were normally present in untreated tissue. They suggested that there is an increase in production of natural cytokinins as a result of the application of exogenous cytokinin. It has been reported that the incorporation of cytokinin into tRNA increases the affinity of the aminoacylated tRNAs, facilitating the codon recognition in the polyribosomal complexes (Gefter and Russel, 1969). Incorporation of BA into ribosomal RNA (rRNA) has been shown by many workers (Armstrong *et al.*, 1976; Teyssendier De La Serve and Jouanneau 1979; Jouanneau et al ., 1984; Teyssendier De La Serve *et al.*, 1982, 1984, 1985). Teyssendier De La Serve *et al.*, (1984) incubated tobacco cells in suspension cultures with 0.4 μM BAP, which was the optimum

concentration for cell division, and 10 μM BAP, which was the cytostatic concentration. They compared the incorporation of BAP with the control, that is with no BAP added. They found that BAP was incorporated into both the poly (A) segment and the coding region of mRNA, in the 0.4 and 10 μM BAP treated samples, in direct proportion to the concentration. Although the low and high concentrations of BAP had different physiological effects, promoting cell division and causing cytostasis respectively, there was no evidence for any change in gene expression as shown by *in vitro* translation. However, these observations suggest that cytokinin could act by altering the structures of transfer or ribosomal or messenger RNA.

Fosket and Tepfer (1978) examined the possibility that some mRNAs are transcribed but not translated in cytokinin deprived soybean cells, and that cytokinin causes these mRNAs to be translated. They grew cells in a medium containing [^{3}H] uridine for 18 h and subsequently transferred the cells either to a medium with zeatin or to a medium without zeatin. They isolated RNA from both cell types and separated the total poly $(A)^+$ RNA using oligo (dT) chromatography. They determined the specific activity of each RNA fraction and found that specific activity of nonpolyadenylated RNA was not changed, specific activity of polysomal poly $(A)^+$ RNA was increased by 79% and specific activity of poly $(A)^+$ RNA was decreased by 40% in cytokinin treated tissue. They interpreted these results by suggesting that mRNAs synthesised in the absence of cytokinin moved to polysomes when treated with the hormone and that mRNAs that were previously in 'cryptic' form were rendered translatable by cytokinin stimulation. Szweykowska *et al*. (1981) showed that cytokinin (kinetin) enhanced protein synthesis by increasing the affinity of ribosomes to mRNAs. They isolated polyribosomes from ^{6}N isopentenyl-adenine (i^6 ade) treated and untreated moss protonema and carried out *in vitro* translation using wheat germ extract. The activity of polyribosomes from cytokinin treated tissue was considerably higher than the control and after 12 h

of i^6 ade treatment the stimulation reached 140%. The level of polyribosomes in both treated and untreated tissues remained the same, as shown by sucrose density gradient. They suggested that cytokinin increases the activity of polyribosomes. *In vitro* translation of ribosomal preparations from pumpkin cotyledons showed that there was a marked increase in protein synthesis in kinetin treated tissues as compared with untreated tissues. Sucrose density centrifugation profiles and electron micrographs showed that the level of monoribosomes decreased and that of polyribosomes increased in kinetin-treated cotyledons. They concluded that cytokinins promote translation by activation of polyribosomes or by increasing the number of polyribosomes through an enhancement of the ribosomal binding into polysome configuration. These two ways of cytokinin action may not occur at the same time in the same cell as in the moss protonema, only activation of polyribosomes was observed whereas in cucumber cotyledons, a stimulation of polyribosome formation was demonstrated.

Most plants when regenerated by tissue culture require a combination of hormones (Skoog and Miller, 1957). Modulation by cytokinin of auxin induced protein synthesis in soybean hypocotyl sections was observed by Zurfluh and Guilfoyle (1982a). They also detected the appearance of new proteins when cytokinin was applied to auxin untreated hypocotyl sections. Eichholz *et al.* (1983) showed that an auxin and cytokinin regulated polypeptide (33 kDa) was abundant in crown gall transformed tobacco tissue. Shinshi *et al.* (1987) also demonstrated that altered gene expression was affected by the combination of auxin and cytokinin. They found that chitinase, a defence related enzyme, was induced by both auxin and cytokinin when applied singly, but the level of chitinase was reduced when auxin was applied in combination with cytokinin.

It is therefore evident from the literature on gene expression influenced by cytokinins that application of cytokinin to plant tissues can affect the synthesis of different polypeptides in different ways, inducing synthesis of some, inhibiting

others, and affecting others quantitatively. It is also evident that cytokinins can act in more than one way, both at transcription and translation. The diversity of effects of cytokinins at the molecular and cellular levels indicates that they may have several sites of action within the cell. Competition between these systems not only for metabolites but also for cytokinins must be considered as a criterion in the regulation of the processes which are under cytokinin control.

1.2.3 Gibberellins and gene expression

GA stimulates the production of hydrolytic enzymes required for the mobilisation of seed storage reserves (Marriot and Northcote, 1975, 1977; Martin and Northcote 1982a, 1982b, 1983). Martin and Northcote (1982a) detected the appearance of rRNA and mRNA in castor bean endosperm during germination and found that GA stimulates iso-citrate lyase activity during germination. Martin *et al.* (1984) examined the changes in levels of mRNA in castor bean endosperms when treated with GA. They found that GA stimulated the rate of transcription non-specifically. They constructed a cDNA library with poly $(A)^+$ RNA extracted from the endosperms of germinating seeds, from which they obtained three endosperm-specific clones. They used these to measure levels of the corresponding transcripts during germination and confirmed that accumulation of all three transcripts was increased by the addition of exogenous GA.

Wasilewska *et al.* (1987) studied the effect of GA in developmental processes in dwarf pea and maize seedlings. They observed that mRNA isolated from GA treated tissues exhibited much higher translational activity. The protein profile of treated seedlings resembled that of untreated seedlings which were two days older, suggesting that GA, in addition to increasing longitudinal growth, could also advance the developmental phase of the seedlings.

1.2.4 Abscisic acid and gene expression

ABA is known to inhibit cell elongation and to decrease RNA and protein

synthesis in certain plants (Walbot, 1978). Marriot and Northcote (1977) showed that ABA inhibited germination and iso-citrate lyase activity and that this inhibition was relieved by the addition of an equimolar concentration of GA. Dommes and Northcote (1985) quantified the mRNA changes resulting from ABA treatment by carrying out *in vitro* translation of poly $(A)^+$ RNA. Using three cDNA clones for iso-citrate lyase, they found that the levels of transcripts during germination were inhibited by application of ABA. ABA also inhibits GA induced alpha amylase activity (Lin and Ho 1986). It is evident from the literature that ABA has an overall antagonistic effect on GA-induced gene expression.

ABA can also have stimulatory effects on mRNA levels. For example, Hong *et al.* (1987) isolated and characterised a cDNA clone from ABA treated barley aleurone layers; they found that this mRNA tripled in quantity within 40 min of ABA treatment and reached a maximum after 8-12 h. The amount of ABA required to induce this mRNA was less than 10^{-9} M, and accumulation of the mRNA increased with increasing ABA concentration. Harada *et al.* (1989) analysed the nucleotide sequence of an mRNA induced by ABA during seed development of *Brassica napus*. This mRNA began to accumulate during late embryogeny and could not be detected after a 24 h period of imbibition. ABA treatment enhanced the accumulation of this polypeptide which had a molecular weight of 30 kDa and an unusual primary structure. They suggested that this polypeptide might have protective functions during desiccation.

1.2.5 Ethylene and gene expression

Ethylene-induced gene expression has been studied by several workers (Christofferson and Laties, 1982; Zurfluh and Guilfoyle, 1982c; Grierson, 1985) and cDNA libraries have been made from poly $(A)^+$ RNA isolated from ethylene treated tissues (Broglie *et al.*, 1986, 1989; Lincoln *et al.*, 1987). Broglie *et al*, (1986) demonstrated ethylene-induced endochitinase activity in bean seedlings.

In vitro translations carried out with poly $(A)^+$ RNA from ethylene treated and untreated tissue showed 20-25 additional polypeptides on 2D gels in the treated tissue. Ethylene also caused quantitative changes in mRNA levels. Broglie *et al.* (1986) found 75-100 fold more chitinase mRNA in ethylene treated tissues than in the control.

Since ethylene production is a common response to wounding (e.g: at the cut edges of explants) and since auxin enhances ethylene formation (Yoshii and Imaseki, 1982), ethylene induced changes in gene expression might be expected to occur in newly initiated tissue cultures, particularly those on media containing auxin. It has been reported, for example, that chitinase and β 1,3-glucanase were induced by the ethylene present in the culture environment (Meins *et al.*, 1990; Grosset *et al.*, 1990). Both of these enzymes were transcriptionally activated in a coordinated manner by ethylene and wounding (Beerhues *et al*, 1990).

1.3 HORMONE-BINDING MOLECULES

Soluble, auxin-binding proteins that stimulate RNA synthesis have been isolated by many workers (e.g., Jacobson, 1981; Van der Zaal *et al*, 1987). Shimomura *et al.* (1988) isolated from maize shoot meristem an auxin-binding protein which was found to be in the endoplasmic reticulum of the cells. Later they constructed a cDNA library from poly $(A)^+$ RNA isolated from three day old seedlings and probed it with an oligonucleotide sequence constructed on the basis of the NH_2-terminal sequence of isolated auxin-binding protein. They deduced the amino acid sequence of the auxin-binding protein from the nucleotide sequence of a full length cDNA, and found that it showed a resemblance to proteins localised in the lumen of the endoplasmic reticulum (Inohara *et al.*, 1989). Klambt (1990) also isolated an auxin-binding protein from maize coleoptile; this protein was associated with plasmalemma and involved in signal transduction from auxin to the site of action, resulting in cell elongation and proton secretion. Similarly, the presence of cytokinin-binding

proteins with high affinity for 6-substituted purines has been reported by many workers (Fox and Gregerson, 1982; Chen, 1989). It has been speculated that the formation of the cytokinin-protein complex is an essential component of the signal transduction chain ending in cytokinin-induced gene expression.

1.4 GENE EXPRESSION DURING SOMATIC EMBRYOGENESIS

Carrot cells can regenerate into plants through somatic embryogenesis. The auxin 2,4-dichloro phenoxy acetic acid (2,4-D) induces carrot cell proliferation, and subsequent transfer of callus to a 2,4-D free medium leads to somatic embryo formation (Steward *et al.*,1958; Reinert, 1959, Lui *et al.*, 1985). Sung and Okimoto (1981, 1983) reported that a protein specific to the embryonic stage was detectable in the carrot cell extracts 4 h after the onset of embryogenesis. Borkird *et al.* (1986) labelled carrot cells in suspension culture with [^{35}S] methionine and extracted proteins during embryogenesis. They confirmed the earlier reports by Sung and Okimoto by carrying out 2D gel electrophoresis. In addition, they observed that the alterations in mRNA production induced by 2,4-D were quantitative rather than qualitative.

Thomas and Wilde (1985, 1987) made a cDNA library using poly $(A)^+$ RNA from carrot embryos and established that 0.5-1% of embryo specific proteins are expressed exclusively in somatic embryos. Cyr *et al.* (1987) studied the developmental modulation of tubulin proteins and the mRNA levels during somatic embryogenesis in cultured carrot cells. They analysed *in vitro* translated products and quantified the tubulin mRNA production by measuring the abundance of tubulin and showed increased production of tubulin during embryogenesis.

Choi *et al.* (1987) made an antiserum against an extract of carrot somatic embryos and used it as a common antibody for a few rare antigens produced at the onset of somatic embryogenesis. They then tested the expression of these antigens in other plant species during embryogenesis. They used somatic

embryos of cassava, peach and maize along with their non-embryogenic callus cultures and differentiated tissues as controls for developmental regulation. They also examined zygotic embryos and leaves of Johnson grass. They detected in all of these embryos the accumulation of a 50 kDa protein which cross-reacted with the common antibody. Since the expression of this antigen was significantly higher in embryos than in callus, they generalised that the genes that are specifically induced in somatic embryogenesis may play a key role in normal plant embryo development.

Rajyalakshmi *et al.* (1991) analysed the soluble proteins in morphogenic callus which gave rise to plantlets through somatic embryogenesis and the non-morphogenic callus of wheat using 2D-PAGE. They found morphogenic callus accumulated polypeptides with Mr 79, 20 and 19 kDa which were not seen in non-morphogenic callus. On the other hand, non-morphogenic callus showed the presence of a protein of Mr 23 kDa, exclusively. In addition, these authors observed a higher expression of several polypeptides in morphogenic callus than in non-morphogenic callus. Out of these polypeptides, two with Mr 55 and 14 kDa were reported to be the large and small subunits of ribulose bisphosphate carboxylase.

1.5 GENE EXPRESSION DURING *IN VITRO* ORGANOGENESIS

Increase of activities of hydrolytic enzymes including alpha amylase, acid phosphatase, catalase and ribonuclease in shoot forming callus as opposed to non-shoot forming callus has been reported by many workers (see review by Thorpe, 1980). Thorpe (1978) demonstrated high levels of phospho-enol pyruvate carboxylase, malic dehydrogenase, glutamic oxaloacetate, transaminase and malic enzyme activity in shoot forming callus, indicating high metabolic activity in shoot forming callus. Beaudoin-Eagan and Thorpe (1985) demonstrated increased activity of tyrosine ammonia lyase (TAL) in shoot forming callus and increased activity of phenylalanine ammonia lyase activity in

non-shoot forming tobacco callus. Since TAL activity was increased during 6-18 days, which corresponded to histogenic events of shoot formation, they suggested the possible role of TAL in shoot forming tobacco cultures. They hypothesised that IAA oxidase was stimulated by the phenolic compounds formed by TAL, thereby reducing the ratio of auxin/cytokinin favouring shoot formation in callus.

Increase in enzyme activity has also been observed during root organogenesis; for example, increased activity of polyphenol oxidase during formation of roots was observed by Habaguchi (1977). Thorpe (1978) reported elevated activities of glutamate, oxaloacetate transaminase and alanine aminopeptidase in *Atropa belladona* cell clumps with roots, indicating higher metabolic activity. Bevan and Northcote (1979) observed a positive correlation between PAL activity and the morphogenetic potential of suspension cultures of *Phaseolus vulgaris.* The increase in activity of these enzymes indicates differences in metabolism between organ-forming and non-organ forming tissues but does not necessarily indicate that these changes are due to change in gene expression.

Mader *et al.* (1975) investigated the differences in isoperoxidases between shoot forming and non-shoot forming callus of tobacco. They observed the disappearance of fast moving anodic peroxidases with a concomitant increase of all other peroxidases. Kim and Kim (1985, 1987) found that the amount of isoperoxidases was greater in non-shoot forming tobacco callus than in shoot forming callus.

Hasegawa *et al.*(1979) showed by fractionating the proteins labelled with tritiated leucine that there was an increased amount of low molecular weight proteins (16-20 kDa) during bud formation from Douglas fir cotyledons. Subsequently, Yasuda *et al.* (1980) reported that these low molecular weight proteins appeared in cotyledons after two days on bud-forming medium and reached a maximum level at the fourth day. Transfer of cotyledons after two

days from bud-forming medium to callus initiation medium resulted in suppression of these low molecular weight proteins, suggesting that these proteins may be associated with early stages of bud formation.

Christianson and Warnick (1988) examined the process of *in vitro* root formation from leaf explants at the molecular level. They isolated mRNA from root regenerating and non regenerating *Convolvulus* cultures and analysed their *in vitro* translation products. They did not find any observable differences in protein profiles between regenerating and non regenerating genotypes. They observed a rapid change in protein profile between the original explant and the explant after 2 days in culture on callus induction medium. Since the protein profile after 2 days in culture remained the same during the rest of the culture period, they concluded that the process of dedifferentiation is completed by the second day in culture. They mentioned that the translation profile changed again only when the cells were 'determined' to form roots and this profile closely resembled the translation profile of mRNA obtained from seedling roots. Since they did not see any differences in mRNA population of root regenerative and non-regenerative genotypes, the second translation profile may not be unique for cultures 'determined' for root organogenesis. However, this work gives evidence supporting changes in gene expression during dedifferentiation, which is the phase preceding organogenesis in an explant or callus.

Reynolds (1990) investigated the changes in proteins during shoot organogenesis from stem segments of *Solanum carolinense* using 2D-PAGE. He identified two polypeptides putatively specific for shoot organogenesis from explants cultured for 2 days on regeneration medium. He observed enhancement and reduction of several polypeptides in shoot producing cultures and enhancement and reduction of several others in callus producing cultures. Since these changes were detected in cultures before differentiation into shoots he suggested that some of these may be specific for shoot regeneration in organogenic cultures.

In order to investigate whether meristem initiation involves changes in proteins and how early the shoot specific proteins are expressed after the meristems are being formed, Renaudin *et al*. (1991) compared the protein patterns of bud forming callus of *Petunia* with callus having no organogenesis. They did not find change of any proteins specifically during meristem initiation and suggested that this may have been due to the low number of cells involved in meristem initiation. They also suggested that since there were no changes in protein composition at the step of meristem formation, the metabolic and cytological changes during meristem initiation may not involve massive changes in gene expression, but a regulatory event may account for the promotive action of cytokinin in meristem formation. They identified a protein which was only present in shoot forming cultures which was found to increase sharply as soon as meristems developed into shoots. Since this protein was present in tissues after meristems were formed and since meristematic tissues were already determined, this protein was thought to be associated with differentiation rather than with a shoot inductive process.

Ry Meeks-Wagner *et al.* (1989) investigated gene expression during the transition from the vegetative to the reproductive state in tobacco. They used an *in vitro* thin cell layer culture system to initiate flowers, roots and shoots. They constructed a cDNA library to mRNA from flower initiating cultures and identified seven genes which appeared only in floral meristems but not in *in vitro* produced shoot or root meristems. They also found that two of these genes were expressed at low levels in incipient floral meristems and at high levels in roots during normal *in vivo* plant development.

Therefore there is evidence for changes of gene expression in terms of changes in mRNA populations, synthesis of novel proteins and differential gene expression during the process of organogenesis. The increase in activity of several enzymes during organogenesis may also be regulated at the transcriptional or posttranscriptional level, although no direct evidence is yet

available.

1.6 OBJECTIVE

The aim was to establish a suitable culture system for shoot organogenesis together with a control system in which shoots were not produced, and then to study the molecular events occurring during the period in culture, in order to investigate whether specific changes in gene expression occur in association with shoot regeneration. The approach was to measure the levels of total and poly $(A)^+$ RNA, then to study whether changes occurred in protein profiles by carrying out *in vivo* labelling experiments and by translating poly $(A)^+$ RNA *in vitro* and analysing the proteins by SDS-PAGE and 2D-PAGE. In addition to providing useful background information, it was hoped that these experiments would identify a time point during the culture period on SIM at which changes in poly $(A)^+$ RNA and protein synthesis occurred. It was then intended to construct a cDNA library using poly $(A)^+$ RNA from this time point to detect genes that are differentially expressed in the two culture systems. Ultimately, it was hoped to use the clones that were identified as differentially expressed to measure the levels of corresponding mRNAs through the culture period.

2 *IN VITRO* CULTURES

2.1 INTRODUCTION

In the field of cell and tissue culture *Nicotiana tabacum* is now considered as a model plant, particularly for the manipulation of organogenesis, since many parts of the plant can be induced to form either roots or shoots *in vitro* (Skoog and Miller, 1957). In attempting to study gene expression during regeneration of shoots in tobacco, it is important to develop a tissue culture system to produce shoots from explants with minimum callus and root formation, to avoid confusion with novel gene expression concerned with other developmental pathways. It is also necessary to have a control system in which the explant does not produce any shoots. Therefore, experiments were first carried out to establish suitable culture systems, one producing shoots and another control system producing no shoots. It was also important that these morphogenetic changes should also be studied at the histological level so that they could be correlated more precisely with the molecular studies.

2.2 MATERIALS AND METHODS

2.2.1 Selection of *in vitro* systems

2.2.1.1 Species and source of explants

The experiments were conducted with *Nicotiana tabacum* var. Xanthi. Seeds of *Nicotiana tabacum* were obtained from the seed collection at the University of Bath and germinated in 'Levington's Universal' compost mixture in a greenhouse at a minimum temperature of 25 °C. Seedlings (4 weeks old) of similar size were transferred to individual pots of 10 cm x 10 cm and grown further in the greenhouse. The youngest fully opened leaves of uniform size were used to provide the explants for tissue culture.

2.2.1.2 Preparation of explants for tissue cultures

The youngest fully opened leaves were removed from the plants and surface sterilised for 10 min with 5% sodium hypochlorite solution containing 2

drops of 'Tween 80' detergent per 100 ml solution. The leaves were subsequently washed with three changes of autoclaved water. Leaf squares of 0.5 cm x 0.5 cm were cut and cultured.

2.2.1.3 Nutrient media and their preparation

a. Basal composition

The basal constituents of all the media were Murashige and Skoog (1962) pre-mixed powder (Flow laboratories, Irvine), 20 g l^{-1} sucrose and 0.7% Oxoid agar (Oxoid Ltd., technical grade) as the gelling agent.

b. callus induction medium (CIM)

The basal medium was supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) in the range 0.25-1.00 mg l^{-1}, as indicated in the individual experiment.

c. Shoot induction medium (SIM)

The basal medium was supplemented with 1.25 mg l^{-1} of 6-benzyl amino purine (BAP).

d. Preparation of media

After combining all the ingredients, but before final dilution, the pH of the media was adjusted to 5.7 using 1 M sodium hydroxide or 1 M hydrochloric acid. Then the solution was made up to the required volume, 0.7% agar was added and the medium was sterilised by autoclaving for 15 min at 1.05 kg cm^{-1}. The medium was cooled to approximately 55 °C and dispensed into sterile Petri-dishes of diameter 5 or 9 cm.

e. Culture environment

All the cultures were incubated at 25 °C±1 °C, under 16 h daily illumination of intensity 30 μmol m^{-2} s^{-1}.

2.2.2 Histological work

2.2.2.1 Fixing of plant material

The plant material was cut into approximately 0.5 x 0.5 cm pieces and fixed in FAA (Formalin:glacial acetic acid:70% ethanol 1:1:2) for at least 18 h.

2.2.2.2 Dehydration

The fixative was replaced by 50% ethanol and after at least 1 h, the specimens were passed through the following series of tertiary butyl alcohol (TBA) solutions with one hour in each solution. The numbers given below represent the percentages by volume.

sol.no	water	95% ethanol	TBA	ethanol
1	50	40	10	-
2	30	50	20	-
3	15	50	35	-
4	-	-	75	25

In order to see the specimens easily during the embedding procedure, they were immersed in a saturated solution of erythrocin in TBA, overnight at 35 °C. The stain was replaced by 100% TBA and left for 2 h at the same temperature to remove any excess stain adhering to the specimens.

2.2.2.3 Embedding the specimens in wax

Molten paraffin wax chips (paraffin wax with ceresin) were poured into the vials containing the specimens. The wax was changed three times at 2 h intervals, to ensure proper infiltration of wax into the tissues. Molten wax was then poured into folded 'paper boats' up to the brim and the specimens were carefully transferred and properly orientated with the help of a pair of forceps. Once the wax at the surface of the boat started to solidify the boats were floated on cold water to complete solidification.

2.2.2.4 Sectioning

The wax block was removed from the paper boat and cut into cubes each containing a specimen with minimum surrounding wax. This was cut into sections of 6 μm thickness using a microtome (Reihert microtome).

2.2.2.5 Mounting

Wax ribbons were floated on warm water to cause expansion. When fully stretched they were placed on glass slides, previously coated with Haupt's adhesive (1 g of gelatin, 2 g of phenol and 15 ml of glycerol in 100 ml of water) and allowed to dry on a warm plate.

2.2.2.6 Staining

The slides were loaded onto carriers and the following schedule was used to stain the sections with 1% (w/v) toluidine blue.

step	reagent	time (minutes)
1 - 3	histoclear	3 x 3
4	histoclear/ethanol (50:50)	3
5	95% ethanol	3
6	70% ethanol	3
7	50% ethanol	3
8	30% ethanol	3
9	water	1
10	toluidine blue	0.75
11 - 12	water	1 x 2
13	30% ethanol	1
14	50% ethanol	0.5
15	70% ethanol	0.5
16	95% ethanol	0.5
17	absolute ethanol	0.5
18	histoclear/ethanol (50:50)	1
19	histoclear	3
20	histoclear	3
21	histoclear	3

A drop of mountant (D.P.X mountant, B.D.H Ltd.,) was placed on each slide and a coverslip was gently lowered, taking care to avoid the trapping of air bubbles. The slides were allowed to air-dry overnight.

2.3 RESULTS

2.3.1 Selection of first-stage medium

Cutting squares from the tissue will itself result in the induction of wound-response proteins (Zurfluh and Guilfoyle, 1980). Therefore, if leaf pieces are cultured on SIM directly, any immediate production of shoot regeneration specific proteins may possibly be masked by the appearance of wound-response proteins. In order to avoid the presence of any wound-response proteins, it was decided to culture the squares initially on a non-regenerative medium for a short time, so that transfer alone to SIM would result in shoot regeneration.

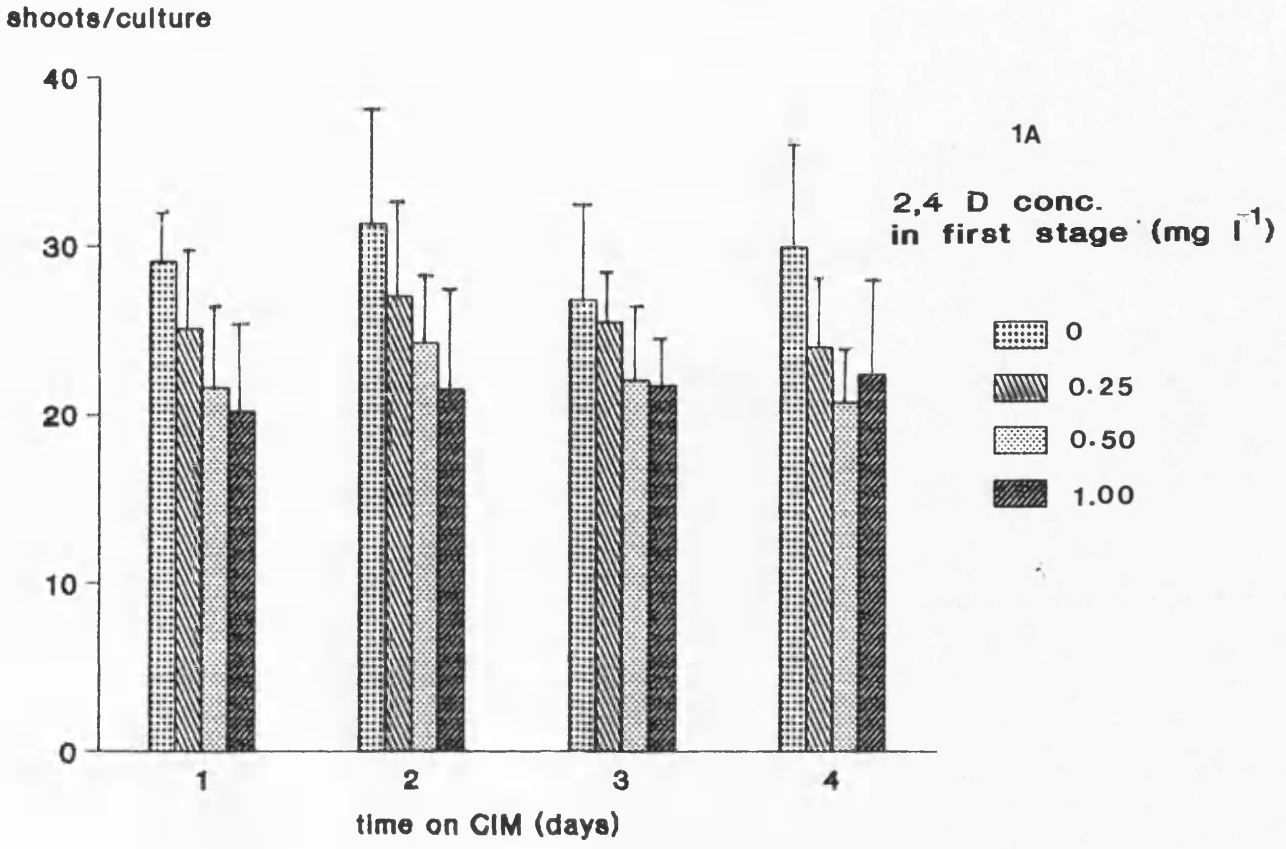
An experiment was carried out to find out the optimum concentration of 2,4-D and length of first-stage culture period, before transferring leaf explants onto SIM. The leaf squares were cultured on media containing 2,4-D concentrations of 0.00, 0.25, 0.50 and 1.00 mg l^{-1} for 1, 2, 3 and 4 days. Four pieces were cultured in a 5 cm diameter Petri dish containing 10 ml of medium and 10 Petri dishes were prepared per treatment. All the leaf squares were transferred on to SIM at the end of the first stage. The fresh weights were recorded and the numbers of shoots, those which could be separated with the help of a pair of fine forceps, were counted after a total period of 16 days of initial culture. The average number of shoots per culture was plotted against the number of days in culture. (Figs. 1A and 1B). Statistical analysis of variance showed that there was no significant difference between the treatments at 95% confidence level.

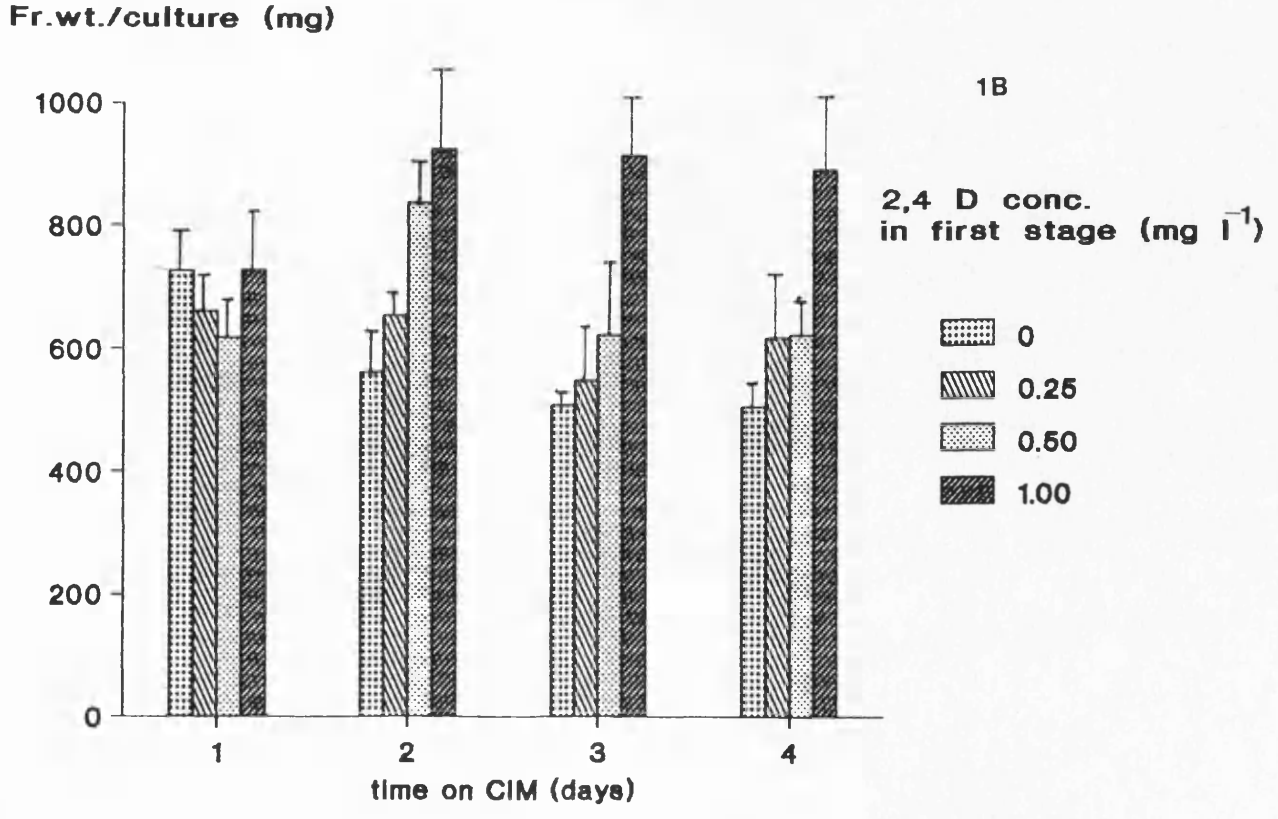
Although culturing the leaf squares on a medium without any hormones or with 0.25 mg l^{-1} 2,4-D for 1-4 days appeared to result in an increase in the mean number of shoots per explant when transferred onto SIM (Fig. 1A), these

Fig 1: Growth of leaf squares on SIM following the initial culture on first-stage CIM containing different concentrations of 2,4-D.

Leaf squares were cultured on CIM containing 0-1.00 mg l^{-1} 2,4-D for 1-4 days and transferred to second-stage SIM. The number of well formed shoots (that can be separated using fine forceps) and fresh weight of 10 cultures (each culture contained 4 explants) were recorded after the total period of 16 days on CIM and SIM.

A: Average number of well formed shoots per culture.
B: Average fresh weight per culture.


shoots/culture
40
30
20
10
0
1
2
3
4
time on CIM (days)
1A
2,4 D conc.
in first stage (mg l⁻¹)
0
0.25
0.50
1.00


Fr.wt./culture (mg)
1000
800
600
400
200
0
1
2
3
4
time on CIM (days)
1B
2,4 D conc.
in first stage (mg l⁻¹)
0
0.25
0.50
1.00

media were not selected to use as the first-stage medium because, when the explants from these media were transferred to second-stage media of the same composition, the production of callus was found to be less than when 0.5 and 1.00 mg l^{-1} 2,4-D were used as first and second-stage media (data not included). Since the control medium was required to show a contrasting morphological character compared with SIM, such as intense callus formation, medium with 0.5 mg l^{-1} 2,4-D was chosen to use as the control for the second-stage medium. Since there was no significant difference on shoot production between the effects of the different lengths of the first-stage culture period, it was decided to leave the squares for 4 days on first-stage CIM before transfer onto SIM for shoot production or to CIM for callus production.

Fig. 1B shows the fresh weight of cultures after 16 days on first and second stage media. As is evident the average fresh weight was higher when leaf squares were grown on 1.00 mg l^{-1} 2,4-D for 2, 3 or 4 days before subculturing onto second-stage SIM. The fresh weight of cultures grown on 1.00 mg l^{-1} 2,4-D for 4 days during the first-stage culture was higher than those grown on 0.5 mg l^{-1} 2,4-D. This increase may be due to callus formation enhanced by higher concentration of 2,4-D, because there was no difference in shoot production between these two treatments (Fig. 1A).

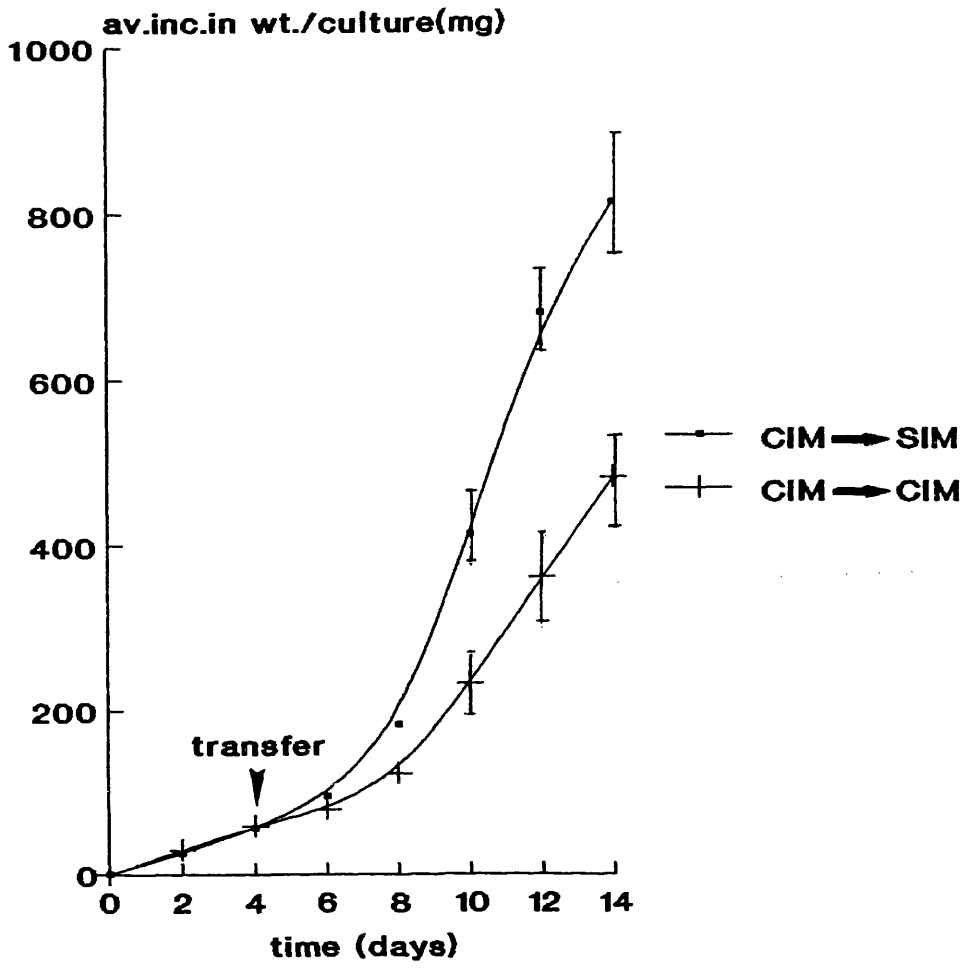
2.3.2 Developmental measurements

0.5 x 0.5 cm squares of leaf tissue (5 squares per Petri dish) were cultured on CIM medium for 4 days and then transferred to either SIM or fresh CIM. The fresh weights of 50 explants (ie. 10 replicate dishes) were recorded every two days and the mean increases in weights of cultures that had been on CIM alone for both first and second stages, and of cultures which had been grown for 4 days on CIM followed by up to 10 days on SIM, were plotted (Fig. 2). There was a greater increase in fresh weight in cultures grown on CIM followed by SIM than those grown on CIM alone (Fig. 2).

Fig 2: Average increase in growth of leaf squares per culture over a period of 14 days.

Leaf squares (5 squares per culture) were cultured on first-stage CIM containing 0.5 mg l^{-1} 2,4-D and transferred to second-stage CIM of the same composition or to SIM. Fresh weights of 10 cultures were recorded at 2 day intervals.

Y axis represents the average increase in weight per culture.


av.inc.in wt./culture(mg)
1000
800
600
400
200
0
transfer
0
2
4
6
8
10
12
14
time (days)
CIM → SIM
CIM → CIM

Plates 1A and 1B show representative samples of explants 8 days after transfer to the respective second-stage media; at this stage of growth SIM cultures showed some developing shoots, when viewed under the stereo microscope, while CIM cultures showed only callus at the cut edges of the explants. The colour of the callus formed by explants on CIM varied from cream, reddish brown to dark grey in appearance. Callus in sufficient quantity to subculture was formed after 14-24 days on CIM. Although the colour of the callus varied, they all produced shoots after 3-4 wks, when subcultured to SIM (data not shown). A continuous, non-synchronous formation of shoots was observed in SIM cultures; vigorously growing shoots were visible in all the 14-24 day old SIM cultures (Plate 1C).

2.3.3. Histological studies

Leaf pieces were cultured on CIM for 4 days and transferred to fresh CIM or to SIM. They were removed from first-stage CIM, second-stage CIM, and SIM cultures at two day intervals and fixed. Then sections were cut and stained as described in section 2.2.2 and examined under the microscope (Olympus BH-2).

The second-stage cultures are denoted by the number of days on a particular medium with specification of the first-stage medium within parentheses. For example, 1d CIM (4d CIM) represents cultures that had been on second-stage CIM for one day following transfer from 4 days on first-stage CIM.

There was some evidence that cell expansion had occurred in the cultures since the individual cells had become larger after two days on first-stage CIM (compare Plates 2 and 3 individual cells are larger in Plate 3). Callus formation was noticeable at the cut edges in cultures that had been on second-stage CIM for two days (Plate 4). Cultures after 4 days on this medium showed evidence of increased cell division especially at the cut end (Plates5 and 6). An area with

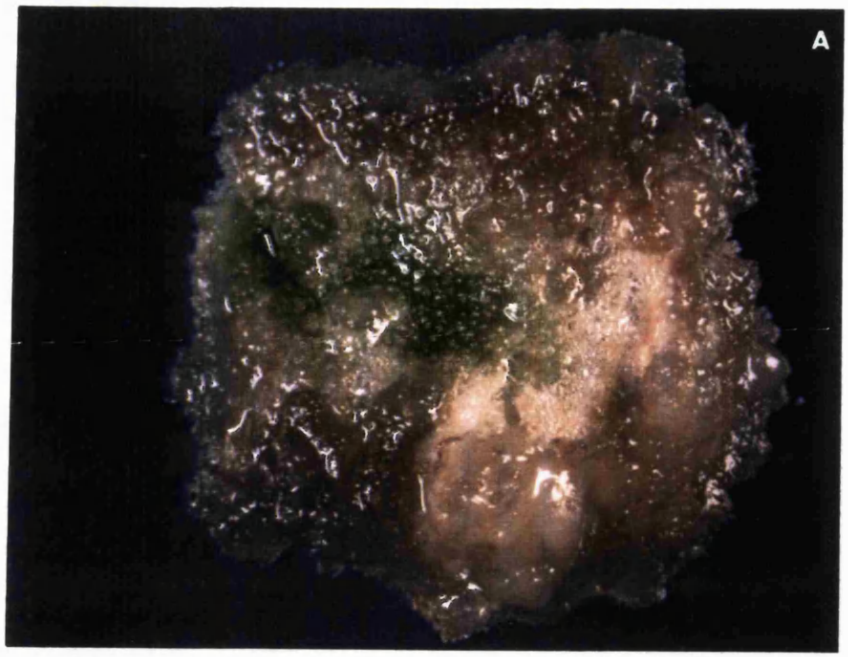
Plate 1: Morphology of explants on second-stage CIM and SIM cultures.

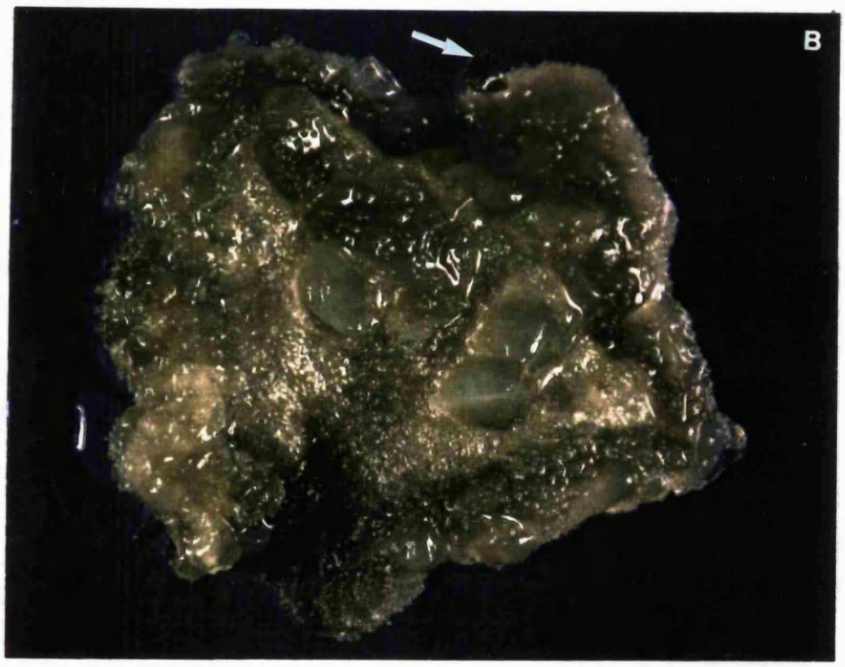
The explants (approximately 0.5 x 0.5 cm squares) were cultured on first-stage CIM for 4 days and transferred to either second-stage CIM or second-stage SIM.

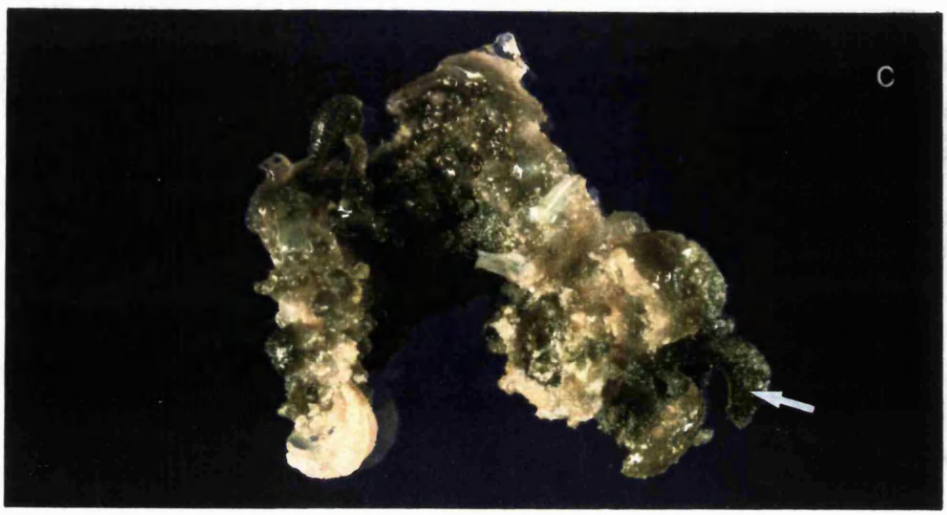
A: after 8 days on second-stage CIM x 8
scale bar = 0.125 cm

B: after 8 days on second-stage SIM x 8; developing shoots (arrowed).
scale bar = 0.125 cm

C: after 14 days on second-stage SIM x 6; developing shoots (arrowed).
scale bar = 0.22 cm


A

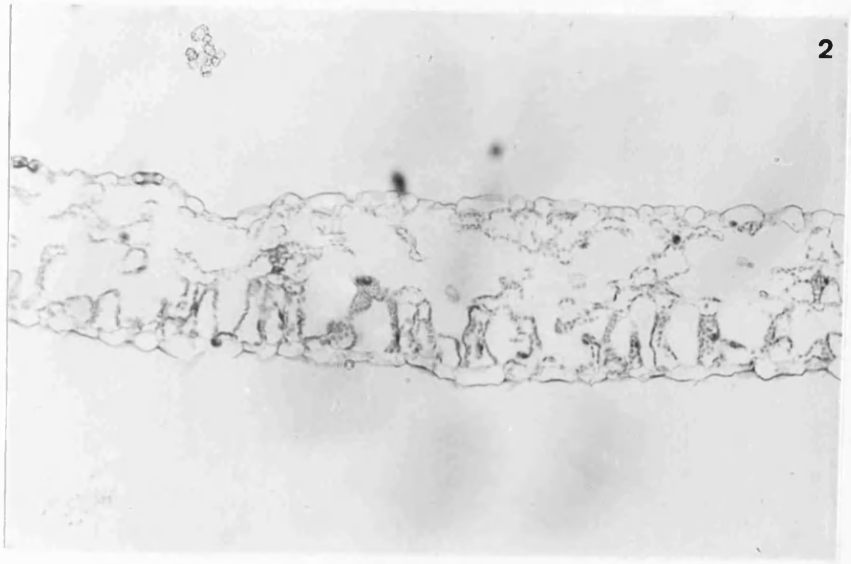

B


C

Plates 2-14 Histology of leaf explants.

Plate 2: Transverse section of a leaf square before culturing (day 0) x 66.
scale bar = 152 μM

Plate 3: Transverse section of a leaf explant after 2 days on first-stage CIM (2d CIM) x 66.
scale bar = 152 μM


2

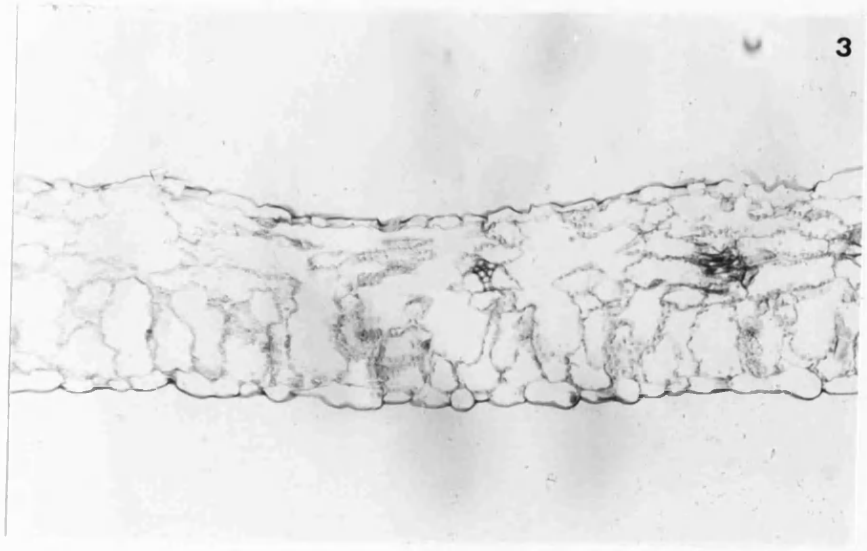
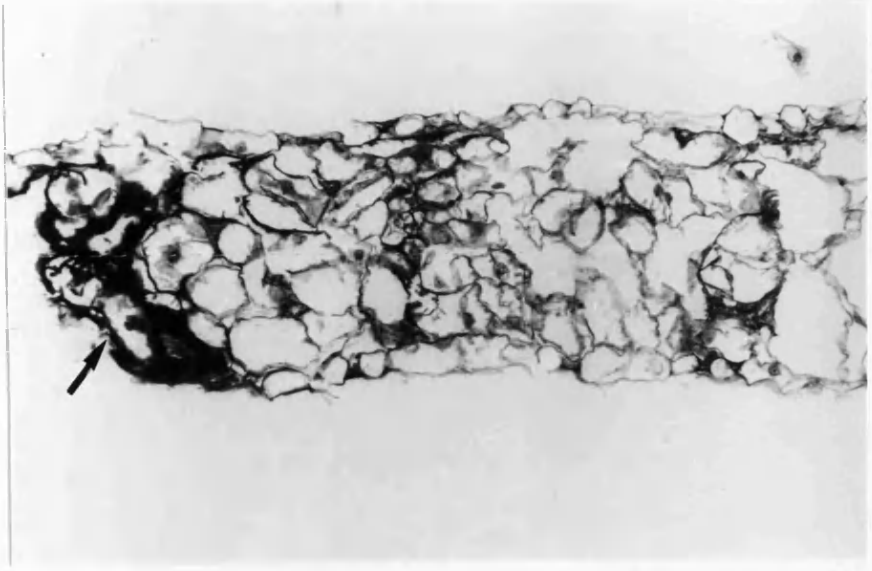

3

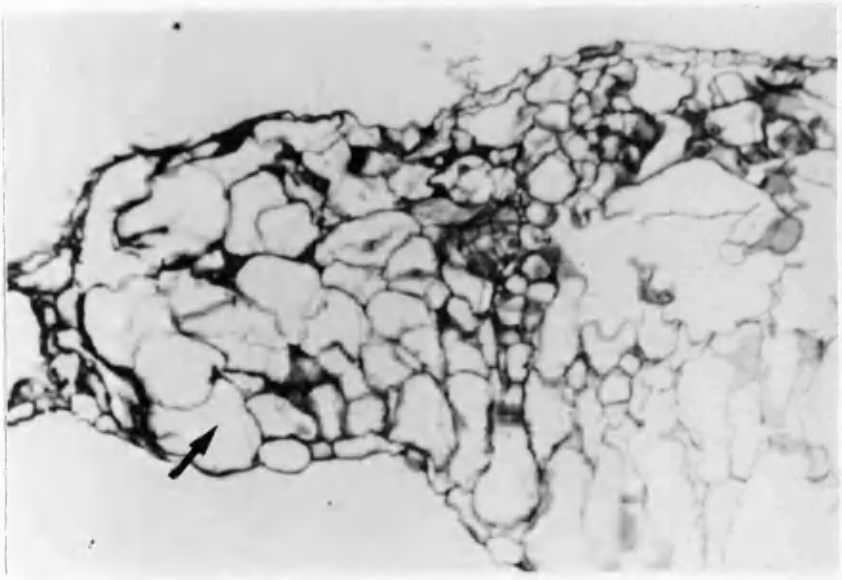
Plate 4: Transverse section of a leaf explant after 2 days on second-stage CIM [2d CIM (4d CIM)] x 165. Cut edge of the explant is marked by an arrow.
scale bar = 60 μM

Plate 5: Transverse section of a leaf explant after 4 days on second-stage CIM [4d CIM (4d CIM)] x 165; large vacuolated cells are visible at the cut end of the explant (arrowed).
scale bar = 60 μM

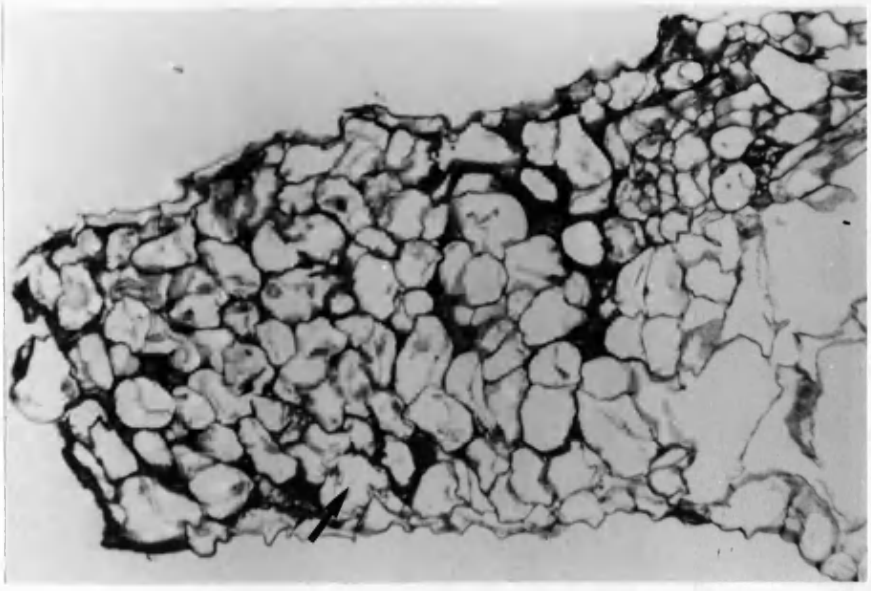
Plate 6: Transverse section of a leaf explant after 6 days on second-stage CIM [6d CIM (4d CIM)] x 165; large vacuolated cells are visible at the cut end of the explant (arrowed).
scale bar = 60 μM



4



5



6

compact newly formed cells as opposed to large vacuolated cells of callus was visible after 8 days (Plate 7), which may correspond to an early stage of root primordia formation. Root primordia were seen in cultures after 12 days of growth on this medium (Plate 8), but there was no evidence of their emergence from the tissues. Although all the explants had a mass of visible callus after 8 days on second-stage CIM, this was not readily visible in stained sections. This may be due to the destruction of loose callus during fixing and staining procedures (Plate 8).

The leaf pieces transferred to SIM showed a continuous series of events leading to shoot organogenesis. Two days after transfer onto SIM, there was enhanced cell division at the cut end (Plate 9). These cells were small and compact as compared to large vacuolated cells observed in 2d CIM (4d CIM) cultures (Plate 4). A rapid increase in cell proliferation accompanied by tracheid formation was observed in the cultures after four days on SIM (Plate 10). At some places of the callus surface a row of darkly stained cells was seen in 4 day SIM cultures. These may be points of origin of shoot primordia (Plate 11). Evidence of shoot primordia can be seen after six days on SIM (Plate 12). Formation of leaf lobes were more prominent in cultures after 8 days on SIM (Plate 13). Shoot apices with leaf primordia were seen after a 12 day period on SIM. These shoot primordia were frequently inter-connected by darkly staining strands of vascular tissue (Plate 14).

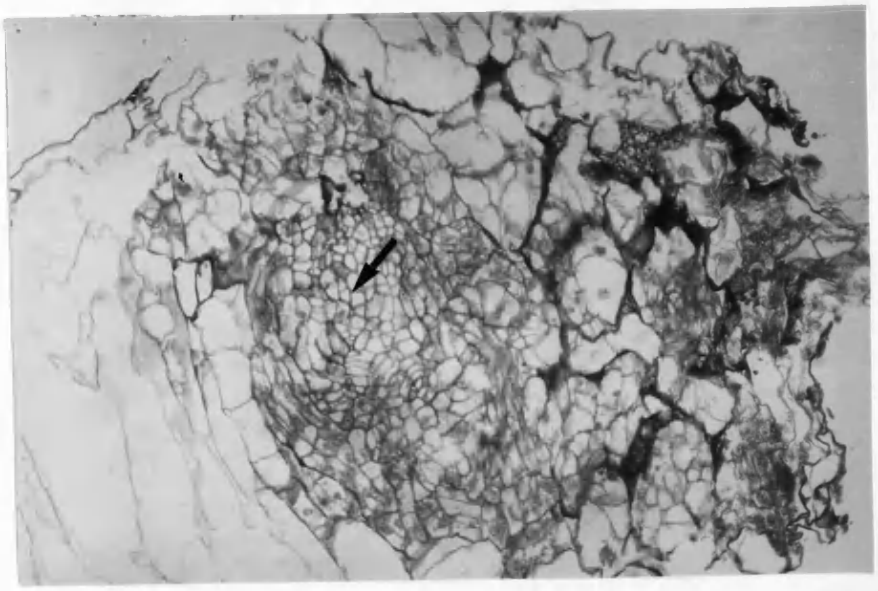
2.4 DISCUSSION

Leaf squares cultured on media containing 0.00-1.00 mg l^{-1} 2,4-D for 1-4 days did not show any significant differences in shoot production when they were transferred to SIM. This may suggest that de-differentiation occurred with or without 2,4-D in tobacco explants and that a sufficient number of cells became competent within one day on the first-stage medium (section 1.1.3) to respond to the stimulatory effect of BAP in SIM for shoot production. Cultures grown on

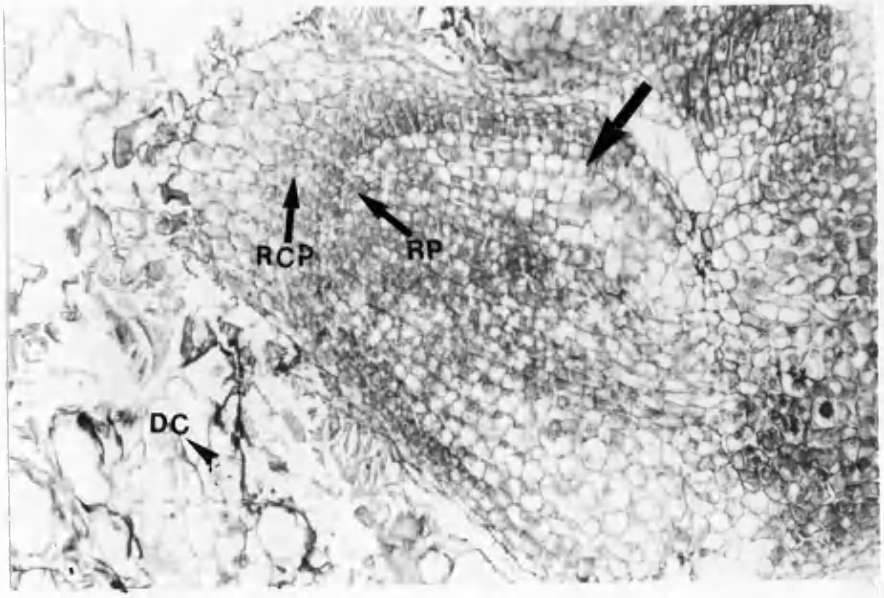
Plate 7: Transverse section of a leaf explant after 8 days on second-stage CIM [8d CIM (4d CIM)] x 66; small compact cells which may correspond to root initials are visible (arrowed).
scale bar = 152 μM

Plate 8: Transverse section of a leaf explant after 12 days on second-stage CIM [12d CIM (4d CIM)] x 66; developing root can be seen within the surrounding callus (arrowed).
scale bar = 152 μM

Abbreviations: DC-damaged cells; RCP-root cap; RP-root primordium



7

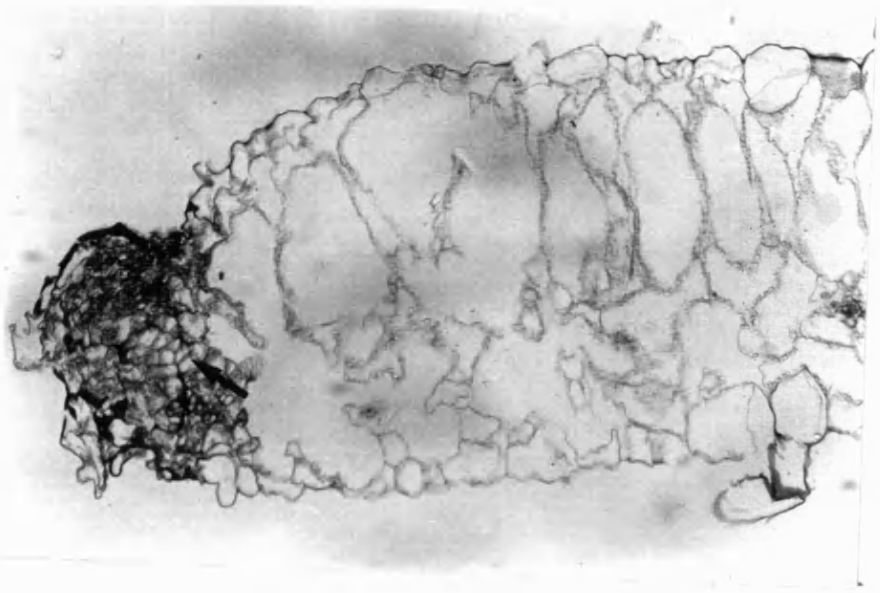


8

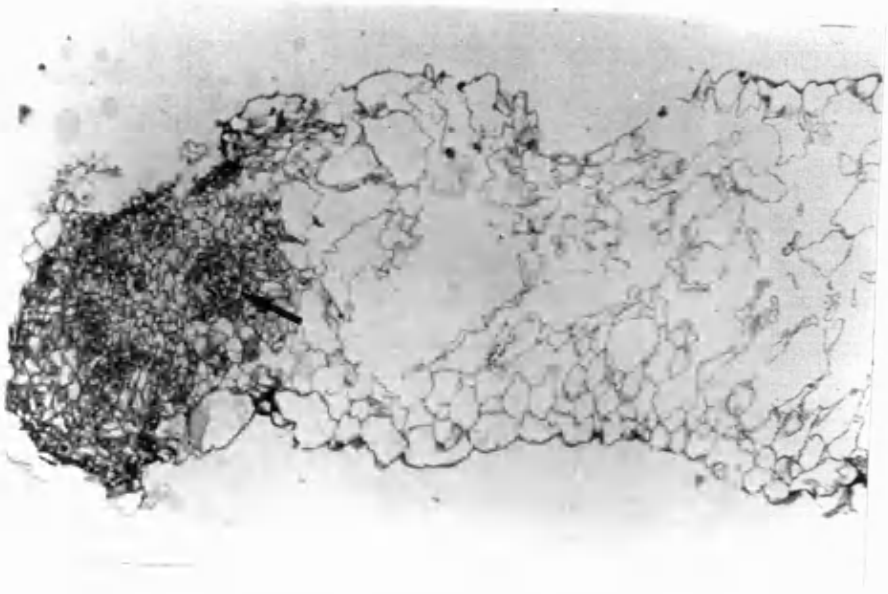
Plate 9: Transverse section of a leaf explant after 2 days on second-stage SIM [2d SIM (4d CIM)] x 66; cell proliferation is visible at the cut end. The newly formed cells are small and compact (arrowed).
scale bar = 152 μM

Plate 10: Transverse section of a leaf explant after 4 days on second-stage SIM [4d SIM (4d CIM)] x 66; centres of darkly stained tissues with tracheids are visible (arrowed).
scale bar = 152 μM

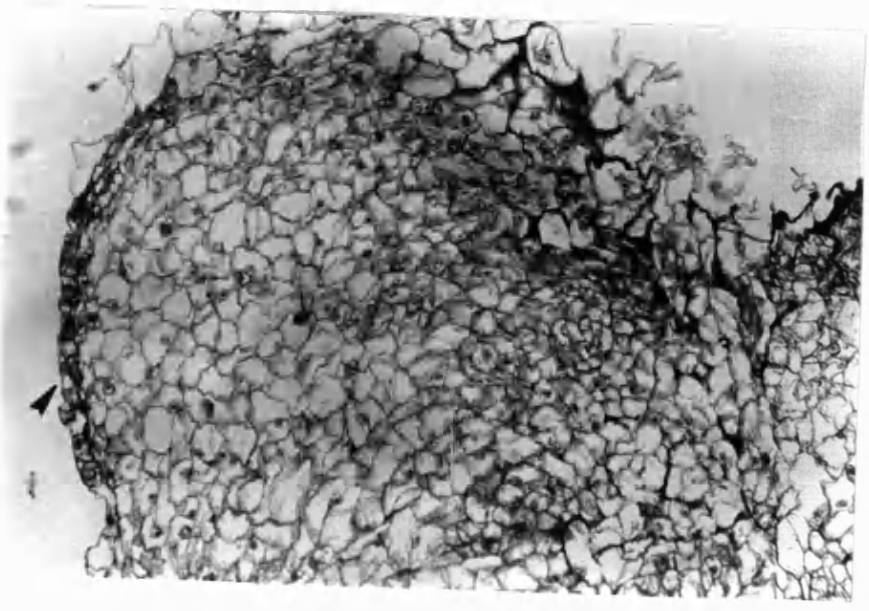
Plate 11: Transverse section of a leaf explant after 4 days on second-stage SIM [(4d SIM (4d CIM)] x 165; a layer of darkly stained cells is visible (arrowed).
scale bar = 60 μM



9



10

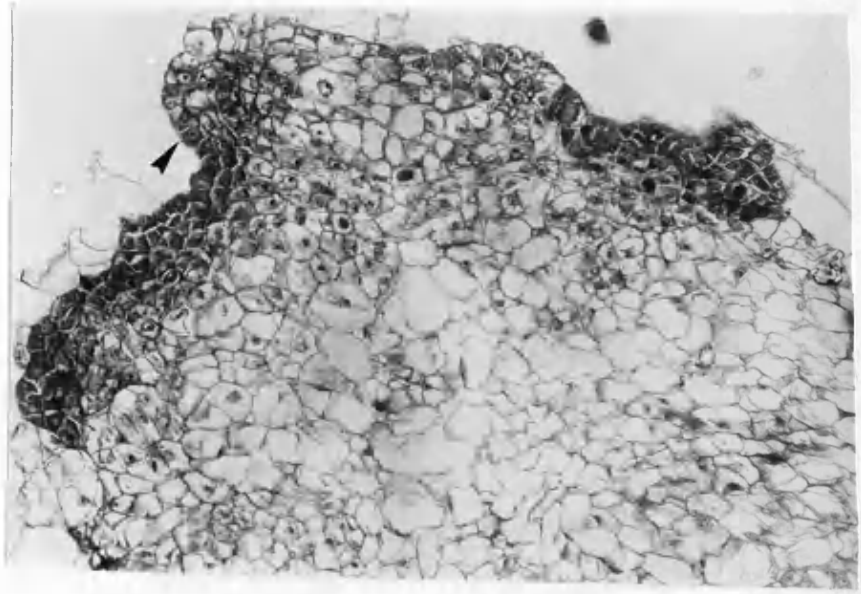


11

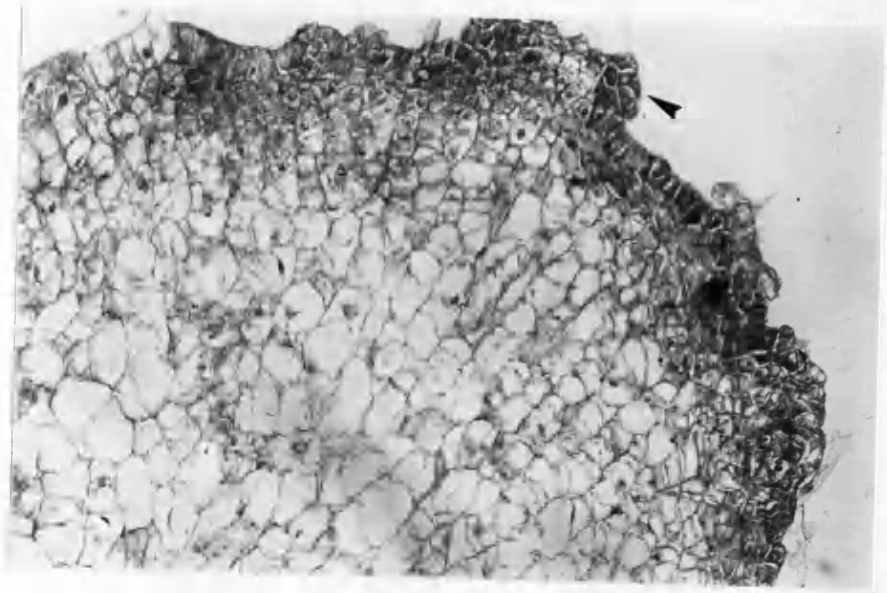
Plate 12: Transverse section of a leaf explant after 6 days on second-stage SIM [6d SIM (4d CIM)] x 165; formation of a leaf primordium is visible(arrowed).
scale bar = 60 μM

Plate 13: Transverse section of a leaf explant after 8 days on second-stage SIM [8d SIM (4d CIM)] x 165; leaf primordia are visible (arrowed).
scale bar = 60 μM

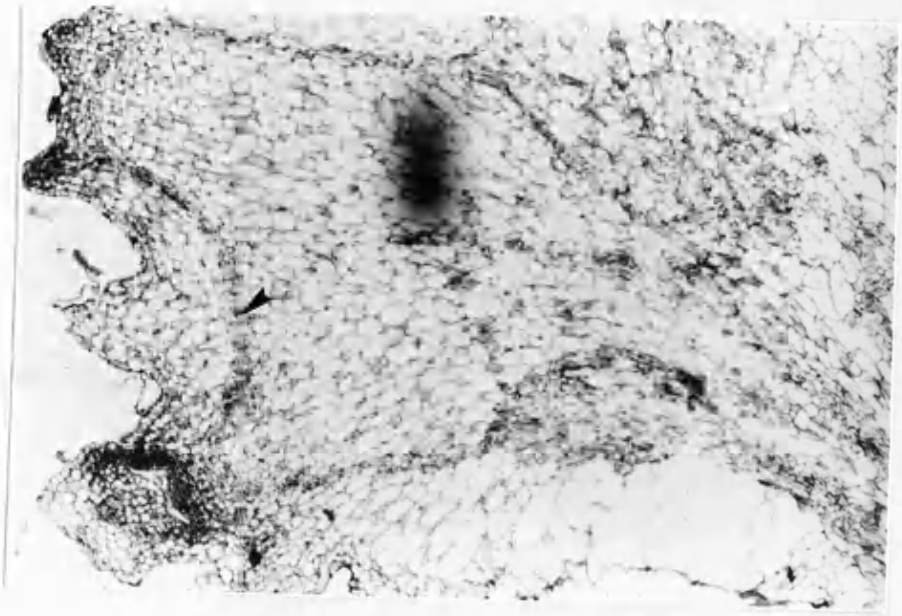
Plate 14: Transverse section of a leaf explant after 12 days on second-stage media [12 SIM (4d CIM)] x 66; vascular strands joining the leaf lobes are visible (arrowed).
scale bar = 152 μM



12



13



14

second-stage CIM produced callus with no visible roots. The colour of the callus was variable, probably due to the accumulation of secondary metabolites such as anthocyanins. The central region in the explant remained green and the callus formation was limited to the cut edges of the explant. Histological studies showed developing roots in some of the cultures after 12 days on second-stage CIM. The sequential steps in root development were not observed in cut sections of explants. This indicates that the frequency of root formation at 0.5 mg l^{-1} 2,4-D was low; callus formation was favoured at this concentration. It has been reported that cultures of tobacco established on 2,4-D media produced what appeared to be root primordia, apparently arrested at different stages of growth (Meins, 1986).

Explants when transferred onto SIM became more greener and shoot formation was observed in 8 days. The histological studies showed no further proliferation of callus after 2 days on SIM. The cells formed on SIM were more compact as compared to those in the callus formed on CIM. Scattered groups of tracheary elements were seen after 4 days on SIM. Meada and Thorpe (1979) observed meristemoid formation in close proximity to groups of xylem elements during shoot regeneration from tobacco callus. The presence of groups of tracheids in cultures after 4 days on SIM may suggest that shoot induction has occurred within 4 days on SIM. Formation of a layer of darkly stained cells at the surface was observed in 4 day old SIM cultures. This layer was contiguous with the outer layer of the callus mass. Similar observations were made by other workers (see section 1.1.4). As suggested by Christianson and Warnick (1988) this layer may correspond to the epidermis of the developing shoot apex and shoot formation was therefore superficial.

It was therefore seen that the culture systems selected gave rise to two different developmental pathways: callus and endogenous root formation on CIM alone and shoot proliferation on CIM followed by transfer to SIM. Hence

it was considered to be appropriate to proceed to analyse the molecular changes occurring in the shoot producing cultures using CIM cultures as the control.

It may, however, be difficult to distinguish between the molecular changes resulting from the morphogenetic events and those resulting from the different hormone treatments, mainly because the number of cells involved in meristemoid formation in response to hormones are very small in number (section 1.1.3), and the number of cells responding to hormones in general may be relatively large. The formation of root primordia in the control medium may also pose the problem of minimising the differences between the two culture systems as it is possible that the initial stages of molecular changes leading to morphogenesis of shoot or root may be very similar.

3 CHANGE IN AMOUNTS OF TOTAL AND POLYADENYLATED RIBONUCLEIC ACID IN *IN VITRO* CULTURES

3.1 INTRODUCTION

The aim of this experiment was to compare the amounts of total ribonucleic acid (RNA) and polyadenylated ribonucleic acid (poly $(A)^+$ RNA) in morphologically different CIM and SIM cultures and to study the relationship between the type of culture and RNA/poly $(A)^+$ RNA accumulation to establish whether the morphological differences observed between the two systems, i.e. callus with endogenous roots on CIM and shoots on SIM, are reflected by changes at the RNA level.

Therefore, in order to find the changes in total and messenger RNA in tobacco explants grown on CIM and SIM (see section 2.2.1.3) isolation of RNAs was carried out from *in vitro* cultures during a 12 day culture period, spanning the entire sequence of morphological changes.

3.2 MATERIALS AND METHODS

It is extremely important to obtain undegraded, biologically active RNA as the entire strategy of research work at the molecular level on gene expression depends on the availability of high quality RNA. The success of RNA preparation is mainly dependent on efficient inhibition and removal of ribonucleases (RNases) during RNA isolation. RNases are extremely stable and almost ubiquitous. Therefore, steps must be taken to eliminate ribonuclease activity from all equipment and solutions used in RNA purification.

3.2.1 General methods

3.2.1.1 Removal of ribonucleases

All the glassware and spatulas were baked (160 ^oC, 4-12 h) and the solutions except those containing Tris, including double distilled water further purified using the 'Milli Q' system (Millipore) were treated with 0.1% diethyl pyrocarbonate (DEPC), shaken thoroughly (10 min) and autoclaved (15 min,

1.05 kg cm^{-2}). The solutions containing Tris were made with DEPC treated water and re-autoclaved. Phenol was treated with 0.1% 8-hydroxyquinoline, a partial inhibitor of ribonucleases. Wherever possible, sterile disposable plasticware free from ribonucleases was used.

3.2.1.2 Precipitation of RNA

To an aqueous solution containing RNA, 3 M sodium acetate (0.1 volume, pH 5.2/5.5) and ethanol (2 volumes) were added. The contents were mixed and left overnight (-20 °C). The RNA was pelleted by centrifugation (either with a Sorvall SS 34 rotor (12000 rpm) or a microfuge (MSE Micro Centaur, 13000 rpm, 4 °C), the pellet was washed with 70% ethanol and dried (5-10 min) in a Speed-vac concentrator (Savant). The pellet was then dissolved in a suitable volume of either TE (pH 8) (Appendix A) or water and stored at -80 °C.

3.2.1.3 Estimation of purity of RNA

a. From ultraviolet absorbance

Optical density was measured at 260 and 280 nm using a Shimadzu UV-260 spectrophotometer. A pure sample will give the ratio of A_{260}:A_{280} > 2.0. Contamination of RNA with DNA or proteins will give a lower ratio typically around 1.8 or less.

b. Using gel electrophoresis

An agarose gel (0.7%) was run in a mini-gel apparatus. Agarose (0.35 g) was boiled with 1 x TBE (50 ml) (Appendix A) , cooled (55 °C) and ethidium bromide (0.5 $\mu g\ ml^{-1}$) was added and poured into the mini gel box with the combs fitted. The combs were removed when the gel had solidified (45 min-1 h) and 1 x TBE (50 ml) containing the same concentration of ethidium bromide was poured onto the gel. To the RNA solution (1 μg in 20 μl), BB loading buffer (2 μl) (Appendix A) was added and loaded onto the gel. Molecular

weight markers prepared as described below were also loaded. The gel was electrophoresed at a constant voltage (40 V, 4-5 h) and observed on a UV-transilluminator. Presence of contaminating DNA will be detected as high molecular weight bands at the top of the gel.

3.2.1.4 Preparation of molecular weight markers

Lambda DNA digested with *Hin* dIII was used as molecular weight markers. The method given below was applied to all the restriction digestions. The restriction enzymes and their buffers were bought from Northumbria Biologicals Ltd. The following components were added in the order given below.

	µl
water	a
10 x enzyme buffer	2
DNA (1 µg)	b
restriction enzyme	1
	20

A minimum of 1 unit of enzyme is required to digest 1 µg of DNA at optimum temperature in 1 h and an excess of enzyme was generally used. The contents of the tube were mixed and centrifuged briefly and incubated (37 ^{o}C for one to several hours). One tenth volume of loading buffer was added when digestion was complete. Two of the fragments of lambda DNA have cohesive termini and to prevent their annealing the digested DNA was heated (65 ^{o}C, 5 min) before loading onto the gel.

3.2.1.5 Denaturing gel

The intactness of the ribosomal RNA reflects the quality of RNA. A non-denaturing gel (such as that detailed above) will show the presence and purity of RNA but not necessarily the quality of RNA. A denaturing gel reveals the integrity of the ribosomal RNA units.

The following method was described in a Nucleic Acids Workshop manual from Hatfield Polytechnic.

The denaturing of RNA was done with a glyoxal and formamide mix in phosphate buffer (GPF mix). Glyoxal was de-ionised by passing through a mixed bed resin AG50-X8(D) to achieve a pH of 7.6. Formamide was de-ionised by stirring with Amberlite monobed resin MB3 (30 min). The GPF mix was prepared (6 ml of glyoxal, 1 ml of formamide, 0.1 ml of 0.5 M phosphate buffer (pH 7.5) and 3.75 ml of water) and stored in 0.5 ml Eppendorf tubes (-80 ^{o}C). An agarose gel (1.1%) was prepared as described for the non-denaturing gel but with 0.01 M phosphate buffer (pH 7.5). The RNA was vacuum dried and the pellet was dissolved to a concentration of 0.5 μg μl^{-1} of RNA in 0.01 M phosphate buffer. Two aliquots (4 μl) were transferred to two Eppendorf tubes, phosphate buffer (8 μl) was added to one and kept on ice. To the other, GPF mix (8 μl) was added, vortexed briefly and incubated (55 ^{o}C, 15 min). Then, 2 μl of loading mix (20% sucrose, 10% Ficoll, 0.01 M EDTA and 1% bromophenol blue in 0.01 M phosphate buffer) was added to each and 8 μl of each sample was run on the gel at constant voltage (100 V, 3 h). The buffer was circulated during the run by using a peristaltic pump. The gel was de-glyoxalated (10 volumes of 0.05 M NaOH and 0.5 μg ml^{-1} ethidium bromide) for 20 min, stained (Tris.HCl, pH 7.5 with 0.5 μg ml^{-1} ethidium bromide) for 40 min, transferred to water and viewed on a UVtransilluminator and photographed (see Appendix C).

3.2.1.6 Quantification of RNA

To estimate the concentration of nucleic acid in solution, a known aliquot was diluted to 1 ml to obtain a dilution factor of 200-1000. The absorbance of this solution at 260 nm was measured. Assuming that an absorbance reading at 260 nm of 1.0 is equivalent to 40 μg ml^{-1} in a light path of 1 cm (Maniatis *et al.*, 1982) the concentration of RNA was calculated.

3.2.2 Preparation of total RNA

The following RNA extraction methods were tried:

Method A - mini-preparations (the laboratory manual, Molecular Biology of Plants course, Cold Spring Harbor, 1986)

Method B - Logemann *et al.* (1987)

Method C - Chambers and Russo (1986)

Method D - Prescott and Martin (1987)

Method D gave the highest yield (see Appendix C), but the RNA contained an insoluble brown pellet. therefore this method was modified as described below.

3.2.2.1 Extraction method D

Tobacco leaves (5 g) were ground well in liquid nitrogen and suspended in two volumes of extraction buffer (Tris.HCl (50 mM), LiCl (150 mM) EDTA (5 mM), pH 9.0). An equal volume of phenol/chloroform prepared according to Maniatis *et al.* (1982) was added and the mixture was constantly shaken until a white emulsion was formed. This was first kept at room temperature (15 min) and then on ice (5 min), shaking occasionally, prior to centrifugation in a Sorvall SS-34 rotor (5000 rpm, 4 °C, 10 min). The aqueous phase was re-extracted with phenol as described before and the upper layer was transferred to a new tube. An equal volume of chloroform/iso-amyl alcohol (24:1) was added, and the mixture was kept on ice (10 min), mixing occasionally. The layers were separated by centrifugation (SS-34, 7000 rpm, 4 °C, 15 min). To the aqueous phase, LiCl was added to a final concentration of 2 M and RNA was precipitated (-20 °C, 3-12 h or 4 °C, overnight). The samples were centrifuged (5000 rpm, 4 °C, 10 min) and the pellet was transferred to an Eppendorf tube, and dissolved in 600 µl water; the RNA precipitation with LiCl was then repeated. The pellet was dissolved in 400 µl of water and ethanol precipitation was carried out (section 3.2.1.2). The pellet was dissolved in water (50 µl) and the RNA concentration was measured. Samples of RNA were run on non denaturing and

denaturing gels as described in sections 3.2.1.3b and 3.2.1.5 respectively. The amounts of RNA obtained by methods A, B, C and D are tabulated and discussed in Appendix C.

3.2.2.2 Removal of polysaccharides

The phenol extracted RNA obtained after centrifugation was treated with ethanol (0.1 volume), chilled on ice (5 min) and centrifuged in a Sorvall SS 34 rotor (5000 rpm, 4 ^{o}C, 10 min) (Mettler 1988). The precipitated polysaccharides were discarded and the supernatant containing RNA was subjected to ethanol precipitation as described in section 3.2.1.2.

The polysaccharides in the RNA samples were removed as follows, by treating the aqueous layer after chloroform/iso-amyl alcohol extraction or the RNA solution after the first or second LiCl precipitation. Alternatively, polysaccharides were removed from the RNA solution after the final ethanol precipitation (section 3.2.1.2), just by centrifugation in a microfuge (13000 rpm, 20 min).

The amount of RNA in each of the supernatants was quantified. The polysaccharide pellet was dissolved in water (400 μl, overnight) and absorbance at 260 nm was measured, in order to observe whether any RNA was lost in the polysaccharide pellet. (Appendix C).

3.2.3 Purification of polyadenylated RNA

Most eukaryotic messenger RNA (mRNA) is polyadenylated after being transcribed from DNA. The 3' end therefore has a poly (A) tail of varying lengths up to 200 nucleotides (Jacobson, 1987). This poly (A) tail can base pair with poly (U) or oligo (dT). The methods of purification of mRNA are based on the pairing of the poly (A) tail with either poly (U) or oligo (dT).

All the precautions required to eliminate ribonucleases (section 3.2.1.1.) were taken when isolating poly $(A)^{+}$ RNA. In addition, Eppendorf tubes, glass

wool and Corex tubes were siliconised according to Maniatis *et al*. (1982), washed thoroughly with warm water and autoclaved.

3.2.3.1 Using messenger affinity paper

Messenger affinity paper (mAP) was purchased from Amersham Inc., U.K. The following protocol from Amersham was followed to isolate poly $(A)^+$ RNA. A piece of mAP (1 cm^2) was pre-wetted with NaCl (0.5 M) and air dried on a sterile filter paper. Total RNA (300 μg in 25 μl of water) was heated (65 ^oC, 5 min), cooled on ice and 5 M NaCl was added to a final concentration of 0.5 M. This solution was applied in small aliquots onto the mAP placed on a sterile Petri dish and left for 5-10 min. The mAP was transferred onto a sterile filter paper for 2 min and washed twice with 0.5 M NaCl (5 ml, 5 min), once in 70% ethanol (2 min) and left to air dry (5 min) on a sterile filter paper, care being taken to prevent complete drying. The mAP was immersed in water (300 μl) heated (70 ^oC, 5 min), vortexed briefly and centrifuged for a few seconds in a microfuge. The poly $(A)^+$ RNA was ethanol precipitated (section 3.2.1.2).

3.2.3.2 Affinity chromatography on oligo (dT) cellulose

The method described by Clemens (1986a) was followed. 100 mg oligo (dT) cellulose (Sigma chemicals) was suspended in the loading buffer (Appendix A) and poured into a column in a siliconised Pasteur pipette containing a siliconised glass wool plug. The column was washed first with water and then with three volumes of a solution containing NaOH (0.1 M) and EDTA (5 mM). The column was finally washed with water until the eluate reached pH 7 and then re-equilibrated by passing through 5 volumes of loading buffer.

The RNA solution (2 $\mu g\ \mu l^{-1}$) was heated (65 ^oC, 5 min), an equal volume of 2 fold strength loading buffer was added, cooled to room temperature and loaded onto the column. The rate of flow through the column was controlled by a clamp on the silicon rubber tubing (about 8 cm in length)

attached to the tip of the Pasteur pipette. The column was washed with loading buffer until the absorbance of the eluate at 260 nm was near zero. The poly $(A)^+$ RNA was eluted with 10 column volumes of elution buffer (Appendix A). The absorbance of the last two fractions were measured to make sure that a value close to 0.000 was obtained. The eluted poly $(A)^+$ RNA was reapplied to the column to minimise any contamination with ribosomal RNA. The poly $(A)^+$ RNA was precipitated as described in section 3.2.1.2 and stored at -80 °C.

In addition, the method by Jacobson (1987) was tried, but the above method was found to be better (see section 5.3.3).

3.2.4 Quantification of poly $(A)^+$ RNA

3.2.4.1 Determination of the amount of poly (A) in poly $(A)^+$ RNA

The procedure described by Slater (1986) was followed. In this method, the polyadenylated sequence [poly (A) tail] at the 3' end of the mRNA was made to hybridise with saturating amounts of [^{3}H]-polyuridylic acid {[^{3}H]-poly (U)}, the excess [^{3}H]-poly (U) was removed by RNase and the hybrid was collected by TCA precipitation. The amount was then quantified by scintillation counting.

An initial experiment was carried out to draw the hybridisation curve with 0.01 μg of commercial poly (A) and increasing amounts of [^{3}H]-poly (U) (Amersham, 500 mCi mmol^{-1}), from 0-200 nCi. All the solutions used in reactions given below were made in 2 x SSC (Appendix B). To six tubes in duplicate, 2 x SSC (2 ml) and commercial poly (A) (0.01 μg) were added. Then, 0, 1, 5, 10, 20, 50, 100 and nCi of [^{3}H]-poly (U) was added and incubated for 3 min at 90 °C and then 60 min at 25 °C. Then 60 μl of RNase (1 mg ml^{-1}) was added to each tube, mixed and incubated (25 °C, 20 min). Subsequently, yeast RNA solution (100 μg), 2'3'uridylic acid solution (100 μg) and 4 ml of 10% trichloroacetic acid (TCA) were added and left on ice (2 h). The precipitate was collected on glass fibre filter discs (Whatman-GF/C 25), washed three times with 5% TCA (10 ml) and twice with absolute ethanol (5 ml). The filters were air

dried, and transferred to scintillation vials, 5 ml of a toluene based scintillant (Optiphase, Pharmacia) was then added and the radioactivity was measured using an LKB Rack Beta scintillation counter. The radioactivity was plotted against the [^{3}H]-poly (U) concentration (Appendix C).

Similarly, the saturation curve for total RNA was drawn following the above method, but with 5 μg of total RNA, instead of commercial poly (A) using 0-200 nCi of [^{3}H]-poly (U) (Appendix C). The optimum concentration of [^{3}H]-poly (U) obtained from the graphs was used to estimate the poly (A) contents in unknown samples. In such cases, a standard curve for commercial poly (A) was obtained by hybridising 0-0.01 μg of poly (A) with the optimum value of [^{3}H]-poly (U) obtained from previous experiments. With experimental samples, an estimated value between 0-0.01 μg of poly (A) (assuming that 1% of total RNA is poly $(A)^+$ RNA and 10% of poly $(A)^+$ RNA is poly (A); Datta *et al*, 1987) was hybridised with the optimum value of [^{3}H]-poly (U). The corresponding concentration was estimated from the standard curve (Appendix C).

3.2.4.2 Determination of the ratio of poly (A) tail to poly $(A)^+$ RNA

The method used to estimate the poly (A) content gives only the amount of poly (A) that is present in RNA. The amount of poly $(A)^+$ RNA is larger, as the poly (A) tail makes up only a certain proportion of the entire mRNA molecule. In order to find the ratio of poly (A) to the entire mRNA molecule, the following method was followed. Poly $(A)^+$ RNA was isolated from total RNA (2000 μg) and the pellet was dissolved in water (1 ml). Aliquots (1 μl and 2.5 μl) from this solution, in duplicate, were used to estimate the amount of poly (A) by the poly (U) hybridisation method; the rest of the solution was used to determine the poly $(A)^+$ RNA concentration using the UV-spectrophotometer. Three samples of tobacco RNA (day 0) were subjected to the above procedures.

The amount of poly $(A)^+$ RNA (as determined by UV absorbance) was divided by the amount of poly (A) in the sample (as estimated by the poly (U)

hybridisation method) to obtain the ratio of poly $(A)^+$ RNA to poly (A) tail. In calculating the amount of poly $(A)^+$ RNA of an unknown sample, the amount of poly (A) was measured by the poly (U) method and this amount was multiplied by the factor obtained as the ratio of poly $(A)^+$ RNA to poly (A) tail.

3.3 RESULTS

3.3.1 Total RNA accumulation in *in vitro* cultures

Leaf pieces were cultured on CIM for 4 days (first-stage) and then transferred to CIM or SIM (second-stage). Leaf squares with a total weight of 4 g were harvested every day from days 1-4 on first-stage CIM (1d CIM-4d CIM) and days 1-8 on second-stage CIM and SIM [1d CIM (4d CIM)-8d CIM (4d CIM) and 1d SIM (4d CIM)-8d SIM (4d CIM)] and stored in liquid nitrogen. RNA was isolated from each of these samples.

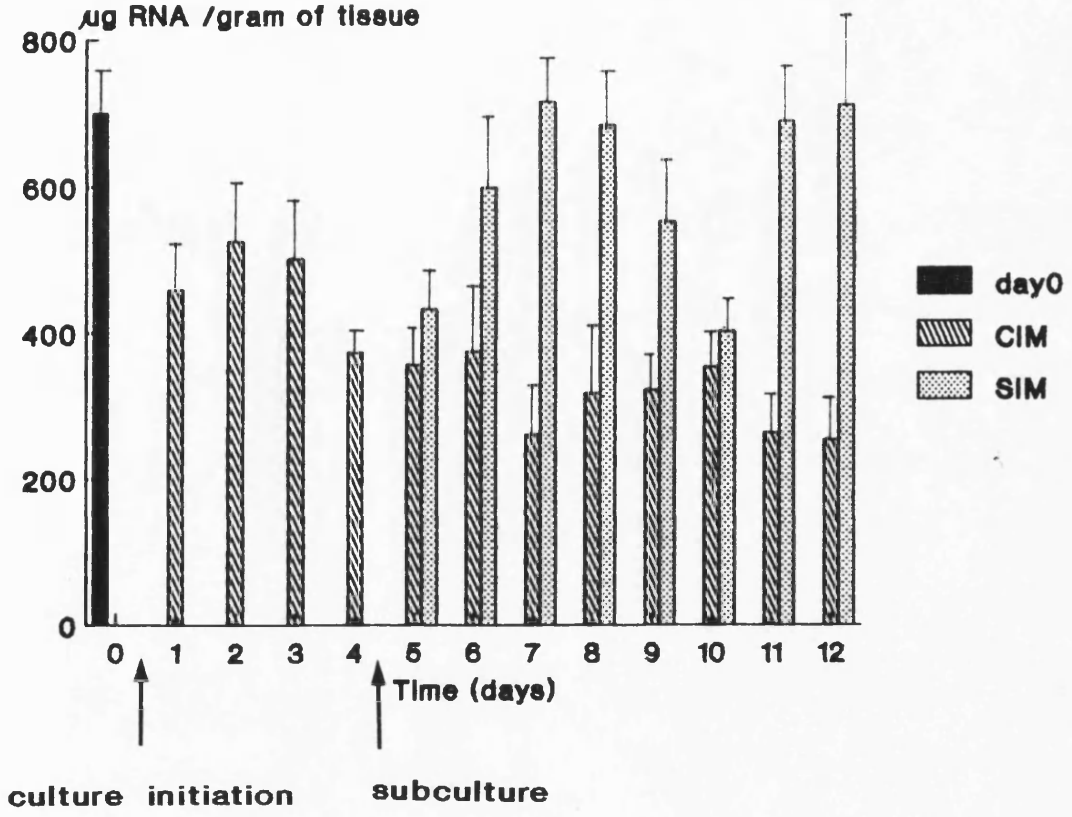
Fig. 3 represents the average amounts of RNA per gram of tissue, isolated from three replicate samples over 12 days. Day 0 denotes the leaf tissues before culturing. Days 1-4 show RNA obtained from first-stage CIM and days 5-12 represent RNA from second-stage CIM and SIM.

The amount of RNA obtained from explants after one day on first-stage CIM was much less than that from day 0 samples. There was a slight increase in amounts of RNA during the second and third day on CIM which appeared to decrease on the fourth day on CIM. Transfer of explants onto fresh CIM did not seem to have any influence on RNA accumulation (days 5-12 in Fig. 3).

The yield of RNA per gram from tissues transferred to second-stage SIM was significantly greater than from those transferred to second-stage CIM, except on the first and sixth days (days 5 and 10 in Fig. 3) after transfer, when there was no significant difference. The amounts of RNA (mean values) at day 2, 3, 4 and 5 on SIM (day 6, 7, 8 and 9 in Fig 3) were greater than RNA at day 1 and 6 on SIM (day 5 and 10 in Fig 3). A sudden increase in amount of RNA was observed at day 7 on SIM (day 11 in Fig. 3).

Fig 3 Average amounts of total RNA isolated from *in vitro* cultures.

RNA was isolated (in three replicates) from day 0 (before culturing), after 1-4 days on first-stage CIM and after a further 1-8 days on second-stage CIM and SIM. The absorbance of RNA solutions of known dilution was measured using a UV-spectrophotometer and the amounts of RNA were calculated assuming that an A_{260} of 1.0 OD was equivalent to 40 μg.


µg RNA /gram of tissue
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (days)
day0
CIM
SIM
culture initiation
subculture

3.3.2 Isolation of poly $(A)^+$ RNA from *in vitro* cultures

3.3.2.1 Determination of the ratio of poly (A) tail to poly $(A)^+$ RNA

The amounts of poly (A^+) RNA and poly (A) obtained from 2000 μg of tobacco total RNA (day 0 samples) are given in Table 1.

sample	*poly (A^+) RNA, μg	**poly (A), μg
A	26.33	2.7
B	24.99	2.5
C	32.30	3.4
mean	27.87	2.87

Table 1 - Amounts of poly $(A)^+$ RNA isolated from each of total RNA samples A, B and C, compared with the amounts of poly (A) in them.

* amount of poly $(A)^+$ RNA isolated from 2000 μg of total RNA, as estimated by OD at 260 nm.

** amount of poly (A) in poly $(A)^+$ RNA isolated from 2000 μg, as estimated by poly (U) hybridisation.

Av. conc. of poly $(A)^+$ RNA in 2000 μg of total RNA = 27.87 μg

Av. poly (A) in 2000 μg of total RNA = 2.87 μg

Therefore, the ratio of poly $(A)^+$ RNA/poly (A) = 27.87/2.87

= 9.71

The average concentration of poly $(A)^+$ RNA divided by the average poly (A) amount gave a value of 9.71. This ratio was assumed to be a constant. The amount of poly $(A)^+$ RNA isolated from *in vitro* cultures was calculated by multiplying the amount of poly (A) by the factor 9.71.

3.3.2.2 Accumulation of poly $(A)^+$ RNA in *in vitro* cultures

The isolation of poly $(A)^+$ RNA was carried out from total RNA extracted from *in vitro* cultures on first-stage CIM (1-4 days) and second-stage CIM and SIM (days 5-12). Three replicates per sample were used in this experiment. The

pattern of poly $(A)^+$ RNA accumulation in second-stage CIM and SIM cultures (Fig. 4) resembled the pattern of total RNA accumulation (Fig. 3).

The percentages of poly $(A)^+$ RNA purified from total RNA isolated from CIM and SIM cultures over the 12 day period are given in Table 2. As is evident the percentages of poly $(A)^+$ RNA obtained from the second-stage SIM cultures were slightly higher than those obtained from second-stage CIM cultures.

Day	CIM	SIM
1	1.20 ± 0.09	
2	1.27 ± 0.06	
3	1.19 ± 0.03	
4	1.12 ± 0.05	
5	1.20 ± 0.13	1.32 ± 0.11
6	1.25 ± 0.07	1.37 ± 0.03
7	1.28 ± 0.08	1.61 ± 0.08
8	1.04 ± 0.04	1.37 ± 0.07
9	1.05 ± 0.15	1.28 ± 0.09
10	1.21 ± 0.08	1.25 ± 0.04
11	1.10 ± 0.06	1.30 ± 0.10
12	1.29 ± 0.09	1.42 ± 0.13

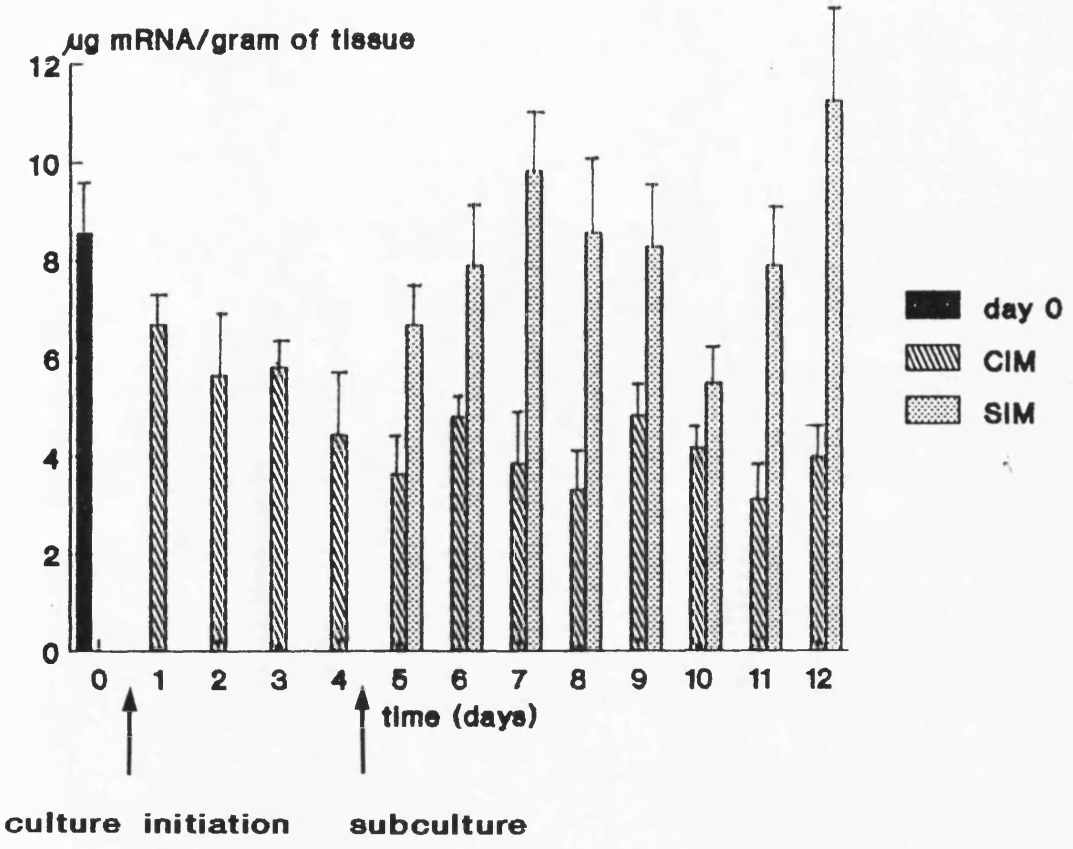
Table 2-Percentage of poly $(A)^+$ RNA purified from total RNA obtained from 1-4 days on first-stage CIM (days 1-4 in the table), and after a further 1-8 days on second-stage CIM or SIM cultures (days 5-12 in the table). The values represent the means of three values ± standard error.

3.4 DISCUSSION

The amount of RNA obtained from tobacco leaf material before culturing (day 0) was much greater than that obtained from explants grown for one day on first-stage CIM. This could be expected as the initial culturing of explants would disturb the normal metabolism found in intact leaves. Although a number of novel mRNA species may be induced by 2,4-D in the medium and the wounding itself, the mRNA species that may be degraded due to hindered growth and

Fig. 4: Average amounts of mRNA obtained from *in vitro* cultures.

Poly $(A)^+$ RNA was purified from total RNA using the oligo (dT) chromatography and the poly (A) content was quantified by the poly (U) hybridisation (section 3.2.4 and Appendix C). The value was multiplied by 9.71 to obtain the amount of poly $(A)^+$ RNA.


µg mRNA/gram of tissue
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12
time (days)
day 0
CIM
SIM
culture initiation
subculture

metabolism can contribute to the decrease in level of RNAs in first day CIM cultures.

Yields of RNA obtained from 1-3 day first-stage CIM cultures were greater than that was obtained from 4 day first-stage CIM cultures. Steward *et al.* (1964) studied the growth and development of cultured carrot cells. They found that total RNA increased during the lag phase (prior to cell division) and dropped rapidly with the onset of cell division. They also found that the cells grew by cell enlargement during lag phase; the enlarged cells stored nucleic acids far in excess of that required for their growth. A similar increase was seen in *in vitro* cultures of tobacco during the first three days. Cell enlargement in explants was prominent during the first two days in culture (histological studies showed this: compare Plates 2 and 3). The increase in RNA during this period on CIM could therefore be due to RNA accumulation prior to cell division.

The amounts of total RNA and poly $(A)^+$ RNA against the time course on second-stage SIM showed that both total and poly $(A)^+$ RNA accumulation increased after transfer of cultures from first-stage CIM onto second-stage SIM with a peak increase at day 3 on SIM (day 7 in Figs. 3 and 4 and Table 2). This would suggest higher rates of transcription and possibly a concomitant increase in protein synthesis in SIM cultures. This increase in transcriptional activity is in accordance with the observations of chapter 2, such as greater increase in fresh weight of explants when they were subcultured to SIM than to CIM, the increased cell division and early appearance of tracheids in SIM cultures as observed from histological studies, and the enhanced chloroplast formation in explants on BAP-containing medium. The observed increases in total and poly $(A)^+$ RNA show that such changes could be brought about by changes in gene expression at the transcriptional level.

Cytokinins are known to activate the synthesis of total RNA and proteins in plant tissues. It has been shown that cytokinin activated production of all types of RNA (rRNA, tRNA and mRNA (Kulaeva, 1981)). He observed

polysome formation as a rapid response to application of BAP to pumpkin cotyledons. He concluded that 70-80% increase of polysome formation was non-transcriptional and 20-30% increase was at transcriptional level due to application of BAP. Neumann and Khokhlova (1981) reported that there was a dramatic increase in total, plastid and cytoplasmic rRNA after BAP treatment to pumpkin cotyledons. Similar results have been obtained by Kinoshita *et al*. (1979) with cucumber cotyledons. Therefore the increases in total RNA observed in SIM cultures may be mainly due to the formation of plastid and ribosomal RNA. The increase in green colour in SIM explants as compared to CIM explants indicates the possibility of increased formation of plastid RNA.

The decline in amounts of RNA/g of tissue obtained from 5d SIM (4d CIM) cultures may be real or could be due to loss of RNA during isolation. To confirm that this decline is genuine, the experiments would have to be repeated. Reynolds (1989) observed that the percentage poly $(A)^+$ RNA in total RNA was greater in BAP-treated stem segments of *Solanum carolinense* than in the 2,4-D-treated cultures. His data also show two peaks in poly $(A)^+$ RNA accumulation in segments cultured on BAP medium; the first, minor peak at day 1 and the second, major peak at day 4. He reported that the second increase in poly $(A)^+$ RNA corresponded to the time at which meristematic zones organised to form shoot apices in these cultures. These data suggest that the observed decline at day 5 could be genuine.

The process of shoot formation occurs in two stages; shoot induction and shoot differentiation. In shoot formation from tobacco leaf explants, it was evident that the total duration for shoot formation was less than 8 days on SIM. It may be possible that RNA accumulation during 1-6 days on SIM corresponded to the shoot induction and differentiation process and the gradual drop in amount of RNA by day 6 was parallel to completion of this and commencement of growth and differentiation. The sudden increase in RNA accumulation at day 7 may correspond to rapid growth of shoots. Alternatively,

the decline at day 6 may have corresponded to the end of inductive process and the marked increase at day 7 to the beginning of differentiation. However, such a well defined inductive process followed by differentiation is highly theoretical.

Transfer of explants onto second-stage CIM showed no increase in total RNA. Histological studies (section 2.3.3) showed large vacuolated callus cells, which may be quiescent as compared to small, nucleated cells observed in SIM cultures. Although some cultures showed endogenous roots by day 12 on second-stage CIM, thus showing that new developmental pathways and therefore probably new patterns of gene expression occur, the scale of the response was much smaller. The lower amounts of RNAs from CIM cultures may reflect the less active growth under CIM conditions. Visible signs of discolouration of explants on CIM indicate the breakdown of chlorophyll. If this process is similar to senescence, which may have been caused by increased production of ethylene by 2,4-D (Sauerbrey *et al.*, 1987), a decline of RNA content with marked fall in ribosomal RNA can be expected. BAP, on the other hand is known to retard senescence. Therefore, it may have prevented such degradation of RNA by BAP in SIM cultures. It may also be possible to some extent that the reason for obtaining lesser amounts of RNA from CIM cultures is due to the method of RNA isolation being more suitable for leaf material than for callus, as difficulty was experienced in grinding frozen explants with callus (callus became 'rocky' when liquid nitrogen was added) compared with shoot regenerating tissues. The high water content of large vacuolated callus cells would have rendered callus rocky when liquid nitrogen was added prior to grinding.

The slight increase in amounts of percentage of poly $(A)^+$ RNA in total RNA from SIM cultures may indicate upregulation of mRNAs by SIM conditions. Although the method used in quantification of poly (A) seemed to be repeatable and therefore accurate, one cannot exclude the possibility that some RNAs had very short tails, and therefore failed to hybridise to the poly (U)

under the experimental conditions (Roshbash and Ford, 1974). Datta *et al.* (1987) determined the amount of poly (A) in poly $(A)^+$ RNA isolated from germinating soy bean axes using poly (U) hybridisation method. He calculated the amount of poly $(A)^+$ RNA by multiplying the poly (A) content by a factor of 14. He assumed an average poly $(A)^+$ RNA of 1400 nucleotides and a poly (A) length of 100 nucleotides. In my experiment a ratio of approximately 10:1 (mRNA to poly (A) tail) was obtained. This method does not determine the absolute length of poly (A) tail, but only the proportion of the mRNA to poly (A) tail.

In quantifying tobacco poly $(A)^+$ RNA, the factor 9.71 obtained as a ratio of poly $(A)^+$ RNA to poly (A) was taken as a constant when calculating the mRNA in the samples. The length of poly (A) can vary according to the age of mRNA (Grierson and Covey, 1985). Samples of different age may contain different proportions of mRNA with varying poly (A) content. Therefore, the method of quantifying mRNA in different *in vitro* samples using the same constant may not be accurate.

4 *DE NOVO* SYNTHESIS OF PROTEINS IN *IN VITRO* CULTURES

4.1 INTRODUCTION

The two morphogenetic effects observed under CIM and SIM culture regimes may be accompanied by the differential synthesis of new proteins and/or by variation in the levels of existing proteins. Such changes in protein complement could possibly be either under the control, directly or indirectly, of a particular hormone in the medium or they could be related to the observed morphogenetic differences. It was therefore intended in this experiment to analyse newly synthesised proteins in explants grown under the two different culture regimes to detect any qualitative or quantitative changes in proteins which may reflect differential gene expression. It was also aimed to observe changes in proteins occurring in SIM cultures over the time course of their appearance, as some of these changes may be related to the histological changes observed (2.3.3) during the process of shoot production.

Changes in gene expression have been studied by many workers by looking at the changes of newly synthesised proteins (e.g: Yasuda *et al.*, 1980; Sung and Okimoto, 1983; Dommes and Northcote, 1985; Ougham, 1987). *In vivo* labelling provides an opportunity to analyse proteins that are being newly synthesised as compared to the analysis of 'cold' proteins which does not distinguish between already existing ones and newly synthesised ones.

Proteins newly synthesised during a short period can be investigated by exposing the cultures to a radio-labelled amino acid. The most commonly used amino acid is [^{35}S] methionine. However, other amino acids such as [^{3}H] leucine and [^{14}C] leucine have also been used (Yasuda *et al.*, 1980). The amino acid is incorporated into the medium in the case of suspension cultures (Johnson-Flanagan and Singh, 1987) or spread on the material for cultures on solid media, such as cotyledons (Yasuda *et al.*, 1980) and germinating seeds (Dommes and Northcote, 1985). After incubating in the presence of the

radioactive amino acid the proteins are extracted and analysed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) or two dimensional gel electrophoresis (2D-PAGE).

4. 2 MATERIALS AND METHODS

4.2.1 Preparation of samples

In vivo labelling of proteins in *in vitro* grown cultures was carried out while the explant was growing on solid medium. The medium immediately surrounding the explant was cut and removed from the Petri-dish and the explant on its solid medium was transferred into a watch glass. [^{35}S] methionine (1347 Ci mmol^{-1}, Amersham) (15 μCi in 100 μl water) was spread on the explant, which was covered with a glass plate and left at room temperature (1-2 h). The explant was then separated from the agar medium, placed in a solid watch glass and 100 μl of extraction buffer (buffer A) (Appendix A) was added. The material was ground well with some fine sand grains (to assist grinding) using the bottom of a 10 ml soda lime test tube. The slurry was transferred to a microfuge tube (1.5 ml) and centrifuged in a microfuge (13,000 rpm, 1 min, room temperature). The supernatant was then transferred into a fresh tube and either used immediately or stored at -80 °C or under liquid nitrogen.

4.2.2 Determination of incorporation of radioactivity

1 μl of protein extract was added to sodium hydroxide (0.5 ml, 1 M) containing hydrogen peroxide (5% v/v). The sample was placed in ice and trichloroacetic acid (TCA) [2 ml of 25% TCA (w/v) with 2% casein hydrolysate (w/v)] was added and left on ice (30 min). The mixture was then filtered through a glass fibre filter disc (Whatman GF/C-25) and washed twice with ice cold TCA [8% (w/v), 3 ml)], using a suction apparatus, to remove unincorporated labelled amino acid. The filter was air dried in a fume cabinet and transferred to a scintillation vial, 5 ml of toluene based scintillant (Optiphase,

Pharmacia) was added and the radioactivity was measured using the [^{14}C] channel in a scintillation counter (LKB Rack beta).

4.2.3 Preparation of molecular weight markers

[^{14}C] methylated proteins (Amersham plc, CFA. 626) (1 μl) were boiled with sample buffer A (5 μl), cooled on ice to room temperature and loaded onto the polyacrylamide gel along with the proteins to be analysed.

4.2.4 Analysis of polypeptides by SDS-PAGE

A discontinuous gel consisting of resolving gel (10%) and stacking gel (4%) was cast in a Bio-Rad Protean II slab gel apparatus. The recipes for the gel are given in Appendix B. The monomer solution, after combining all the reagents except SDS, ammonium persulphate (APS) and N,N,N',N' tetramethyl ethylene diamine (TEMED), was prepared and de-gassed under vacuum until bubbling ceased. Then, SDS, APS and TEMED were added and the gel solution was poured into the Protean II gel apparatus using a glass pipette. This resolving gel was overlaid with water-saturated iso-butanol and allowed to polymerise (30-45 min). The overlay solvent was removed and the gel was washed with water (4-5 times) until all iso-butanol was removed. The gel comb was placed at an angle (10^{o}) between the plates to allow air bubbles to escape and the stacking gel, prepared in the same manner as the resolving gel, was poured. The gel was left to polymerise (30 min), the comb was removed, the wells were washed with water and filled with the gel running buffer (Appendix A).

Aliquots of samples with the same amount of acid precipitable radioactivity were made up to 20 μl with buffer A (Laemmli, 1970) and the proteins were denatured by heating in a water bath (95 ^{o}C, 4 min). The samples were cooled to room temperature and loaded onto a polyacrylamide gel. The gel was electrophoresed at constant current (4-5 h). For a 0.75 mm thick gel, the current was set at 13 mA through the stacking gel and 18 mA through the

resolving gel. For a 1.5 mm gel, the current was set at 18 mA through the stacking gel and 24 mA through the resolving gel. The gel was carefully removed from the glass plates and fixed for 30 min in a solution of methanol (40%) and acetic acid (10%).

4.2.5 Autoradiography and fluorography

After fixing the proteins, the gel was transferred onto Whatman 3MM paper, covered with cling film (Saran wrap) and dried in a Bio Rad gel drier (60 °C, 1 h). The cling film was removed and the dried gel subjected to autoradiography (-70 °C) using Kodak AX or Fuji RX film in a cassette containing intensifying screens (Dupont Lightning Plus). For fluorography, after fixing the proteins, the gel was immersed in Amplify (Amersham) for 15-30 min to impregnate the gel with fluor, dried and subjected to autoradiography as above.

4.3 RESULTS

4.3.1 Protein synthesis in first-stage cultures

Leaf explants cultured for 1-4 days on a medium with no hormones (NHM), or on a medium with 2,4-D (CIM), when transferred to SIM, did not show any significant difference in shoot production (Chapter 2, Fig. 1). This indicated that 1-4 days on different first-stage media had no visible effect on shoot production per explant. In order to investigate whether there was any variation in the proteins produced in cultures grown on different first-stage media, the following experiment was carried out. Leaf pieces were cultured for 1-4 days on MS basal medium containing no hormones (NHM), on medium containing 0.5 mg l^{-1} 2,4-D (CIM) and on medium containing 1.25 mg l^{-1} BAP (SIM). *In vivo* labelling was carried out with cultures grown on these first-stage media and the labelled proteins were analysed using SDS-PAGE to determine whether the different media induced different patterns of protein

synthesis. Plate 15 represents the autoradiograph of the gel. The observations are given below:

Lane	Sample	Change in proteins
1	mol. wt. markers	-
2	day 0	-
3	1d NHM	same as lane 2
4	1d CIM	same as lane 2
5	1d SIM	same as lane 2
6	2d NHM	band A faint; B prominent; bands D, E, F not seen as discrete bands; band G faintly visible
7	2d CIM	A, B not visible; C faded; D, E, F, G same as in 6
8	2d SIM	C prominent; A, B not visible; D, E ,F same as in 6; G not apparent
9	3d NHM	B prominent; A not visible; D, E ,F ,G same as in 6
10	3d CIM	A, B not visible; D, E, F same as in 6; G visible
11	3d SIM	A, B, D, E, F same as in 10; G not apparent
12	4d NHM	poor labelling
13	4d CIM	C very faint; D, E ,F same as in 6; G visible
14	4d SIM	C very prominent; D, E ,F same as in 6; G not apparent

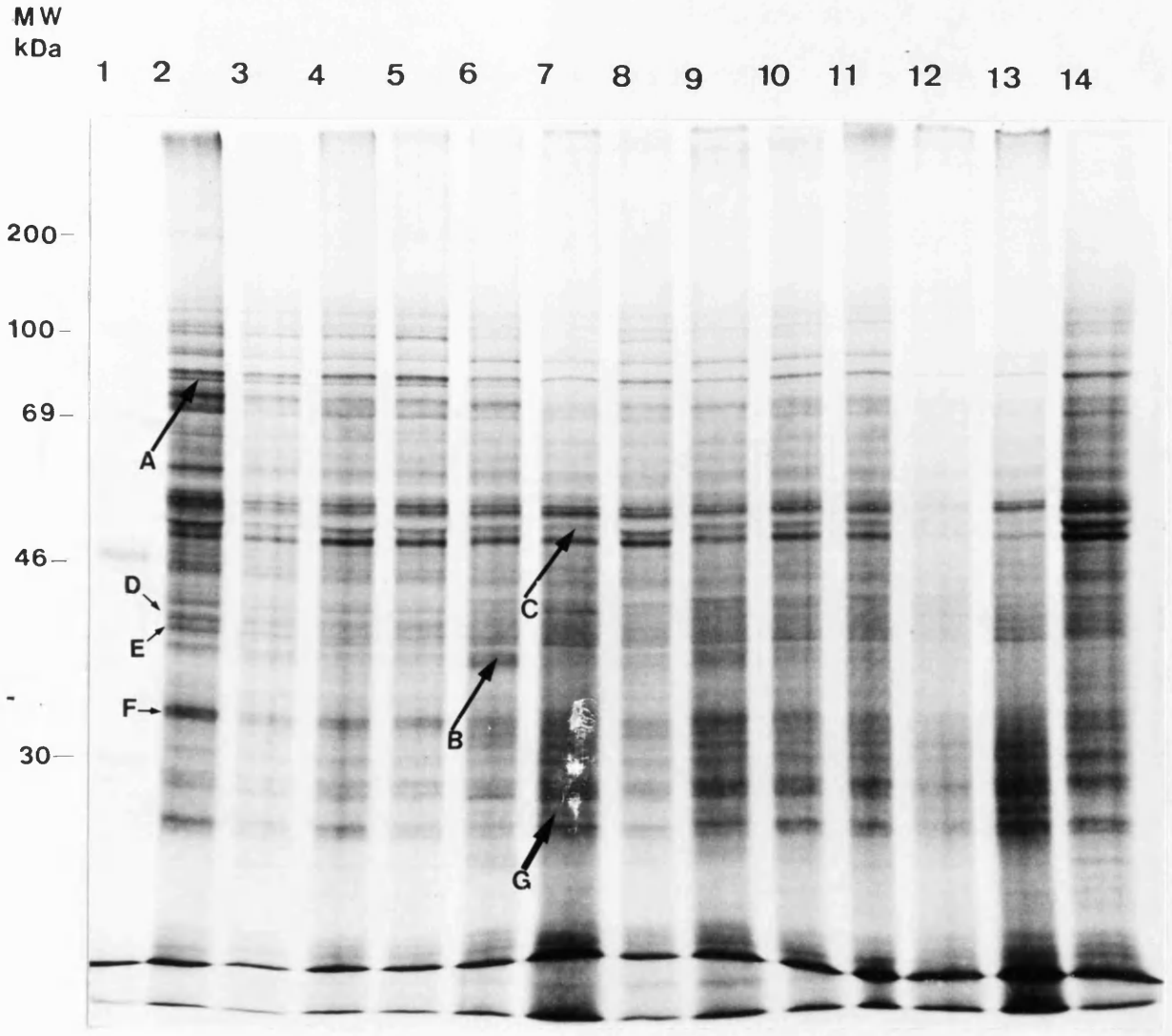
Comparison of banding patterns of cultures grown on NHM, CIM and SIM for 4 days showed the following differences. Band A (Mr, approximately 77.6 kDa) which was very prominent in day 0 samples (before culturing) faded with time under all three *in vitro* conditions. Bands D, E and F (Mr 39.8, 38 and 32.4 kDa) which were seen as discrete bands in day 0 samples remained the same in day 1 NHM, CIM and SIM cultures, but were replaced by several bands of close molecular weights in day 2, 3 and 4 NHM, CIM and SIM cultures.

Plate 15: Analysis of proteins in first-stage cultures.

Explants grown for 1-4 days on NHM, CIM and SIM were pulse-labelled by spreading [^{35}S] methionine 15 μCi/100 μl) on the explant for 1 h. The extracted proteins were subjected to SDS-PAGE followed by fluorography.

lane	sample
1	[^{14}C] molecular weight markers
2	day 0 (before culturing)
3	1d NHM
4	1d CIM
5	1d SIM
6	2d NHM
7	2d CIM
8	2d SIM
9	3d NHM
10	3d CIM
11	3d SIM
12	4d NHM
13	4d CIM
14	4d SIM

Bands A, D, E and F (Mr=approximately 77.6, 39.8, 38 and 32.3 kDa respectively) apparent in day 0 cultures are not visible in 2d CIM, 2d SIM and 3d SIM cultures. Band B (Mr=approximately 34.7 kDa) is prominent in NHM cultures. Band C (Mr=approximately 52.5 kDa) is more conspicuous in SIM cultures. Band G (Mr=approximately 27.5 kDa) is visible only in NHM and CIM cultures and is not visible in SIM cultures.


MW
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
14
200
100
69
46
30
A
B
C
D
E
F
G

Band B (Mr, approximately 34.7 kDa) was most prominent in day 2 and 3 NHM samples. Band C (Mr, approximately 52.5 kDa) that was present in day 0 cultures tended to decrease initially in all cultures and recovered in day 2 NHM and SIM samples but not in CIM cultures. Band G (Mr, approximately 27.5 kDa) which was not visible at day 0 appeared as a faint band in NHM cultures and more intensely in CIM cultures. This band was not apparent in SIM cultures.

4.3.2 Protein synthesis in second-stage cultures

The leaf squares at the end of the first-stage culture period on NHM, CIM and SIM were transferred onto CIM and SIM. *In vivo* labelling was carried out after one and two days on these media in order to determine whether there was any change in protein profile due to the transfer onto fresh media.

The second-stage cultures are denoted by the number of days on a particular medium with specification of the first-stage medium within parentheses. For example, 1d CIM (4d CIM) represents the cultures that have been on second-stage CIM for 1 day following transfer from 4 days on CIM (first-stage medium).

Plate 16 shows the proteins analysed by SDS-PAGE. The observations are tabulated below. Unfortunately, the loading on this gel was much more uneven than on the previous one. Therefore, the making of categorical statements about the presence or absence of some bands, especially high molecular weight bands is fraught with difficulty.

Lane	Sample	Changes in protein
1	day 0	-
2	4d NHM	-
3	1d CIM (4d NHM)	band G visible
4	1d SIM (4d NHM)	band G not apparent
5	2d CIM (4d NHM)	band G visible
6	2d SIM (4d NHM)	band C prominent; band G not apparent
7	4d CIM	bands C and H faint
8	1d CIM (4d CIM)	bands C and H faint; band G visible
9	1d SIM (4d CIM)	band H prominent
10	2d CIM (4d CIM)	band C faint; band H faint
11	2d SIM (4d CIM)	bands C and H are clear; band G not apparent
12	4d SIM	bands C, H are very prominent; G not apparent; band I visible
13	1d CIM (4d SIM)	same as lane 12
14	1d SIM (4d SIM)	same as lane 12
15	2d CIM (4d SIM)	same as lane 12
16	2d SIM (4d SIM)	banding pattern not clear.

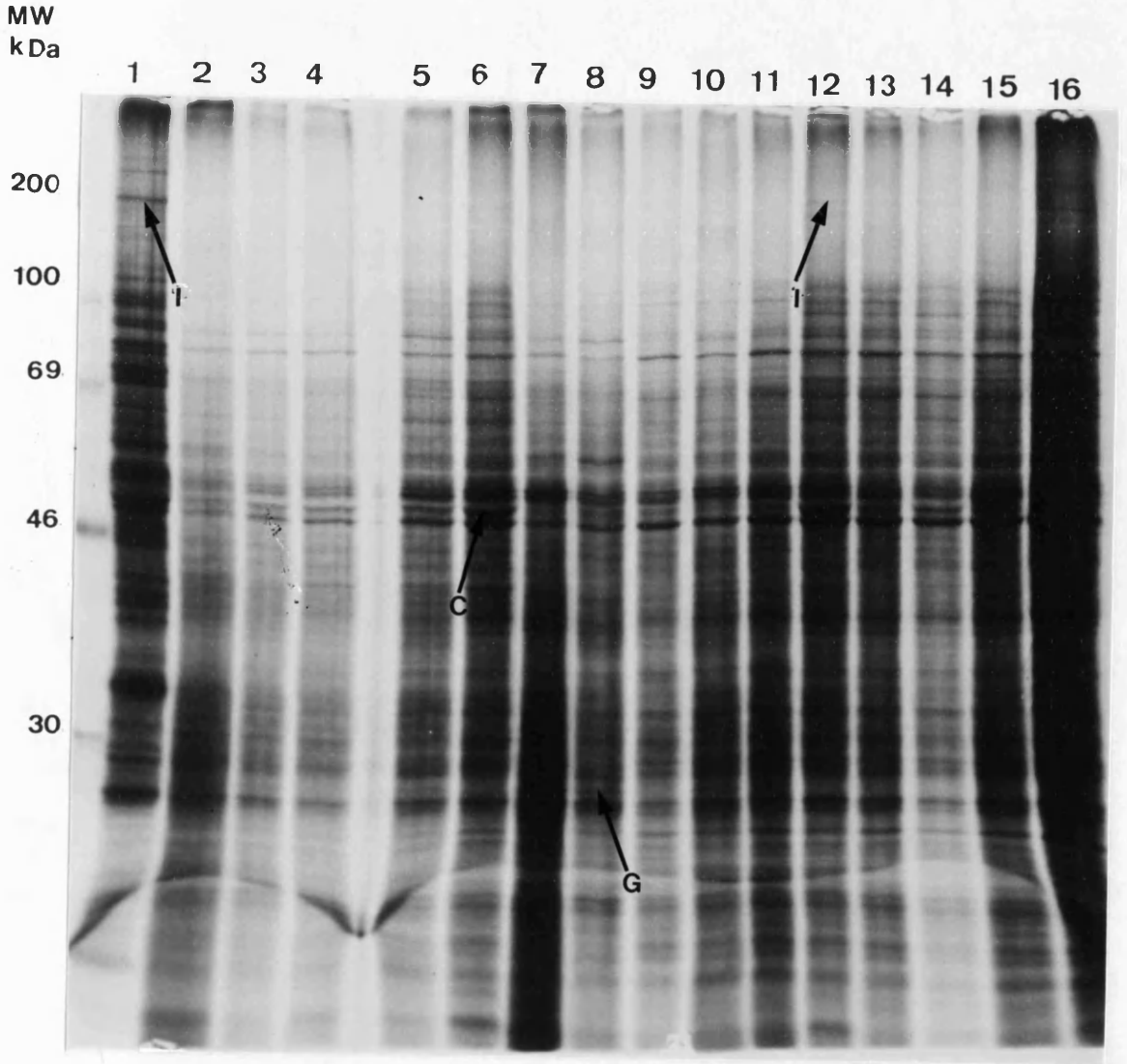
The results listed above showed that the protein bands C and H (Mr, approximately 52.5, and 69 kDa respectively) were more prominent in all SIM cultures. Band C which reduced in intensity during the first-stage on CIM, was maintained at a low level when transferred to fresh CIM, but synthesis was increased on transfer to SIM. Increase in intensity of band H was observed in cultures grown for 4 days on SIM, and in cultures that were transferred to SIM from CIM. Band G (Mr, 27.5 kDa) was visible in CIM

Plate 16: Analysis of proteins in second-stage cultures.

Leaf explants were transferred from first-stage NHM, CIM and SIM to second-stage CIM and SIM. Pulse labelling was carried out with day 0 samples (before culturing), 4d NHM, 4d CIM, 4d SIM and 1-2 day second-stage CIM and SIM cultures by spreading [^{35}S] methionine (15 μCi/100 μl) and leaving for 1 h. Proteins were extracted and subjected to SDS-PAGE followed by fluorography.

lane	sample
1	day 0 (before culturing)
2	4d NHM
3	1d CIM (4d NHM)
4	1d SIM (4d NHM)
5	2d CIM (4d NHM)
6	2d SIM (4d NHM)
7	4d CIM
8	1d CIM (4d CIM)
9	1d SIM (4d CIM)
10	2d CIM (4d CIM)
11	2d SIM (4d CIM)
12	4d SIM
13	1d CIM (4d SIM)
14	1d SIM (4d SIM)
15	2d CIM (4d SIM)
16	2d SIM (4d SIM)

Band C (Mr=approximately 52.5 kDa) tended to disappear on CIM and is prominent in SIM cultures. Band G (Mr=approximately 27.5 kDa) is visible in CIM cultures but not in SIM cultures. Band I (Mr=approximately 147.2 kDa) which is present in day 0 samples is visible in 2 day second-stage SIM samples, which may be due to loading variation. Band H (Mr=approximately 69 kDa) is prominent in SIM cultures, one day after transfer from CIM. Band H is only faintly visible in second-stage CIM cultures.


MW
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
200
100
69
46
30
I
I
C
G

cultures and was not apparent in SIM cultures. Although band I (Mr, 147.2 kDa) was visible only in SIM cultures, it must be emphasised that this band may not be visible in other lanes due to loading variations. The pattern of the protein profile remained the same when cultures after 4 days on first stage SIM were transferred to fresh SIM or to CIM (compare lane 12 with 13 and 14).

4.3.3 Protein synthesis in second-stage SIM and CIM cultures

In order to investigate the changes in protein synthesis in cultures after transfer to CIM and SIM, *in vivo* labelling of proteins was carried out with *in vitro* cultures, after 0.25-4 h, 1-8 days and 12-24 days on the two media.

4.3.3.1 During the first four hours after transfer

Leaf squares cultured on CIM were transferred to CIM and SIM and *in vivo* labelling was carried out after 0.25, 0.50, 1, 2 and 4 h. As apparent from Plate 17 there was no difference in the protein profiles obtained after an incubation period of 1 h with labelled methionine.

4.3.3.2 During 1-8 days

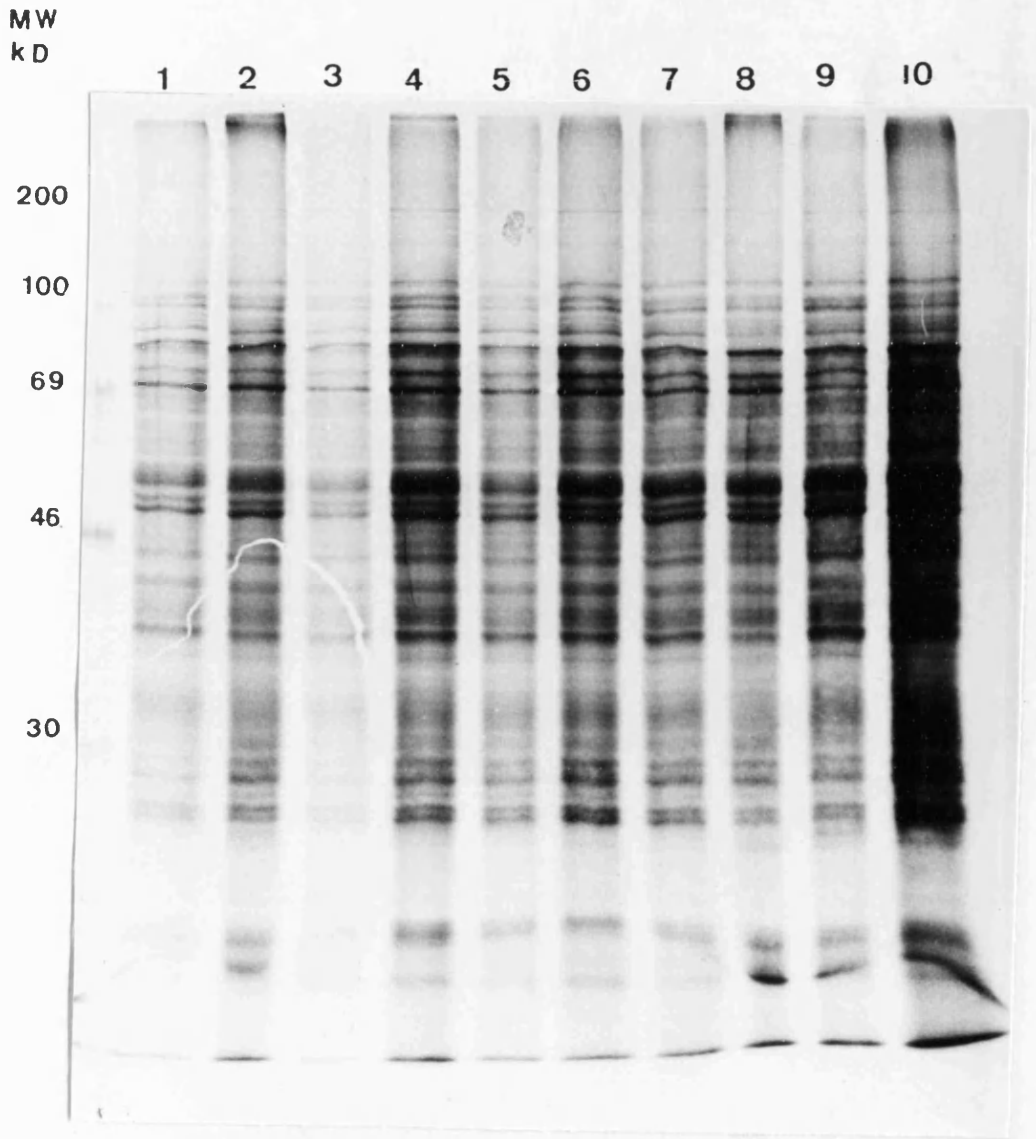
The experiment was continued with cultures from 1-8 days on second-stage CIM and SIM considering the 4d CIM (end of first-stage) samples as the control. Plate 18 represents the autoradiograph of the SDS-PAGE carried out with day 1-5 CIM and SIM samples, the observations of which are given below. The radioactive incorporation was found to be higher in tissues transferred to SIM as compared to second-stage CIM. For example, 1d SIM (4d CIM) samples showed 7326000 cpm whereas 1d CIM (4d CIM) samples showed 2150500 cpm g^{-1} of tissue. It must be pointed out that on this plate (Plate 18) some bands, especially higher molecular weight ones, may not be visible due to variations in loading.

Plate 17: Analysis of proteins synthesised during 0.25 to 4 h period on second-stage CIM and SIM cultures.

Leaf explants were transferred from first stage CIM to second stage CIM and SIM and pulse labelling was carried out after 0.25, 0.5, 1 and 4 h period, using [^{35}S] methionine (15 μCi/100 μl, 1 h). Proteins were extracted and analysed by SDS-PAGE followed by fluorography.

lane	samples
1	0.25 h CIM (4d CIM)
2	0.25 h SIM (4d CIM)
3	0.5 h CIM (4d CIM)
4	0.5 h SIM (4d CIM)
5	1 h CIM (4d CIM)
6	1 h SIM (4d CIM)
7	2 h CIM (4d CIM)
8	2 h SIM (4d CIM)
9	4 h CIM (4d CIM)
10	4 h SIM (4d CIM)

The banding pattern is similar in all the tracks.


MW
kD
1
2
3
4
5
6
7
8
9
10
200
100
69
46
30

Lane	Sample	Change in proteins
1	4d CIM	-
2	1d SIM (4d CIM)	band G not visible; H and C faintly visible
3	1d CIM (4d CIM)	band G visible; H visible; C faintly visible
4	2d SIM (4d CIM)	G not visible;H and C visible
5	2d CIM (4d CIM)	G visible; H and C faint
6	3d SIM (4d CIM)	same as lane 4
7	3d CIM (4d CIM)	same as lane 5
8	4d SIM (4d CIM)	same as lane 4
9	4d CIM (4d CIM)	same as lane 5
10	5d SIM (4d CIM)	same as lane 4
11	5d CIM (4d CIM)	banding pattern is not clear

The results listed above (Plate 18) indicated that bands C (Mr, approximately 52.5 kDa) and H (Mr, approximately 69 kDa) were more prominent in 1-5d SIM (4d CIM) cultures than in 1-5d CIM (4d CIM) cultures. Band G (Mr approximately 27.5 kDa) was only visible in CIM cultures. Plate 19 represents the gel run with *in vivo* labelled proteins from the rest of the *in vitro* cultures (i.e 6-8 day second-stage CIM and SIM cultures). There was higher intensity of band C in SIM cultures as compared to CIM cultures. Band G which was present in 1-5d CIM (4d CIM) cultures was not visible in 6d SIM (4d CIM) cultures. It is difficult to determine whether this band is present or absent in other samples because of the intense background in that region. Band H which was prominent in 6d SIM (4d CIM) and 7d SIM (4d CIM) cultures was less prominent in 8d SIM (4d CIM) cultures and also in CIM cultures.

Plate 18: Analysis of proteins synthesised during 1-5 days on second-stage CIM and SIM.

Pulse labelling was carried out with cultures grown for 1-5 days on second-stage CIM and SIM using [^{35}S] methionine (15 μCi/100 μl, 1h). Proteins were extracted and analysed by SDS-PAGE and fluorography.

lane	sample
1	4d CIM
2	1d SIM (4d CIM)
3	1d CIM (4d CIM)
4	2d SIM (4d CIM)
5	2d CIM (4d CIM)
6	3d SIM (4d CIM)
7	3d CIM (4d CIM)
8	4d SIM (4d CIM)
9	4d CIM (4d CIM)
10	5d SIM (4d CIM)
11	5d CIM (4d CIM)

Bands C (Mr=approximately 52.5 kDa) and H (Mr=approximately 69 kDa) are more prominent in SIM cultures than in CIM cultures. Band G (Mr=approximately 27.5 kDa) is visible in CIM cultures.

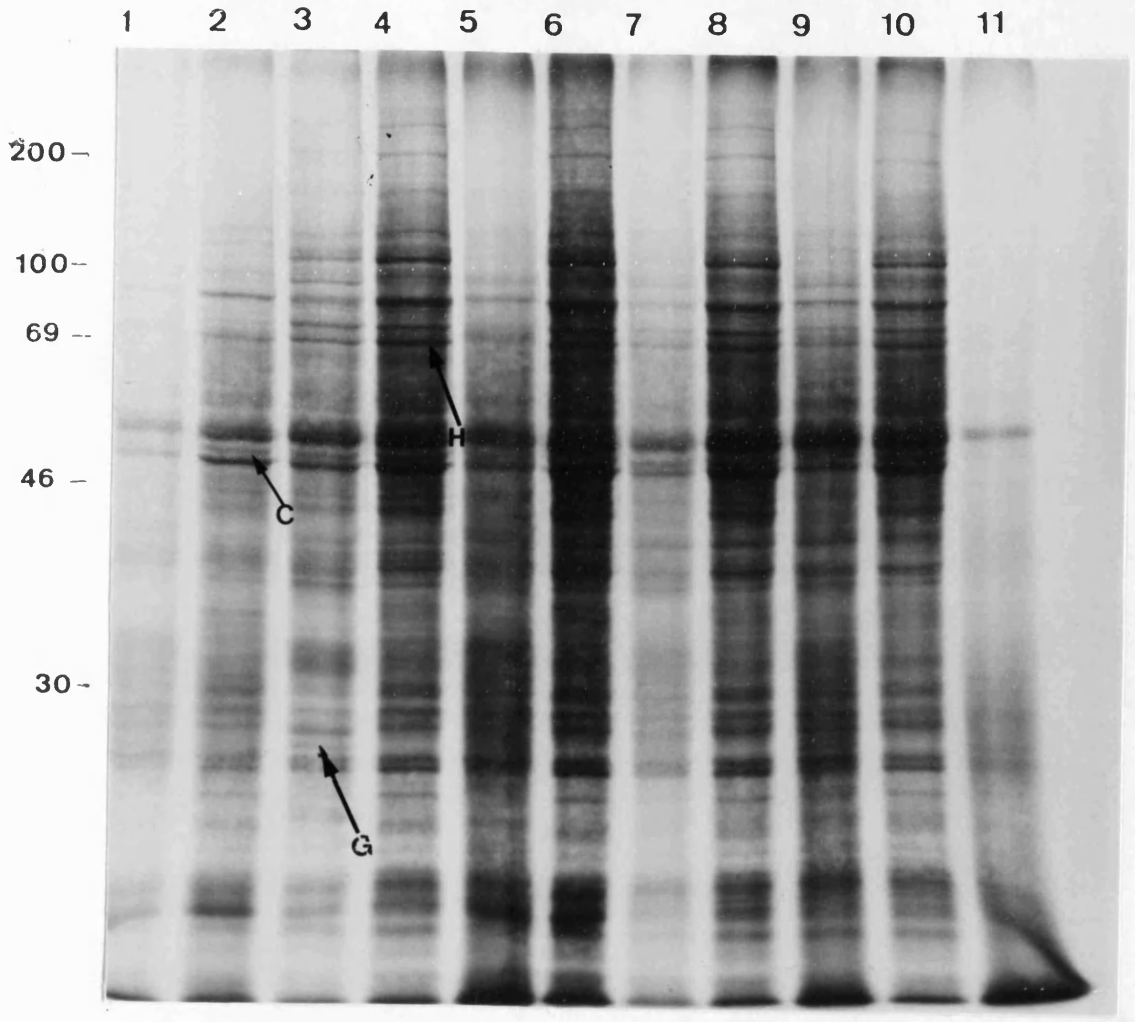
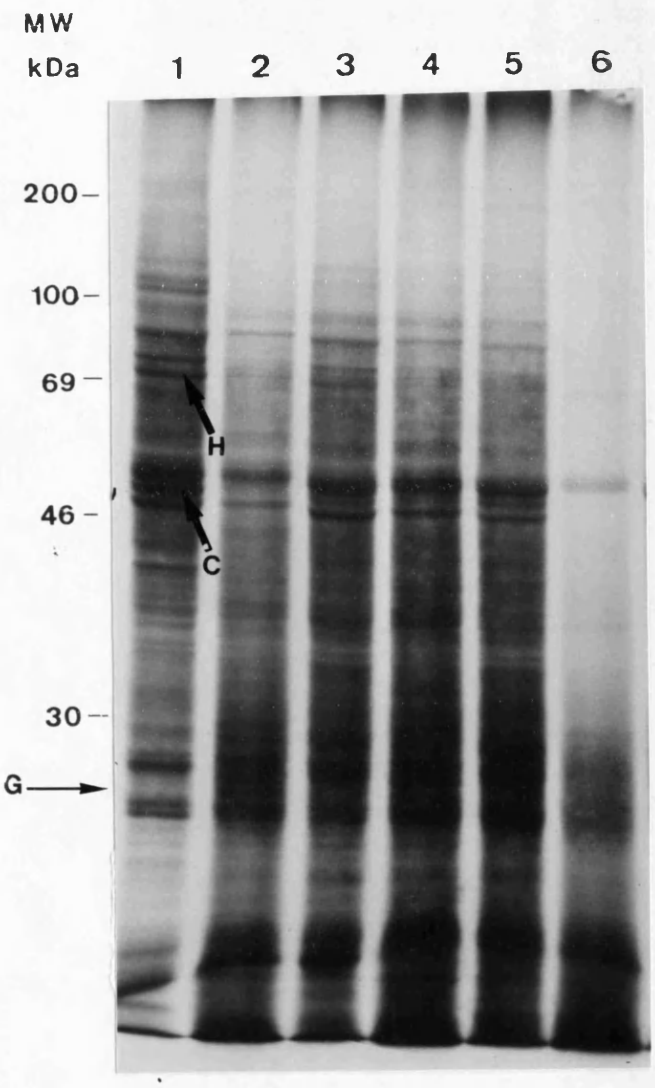

1
2
3
4
5
6
7
8
9
10
11
200
100
69
46
30
H
C
G

Plate 19: Analysis of proteins synthesised during the 6-8 day period on second-stage CIM and SIM.

Pulse labelling was carried out with cultures grown for 6-8 days on second-stage CIM and SIM cultures using [^{35}S] methionine (15 μCi/100 μl, 1h). Proteins were extracted and analysed by SDS-PAGE and fluorography.

lane	sample
1	6d SIM (4d CIM)
2	6d CIM (4d CIM)
3	7d SIM (4d CIM)
4	7d CIM (4d CIM)
5	8d SIM (4d CIM)
6	8d CIM (4d CIM)

Bands C (Mr=approximately 52.5 kDa) and H (Mr=approximately 69 kDa) are prominent in SIM cultures and are faint in CIM cultures. Band G is not apparent in 6d SIM (4d CIM) cultures; presence or absence of band G in lanes 2-6 is not obvious because of the background in that region.


MW
kDa
1
2
3
4
5
6
200
100
69
46
30
H
C
G

4.3.3.3 During 12-24 days

In order to compare the protein profiles in CIM cultures with callus and SIM cultures with shoots, *in vivo* labelling of 12-24 day second-stage CIM and SIM cultures was carried out. Plate 20 shows the proteins in CIM cultures and Plate 21 shows that of SIM cultures, analysed by SDS-PAGE. Band C (Mr, approximately 52.5 kDa) was not visible in all CIM cultures but was present in some of the SIM cultures with shoots (12-24 days after transfer onto CIM or SIM). When 1 μl of protein extract obtained from equal amounts of explants labelled with equal amounts of [^{35}S] methionine was tested for radioactive incorporation, the radioactive counts were much higher in SIM samples than in CIM samples. A much clearer banding pattern of high molecular proteins was obtained with SIM samples as compared with CIM samples (compare Plates 20 and 21). Band G which was visible in 2-4 days first-stage and in 1-5 days second-stage CIM cultures was not visible in both 12-24 days SIM and 12-24 days CIM cultures.

4.4 DISCUSSION

When interpreting the autoradiographs shown in the results section, it is important to bear a number of points in mind. Although equal number of counts were loaded onto each track of the gel for SDS-PAGE, the intensities of protein profiles obtained were not uniform. The samples were stored at -80 °C or under liquid nitrogen until the radioactivity counts for the samples were obtained. Some samples were found to change to a dark brown colour during storage and thawing, which could have been due to degradation of proteins. Since most of the samples, when thawed, showed a slight curdy appearance, they were centrifuged briefly in a microfuge, and aliquots were removed from the supernatant for electrophoresis. This may have contributed to the error of considering same number of counts in the supernatant before and after freezing. Hence attempts were made to compare the lanes with similar intensities.

Plate 20: Analysis of proteins from 12-24 days old second-stage CIM cultures.

12-24 days old second-stage CIM cultures were labelled with [^{35}S] methionine (15 μCi/100 μl, 1 h) and subjected to SDS-PAGE followed by fluorography.

lane	sample
1	12d CIM (4d CIM)
2	13d CIM (4d CIM)
3	14d CIM (4d CIM)
4	15d CIM (4d CIM)
5	16d CIM (4d CIM)
6	17d CIM (4d CIM)
7	18d CIM (4d CIM)
8	19d CIM (4d CIM)
9	20d CIM (4d CIM)
10	21d CIM (4d CIM)
11	22d CIM (4d CIM)
12	23d CIM (4d CIM)
13	24d CIM (4d CIM)

Band C (Mr=approximately 52.5 kDa) and band G (Mr=approximately 27.5 kDa) are not visible. Positions of bands C and G are marked by arrows.

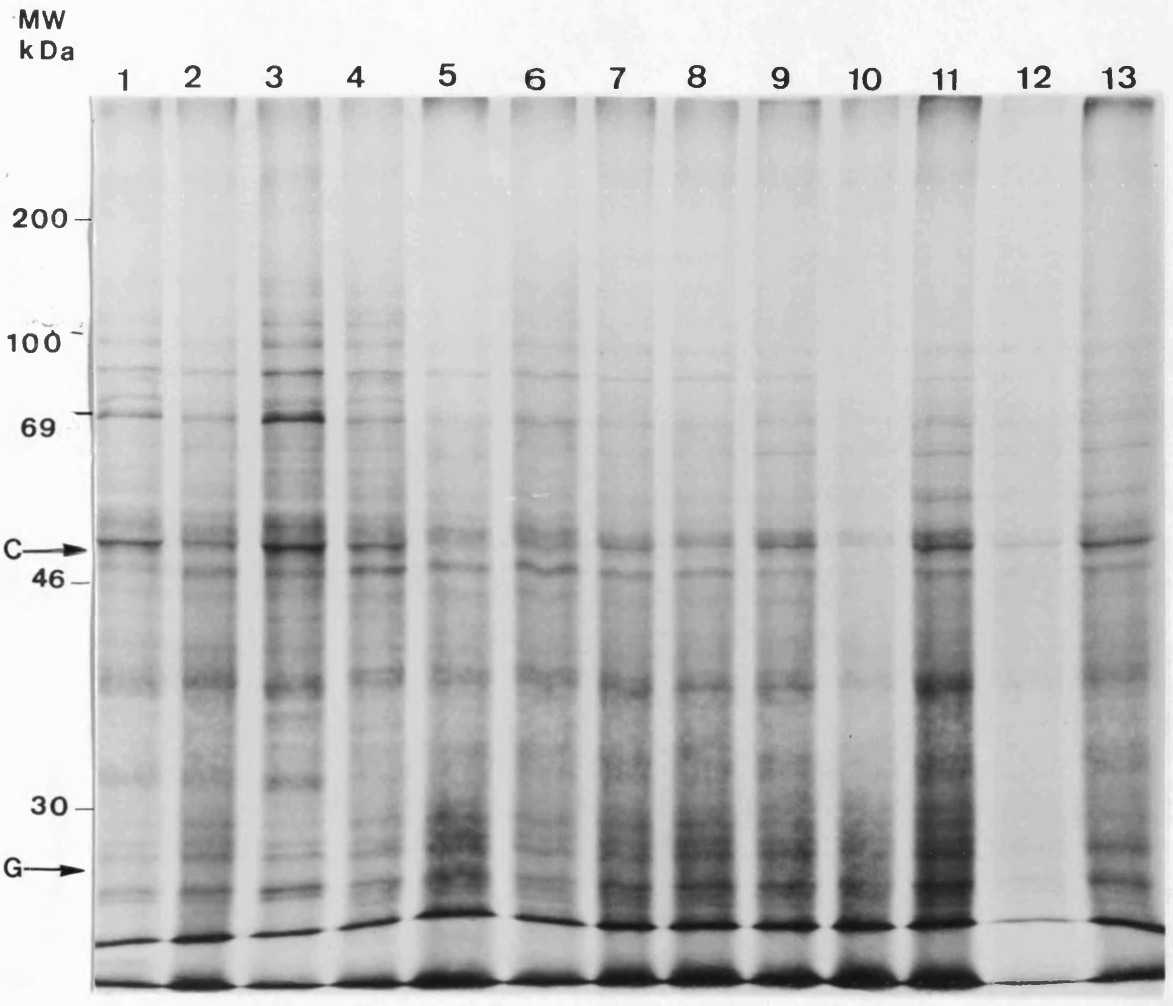
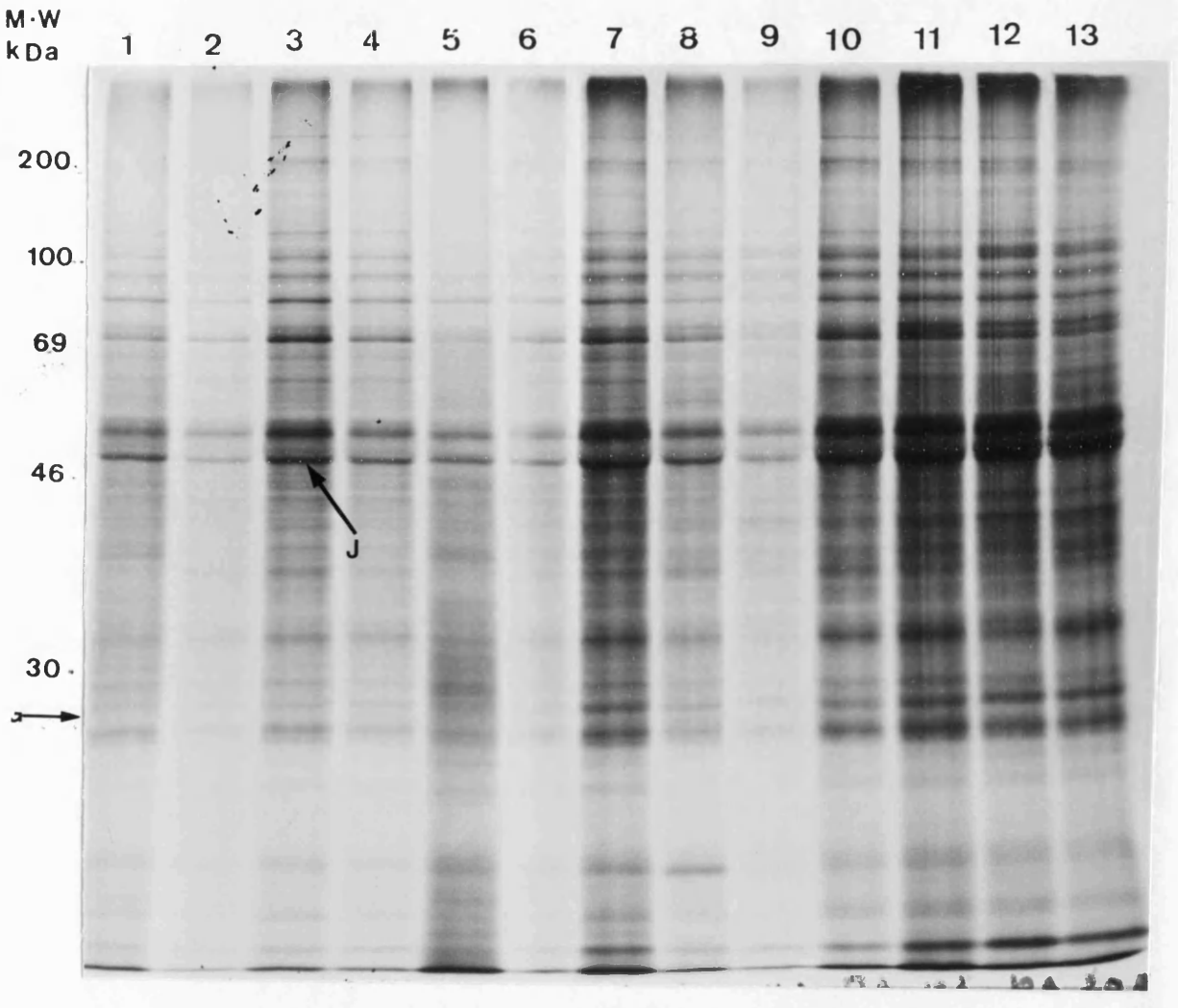

MW
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
200
100
69
C
46
30
G

Plate 21: Analysis of proteins from 12-24 days old second-stage SIM cultures.

10-24 days old second-stage SIM cultures were labelled with [^{35}S] methionine (15 μCi/100 μl, 1 h) and subjected to SDS-PAGE followed by fluorography.

lane	sample
1	12d SIM (4d CIM)
2	13d SIM (4d CIM)
3	14d SIM (4d CIM)
4	15d SIM (4d CIM)
5	16d SIM (4d CIM)
6	17d SIM (4d CIM)
7	18d SIM (4d CIM)
8	19d SIM (4d CIM)
9	20d SIM (4d CIM)
10	21d SIM (4d CIM)
11	22d SIM (4d CIM)
12	23d SIM (4d CIM)
13	24d SIM (4d CIM)

Band C (Mr=approximately 52.5 kDa) is visible only in some samples. In other samples this band is not visible; this may be due to comigration with band J. High molecular weight bands (Mr > 100 kDa) are prominent. Band G (Mr=approximately 27.5 kDa) is not visible. Position of band G is marked by an arrow.


M·W
kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
200
100
69
46
30
J

Absence of a band on an autoradiograph may not necessarily be due lack of that particular protein but because there would have been insufficient radioactivity to expose the film. The sensitivity of the film could have been improved by preflashing it. In tracks with similar overall levels of radioactivity, though, it is valid to compare relative band intensities visually. The absolute levels of radioactivity cannot be quantified using a densitometer, if the film has not been preflashed.

Generally, SIM cultures gave protein profiles with sharper bands than those given by CIM cultures. Also, the radioactive counts per gram of tissue of CIM samples were always lower than those of SIM samples. Several authors have reported an increase in total protein content prior to bud initiation (Hasegawa *et al.* 1979; Thorpe, 1980; Reynolds, 1990; Renaudin *et al.* 1991). This suggests that there is more active protein synthesis in vigorously growing shoot cultures than in callus cultures. Fosket and Tepfer (1978) found that when soybean cells were treated with [^{3}H] uridine, the specific activity of polysomal poly $(A)^{+}$ RNA increased by 79% and that of non polyribosomal poly $(A)^{+}$ RNA was decreased by 40%. They interpreted these results as indicating that mRNA species synthesised in the absence of cytokinin are moved into polysomes when the cells were treated with cytokinins, and that cytokinins somehow render untranslatable RNA translatable by an unknown mechanism. Therefore the higher incorporation of [^{35}S] methionine by SIM cultures may be due to enhancement of translation by BAP. However, the possibility cannot be ruled out that because of its consistency, the callus tissue (cultured on CIM) was less permeable to the radioactive methionine than was tissue cultured on SIM.

Culturing leaf squares on any of the first-stage media NHM, CIM or SIM led to qualitative differences in protein profiles, for example, the disappearance of several polypeptides that were present in day 0 samples (leaf squares just after cutting). The day 0 sample represents the proteins synthesised in the cut leaf square within 1 h after cutting. Some of these polypeptides may be wound

induced. These wound response proteins may have disappeared after one to two days in culture. Zurfluh and Guilfoyle (1980, 1981) reported the appearance of wound induced proteins which masked the auxin induced proteins during 1-2 h after application. Disappearance of some of the polypeptides and appearance of others under *in vitro* conditions may be due to the process of dedifferentiation, where the cells stop carrying out specialised functions and revert back to the lowest level of organisation. The protein profile after two days on all three media more or less remained the same except for minor quantitative differences. Christianson and Warnick (1988) reported that in *Convolvulus* cultures dedifferentiation was completed after two days, as the protein profile after 2 days in culture remained the same throughout the culture period. It is possible that band G, which was visible in all CIM cultures, is auxin induced. However, band G was also faintly visible in NHM cultures. Band G was not visible in SIM cultures under the conditions used here. Renaudin *et al*, (1991) reported the presence of a protein of Mr 27.9 kDa in callus cultures (used as the control) which was not found in shoot forming cultures of *Petunia hybrida* This may suggest that this protein is specific for callus formation. The similarity in size of this protein with the Mr 27.5 kDa one (band G) reported in this chapter, and the similarities in their expression patterns, suggest that they may be the same protein.

Band B, which became prominent in days 2 and 3 on the medium with no hormones (NHM) only may be due to stress or some other factor influential under *in vitro* conditions in media without any hormones. Band C was present in all three culture regimes; its gradual fading in CIM cultures may indicate that 2,4-D inhibits synthesis of this particular protein (proteins).

The experiments carried out using *in vivo* labelling of *in vitro* cultures that had been transferred from first-stage CIM showed that at least one protein increased in quantity in those cultures transferred to SIM for shoot production (Plate 16, band H). The increased production of this band was apparent 2 days

after transfer onto SIM and continued throughout the time course. One protein band, G, which was present in CIM and NHM cultures was absent in SIM cultutes which may indicate that there is also suppression of some proteins under SIM conditions. These observed differences may be related to the anatomical changes occurring under the influence of the two different hormonal regimes, 2,4-D in CIM and BAP in SIM. Differences in tissue types do not necessarily have to be based on qualitative differences: It has been speculated that there could be more quantitative changes in proteins than qualitative ones between root and shoot development because the two types differ in the proportions of various tissues rather than in the types of cells present (Fukuda and Komamaine, 1983). This indicates a great similarity between root and shoot organogenesis. Hence the changes observed in *in vivo* labelling experiments, although they appeared to be minor, could be related to changes in gene expression during the process of shoot production. However, the possibility that these changes are a general response to the two hormonal regimes, but not related to any of the anatomical differences observed in the two culture systems, cannot be excluded.

The molecular weights of these polypeptides differed from those reported by other authors (see section 1.5). Renaudin *et al.* (1991) compared the proteins of *Petunia* callus cultured on high concentration of BAP for bud formation with callus cultured on low concentration of BAP, and reported the appearance of a polypeptide of 22 kDa which increased sharply as soon as the meristems develop into buds. This polypeptide was different in its molecular weight and expression pattern from those detected by Reynolds (1990) and Stabel *et al.* (1990). Renaudin *et al.* (1991) mentioned that this could be expected as cytokinin-controlled stages in developmental pathway can differ according to the experimental material and not as a common response to cytokinin. Since cytokinin is known to promote cell division, differentiation and plastid development, it may accordingly activate several enzymes participating in a wide range of metabolic reactions depending on the stage of development.

5 ANALYSIS OF mRNA POPULATIONS UNDER DIFFERENT HORMONE REGIMES AND DURING SHOOT ORGANOGENESIS

5.1 INTRODUCTION

If proteins have a major role in initiating the stages described in organogenesis (1.1.1) mediated by hormones, then hormonally induced changes prior to the onset of these phenomena should be detectable. It was aimed in this experiment to investigate whether there are changes in mRNA population during the process of organogenesis under different hormone regimes. These changes, qualitative or quantitative, may start at a particular time point during the time course. It is important to identify this time point to know when to isolate mRNA for construction of a cDNA library, in order to ascertain changes in gene expression during shoot organogenesis. In addition, before proceeding to cDNA cloning, the purity and quality of the poly $(A)^+$ RNA has to be confirmed.

The technique of *in vitro* translation allows changes in mRNA populations to be followed quantitatively and qualitatively. Differential gene expression in terms of changing populations of poly $(A)^+$ RNA over a time course can be investigated by carrying out *in vitro* translation and examining the corresponding polypeptides on SDS-PAGE. *In vitro* translation also provides a method to confirm the quality of poly $(A)^+$ RNA.

There are many examples, where *in vitro* translations have enabled the detection of differences in gene expression. Some of these include changes in gene expression induced by growth regulators (Theologis and Ray, 1982a,b; Zurfluh and Guilfoyle, 1982a,b,c; Dommes and Northcote, 1985), during leaf development (Ougham, 1987), during floral development (Gasser *et al.*, 1988), during fruit development (Lincoln *et al*, 1987) and during somatic embryogenesis (Sung and Okimoto, 1983).

5.2 MATERIALS AND METHODS

5.2.1 *In vitro* translation of poly $(A)^+$ RNA

There are two systems commonly used for *in vitro* translation of mRNA: those based on rabbit reticulocyte lysates (Pelham and Jackson, 1976) and the wheat germ system (Marcu and Dudock, 1974). In both systems only minute amounts of proteins are synthesised and hence a radioactive amino acid is routinely included to enable the detection of the translation products. The proteins produced are then analysed by SDS-PAGE and/or 2D-PAGE followed by fluorography as described in section 4.2.4.

The rabbit reticulocyte lysate systems N. 150 and N. 90 (Amersham plc.) and the wheat germ system were used to carry out translation of tobacco poly $(A)^+$ RNA. The precautions outlined in section 3.2.1.1 were taken to avoid any ribonuclease contamination.

5.2.1.1 Rabbit reticulocyte lysate system N. 150

The rabbit reticulocyte lysate system (RRLS) N. 150 is amino acid depleted. It does not contain potassium and magnesium ions at their optimum concentrations. Therefore, amino acids, K^+ and Mg^{2+} have to be supplied exogenously. The rabbit reticulocyte lysate N.150, [^{35}S] methionine (15 μCi μl^{-1}), potassium acetate (2 M) and magnesium acetate (1 M) were purchased from Amersham plc. A reaction was set up on ice as follows.

	μl
lysate	35.0
potassium acetate (2 M)	2.5
amino acid mixture (1 mM)	2.5
mRNA (1 μg)	1.0
[^{35}S] methionine	2.0
water	7.0
	50.0

The mixture was incubated in a water bath (30 ^{o}C, 1h). Two 1 μl samples of translation mix were then withdrawn for measurement of radioactive incorporation (4.2.5). 10 μl of sample buffer A (Appendix A) was added to 5 μl

of translation mix, heated (95 °C, 4 min) and subjected to SDS-PAGE and fluorography (4.2.4).

5.2.1.2 Wheat germ system

Wheat germ extract (Amersham plc.) and the recipe for *in vitro* translations were kindly given by Dr Colin Lazarus at Bristol University. A reaction was set up by mixing the following ingredients.

	µl
wheat germ extract	10
1 M potassium acetate	1
1 mM amino acid mix	2
RNasin	1
tobacco mRNA (1 µg)	1
[^{35}S] methionine (15 µCi)	1
water	4
	20

The mixture was incubated (25 °C, 1 h). Then the reaction was stopped by adding SDS (1 µl of 20%) and boiling (2 min). An aliquot (1 µl) from the reaction mix was withdrawn in duplicate to measure the radioactive incorporation. Sample buffer A (5 µl, Appendix A) was added to the translation mix (1 µl), heated (95 °C, 4 min) and subjected to SDS-PAGE and fluorography.

5.2.1.3 Rabbit reticulocyte lysate system N. 90

Rabbit reticulocyte lysate N. 90 contains the cold amino acid pool with concentrated Mg^{2+} and K^{+}. The following reaction was set up on ice to carry out *in vitro* translations.

	µl
lysate (N. 90)	8
RNasin	1
mRNA (up to 1 µg)	1
[^{35}S] methionine (15 µCi)	1
	11

The mixture was incubated (30 °C, 1 h). The reaction was stopped by adding SDS (1 μl of 20%) and boiling (2 min). An aliqout (1 μl) in duplicate was used to determine the radioactive incorporation. Sample buffer A (5 μl, Appendix A) was added to the translation mix (1 μl), heated (95 °C, 4 min) and subjected to SDS-PAGE and fluorography.

5.2.1.4 Two dimensional SDS-PAGE

In two dimensional SDS-PAGE (2D-PAGE), isoelectrofocusing of proteins was carried out prior to one dimensional SDS-PAGE. The Protean II (Bio Rad) gel apparatus was used to carry out 2D-PAGE. The method described by O'Farrell (1974) and the protocol given in the Protean II manual were followed. The gel was silver stained (Sammons *et al.*, 1981), destained and dried (60 °C, 1 h) prior to fluorography.

5.3 Optimisation of translation systems

5.3.1. Rabbit reticulocyte lysate system N. 150

Tobacco poly $(A)^+$ RNA (0, 50, 100, 200, 400 and 1000 ng) was translated *in vitro* using RRLS-N. 150. Castor bean poly $(A)^+$ RNA (1000 ng), which was found to be translatable (Martin *et al.*, 1984) was used as the control. The success of translations was determined by measuring radioactive incorporation and SDS-PAGE.

The radioactive counts of all the samples lay between 1100 and 1950 cpm indicating that little or no translation had occurred. The autoradiograph showed only the bands corresponding to the [^{14}C] methylated proteins used as the molecular weight marker. Even the castor bean mRNA which had translated in another system failed to translate. These results indicated that RRLS-N. 150 needed optimisation of K^+ and Mg^{2+}. In order to find the optimum K^+ concentration, translation of tobacco mRNA (1 μg) was carried out with 750, 1000, 1250, 1750 and 2000 mM K^+. The radioactive counts were found to lie between 211 and 1091 cpm, showing that the translation had not been

successful. SDS-PAGE of samples confirmed this. These results led to the suspicion that perhaps Mg^{2+} was not at its optimum. Therefore the optimisation of magnesium was carried out leaving K^+ constant. Translation of tobacco poly $(A)^+$ RNA (1 μg) was carried out with 0 (water), 2.5, 5.0, 7.5, 10.0 and 12.5 mM magnesium acetate. The radioactive counts in all samples were in the range of 409-1308 cpm, showing that *in vitro* translation had not occurred. The autoradiograph of the gel showed black smears with no visible separation into bands (Plate 22).

5.3.2 Wheat germ system

Tobacco poly $(A)^+$ RNA (300 ng), isolated by oligo (dT) chromatography and by the mAP method was used as the experimental material in carrying out *in vitro* translations. The success of the reaction was determined by measuring radioactivity and by SDS-PAGE. Table 3 shows the radioactive incorporation of the samples tested.

mRNA	cpm
0	41.5
mAP purified	22732.15
oligo (dT) purified	31950.68

Table 3 -Average radioactive incorporation of 1 μl of translation mix from the reactions primed by poly $(A)^+$ RNA isolated using mAP or oligo (dT). Sample 0 denotes the control reaction with no mRNA.

The high incorporation of $[^{35}S]$ methionine into proteins showed that the efficiency of translation was very high. The mRNA isolated using oligo (dT) gave a higher value for radioactive incorporation than mAP purified poly $(A)^+$ RNA, indicating better quality of poly $(A)^+$ RNA from the former system. Plate 23 represents the autoradiograph of the gel. It was seen that the

Plate 22: Optimisation of RRLS-N.150 .

1 μg of tobacco poly $(A)^+$ RNA was translated using RRLS-N.150 with varying concentrations of magnesium acetate. 5 μl from each sample was subjected to SDS-PAGE and fluorography.

Lanes 1-6: 0, 2.5, 5.0, 7.5, 10.0 and 12.5 mM magnesium acetate, lane 7: [^{14}C] molecular weight markers.

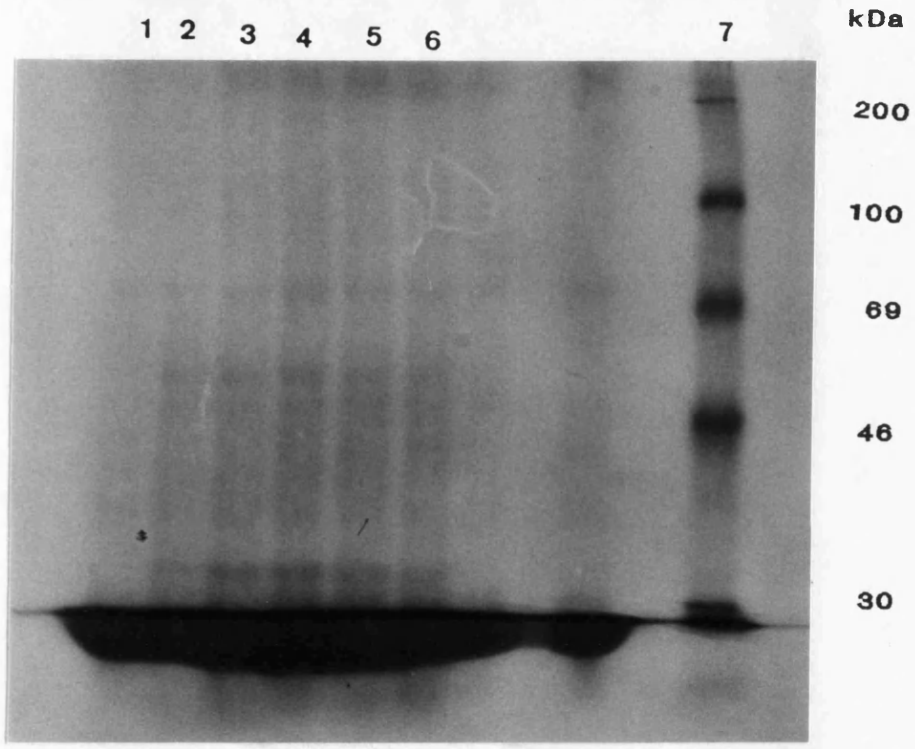
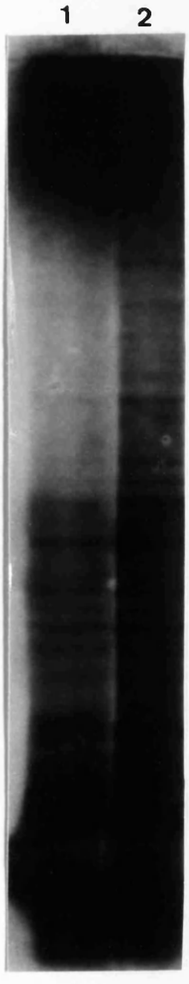

1 2 3 4 5 6
7
kDa
200
100
69
46
30

Plate 23: *In vitro* translation of tobacco mRNA by wheat germ system.

300 ng of tobacco poly $(A)^+$ RNA purified using the mAP or the oligo (dT) method was translated using wheat germ extract. 1 μl from each sample was analysed by SDS-PAGE.

Lane 1: translated product of mRNA purified by the mAP method; lane 2: translated product of mRNA purified by the oligo (dT) method.


1
2

intensities of bands in lane 2 were higher than that of lane 1, indicating that translation was more successful with poly $(A)^+$ RNA isolated by the oligo (dT) method.

5.3.3 Rabbit reticulocyte lysate system-N. 90

In vitro translations of tobacco mRNA isolated by the oligo (dT) method and the mAP method were carried out using RRLS-N.90. Two samples of poly $(A)^+$ RNA isolated by two different methods of oligo (dT) chromatography were compared; a. by Clemens (1986b) and b. by Jacobson (1987). 1 μg of TMV mRNA (Amersham plc.) was used as a control of the *in vitro* translation procedures. Plate 24 (on page 93) represents the autoradiograph of the gel. Table 4 shows the incorporation of $[^{35}S]$ methionine by various samples.

mRNA	cpm
0	39.8
oligo (dT) (a)	40729.47
mAP	29685.47
oligo (dT) (b)	37816.0

Table 4-Average radioactive incorporation in 1 μl samples withdrawn from the translated products of the control (water-0), poly $(A)^+$ RNA isolated by Clemens' method (a), by the mAP method and by Jacobson's method (b).

As evident by the radioactive incorporation and the fluorograph, tobacco mRNA isolated by oligo (dT) chromatography gave better results than mRNA isolated by the mAP method. Out of the two methods tested for purification of mRNA by oligo (dT) chromatography, the method developed by Clemens (1986b) gave sharper bands with higher molecular weight polypeptides. Hence, this method was selected for the routine purification of tobacco mRNA. The *in vitro* translation products of TMV mRNA used as the control can be seen in lane 1 of Plate 24, 1 μg of the above was used as opposed to 300 ng of the

experimental samples. The separation of the bands cannot be seen in lane 1 due to overloading the track with polypeptides of very high radioactive incorporation.

5.3.4 Comparison of wheat germ and RRLS-N. 90

Tobacco mRNA (300 ng) was translated separately using wheat germ and RRLS-N. 90 systems. Plates 25 and 24 represent the results of *in vitro* translations by wheat germ and RRLS-N. 90 systems respectively. There was better separation of protein bands by SDS-PAGE with no background signals of radioactivity in Plate 24 as compared to Plate 25 indicating that RRLS-N. 90 was better than the wheat germ system. Since RRLS-N. 90 gave the best results in translating tobacco poly $(A)^+$ RNA to proteins, it was decided to use this system in future experiments.

Occasionally, some samples of tobacco mRNA failed to translate into proteins *in vitro*. The quality of such samples were increased by reprecipitating mRNA with potassium acetate. However, the yield of mRNA was higher when precipitation was carried out with sodium acetate than with potassium acetate. Because there were only a few samples that failed to translate, precipitation was routinely carried out with sodium acetate.

5.4 RESULTS

5.4.1 *In vitro* translations of poly $(A)^+$ RNA isolated from tissue cultures

5.4.1.1 Poly $(A)^+$ RNA from SIM cultures

In order to investigate any differences in mRNA populations during organogenesis, *in vitro* translations were carried out with the poly $(A)^+$ RNA isolated from cultures after transfer to shoot induction medium (SIM). Since appearance of a shoot primordium was observed in cultures after 6 days on SIM (section 2.3.3, Plate 12), it was assumed that shoot induction is complete by then. Therefore, tobacco poly $(A)^+$ RNA (300 ng) isolated from each of the 1d SIM (4d CIM) to 6d SIM (4d CIM) cultures was translated *in vitro* using

Plate 24: *In vitro* translation of tobacco poly $(A)^+$ RNA using RRLS-N.90

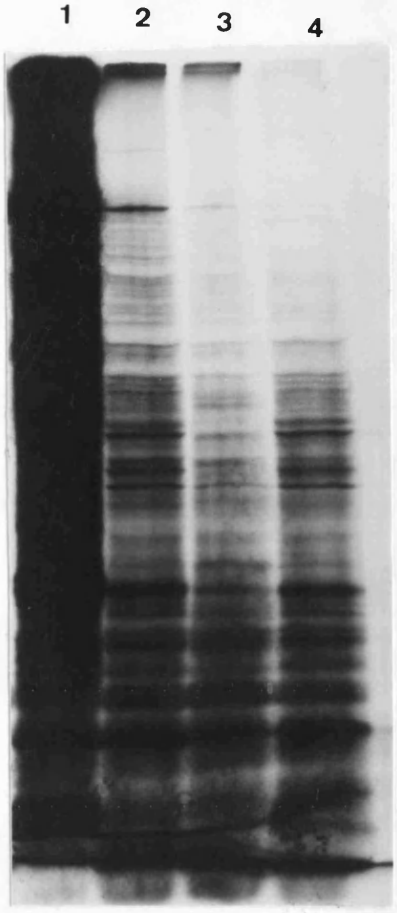
Poly $(A)^+$ RNA (300 ng) isolated by oligo (dT) chromatography and the mAP method along with TMV mRNA (1 μg) was translated *in vitro* and 1 μl from each sample was subjected to SDS-PAGE and fluorography.

Lane 1: TMV mRNA; lane 2: poly $(A)^+$ RNA purified by the oligo (dT) chromatography method according to Clemans; lane 3: poly $(A)^+$ RNA purified by the mAP method; lane 4: poly $(A)^+$ RNA purified by the oligo (dT) method according to Jacobson.

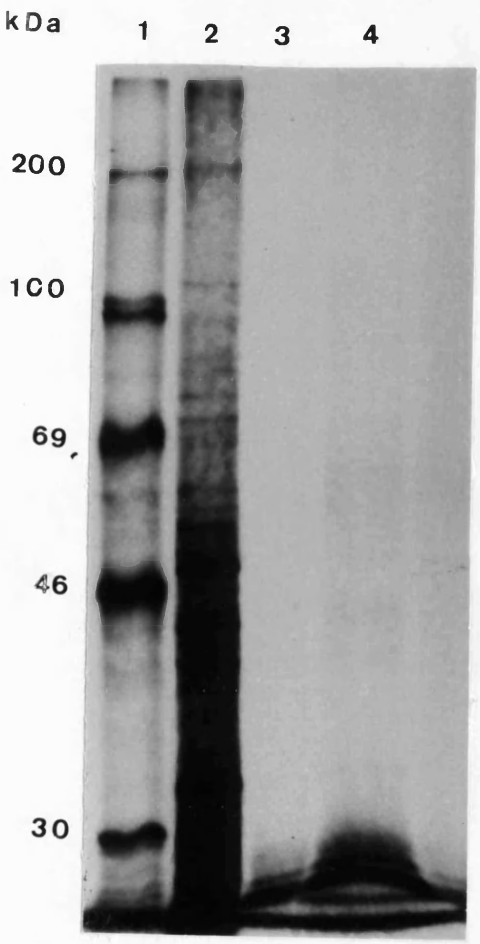
Plate 25: Comparison of translation products obtained using RRLS-N.150 and the wheat germ system (WGS)

300 ng of tobacco poly $(A)^+$ RNA purified by oligo (dT) chromatography was translated *in vitro* by RRLS-N.150 and WGS, and 1 μl from each sample was analysed by SDS-PAGE.

Lane 1: molecular weight markers; lane 2: tobacco poly $(A)^+$ RNA translated by WGS; lanes 3 and 4: poly $(A)^+$ RNA translated by RRLS-N.150. There were no bands in lanes 3 and 4 indicating the lack of translation by RRLS-N.150.



24



25

RRLS-N. 90. The same amount of poly $(A)^+$ RNA from 2d CIM (4d CIM) cultures (see section 4.3.3) and day 0 (before culturing) cultures was also translated *in vitro* along with the experimental samples. The translated products were fractionated by SDS-PAGE and subjected to fluorography.

Plate 26 shows the results of this experiment. Lane 2 represents the protein bands of the translated products of mRNA isolated from normal tobacco tissues before culturing. There were many bands unique to day 0 (a,b,c,d) which were not present in any of the *in vitro* cultures (lanes 2-11). The band X (Mr, approximately 39 kDa) only appeared on cultures that had been grown for 2 days on CIM (lane 3). The experiment was repeated with mRNA isolated from duplicate samples and the translated products appeared identical to Plate 26 when analysed by SDS-PAGE.

The translated products of mRNA isolated from 2d CIM (4d CIM) and 2d SIM (4d CIM) cultures were subjected to 2D-PAGE (see section 5.2.1.4). The gels showed most of the proteins confined to one corner, suggesting poor separation of proteins by the iso-electric focusing gel. The analysis of translation products by 2D-PAGE was repeated according to the protocol given in the Protean II manual. There were more bands than in previous attempts but there was no proper fractionation of proteins (Plate 27).

5.4.1.2 Poly $(A)^+$ RNA from shoot and callus cultures

In order to see whether there are any differences in mRNA populations between explants with prominent callus and explants with prominent shoots, the following experiment was conducted. Poly $(A)^+$ RNA (300 ng) isolated from 10d CIM (4d CIM)-13d CIM (4d CIM) and 10d SIM (4d CIM)-13d SIM (4d CIM) cultures was translated using RRLS-N. 90 and analysed by SDS-PAGE. Out of the 4 samples of CIM only the 12d CIM (4d CIM) sample was successfully translated into proteins (Plate 28) (see section 5.3.4). Comparison of this sample with 10d SIM (4d CIM)-13d SIM (4d CIM) samples showed that

Plate 26: *In vitro* translation of poly $(A)^+$ RNA isolated from *in vitro* cultures.

300 ng of tobacco poly $(A)^+$ RNA obtained from tobacco leaves before culturing (day 0), 1-8 days second-stage SIM cultures [1d SIM (4d CIM)-8d SIM (4d CIM)] and 2 day second-stage CIM cultures [2d CIM (4d CIM)] were translated *in vitro* using RRLS-N.90 and 1 μl from each sample was analysed by SDS-PAGE.

lane	sample
1	[^{14}C] molecular weight markers
2	day 0 (leaf tissue before culturing)
3	2d CIM (4d CIM)
4	1d SIM (4d CIM)
5	2d SIM (4d CIM)
6	3d SIM (4d CIM)
7	4d SIM (4d CIM)
8	5d SIM (4d CIM)
9	6d SIM (4d CIM)
10	7d SIM (4d CIM)
11	8d SIM (4d CIM)

The bands a,b,c and d seen in day 0 sample (lane 2) are not detectable in the rest of the lanes. The band X (Mr=approximately 39 kDa) is apparent in lane 3 corresponding to 2d CIM (4d CIM) cultures. lane 5 representing 2 day second-stage SIM cultures shows higher intensity of bands.

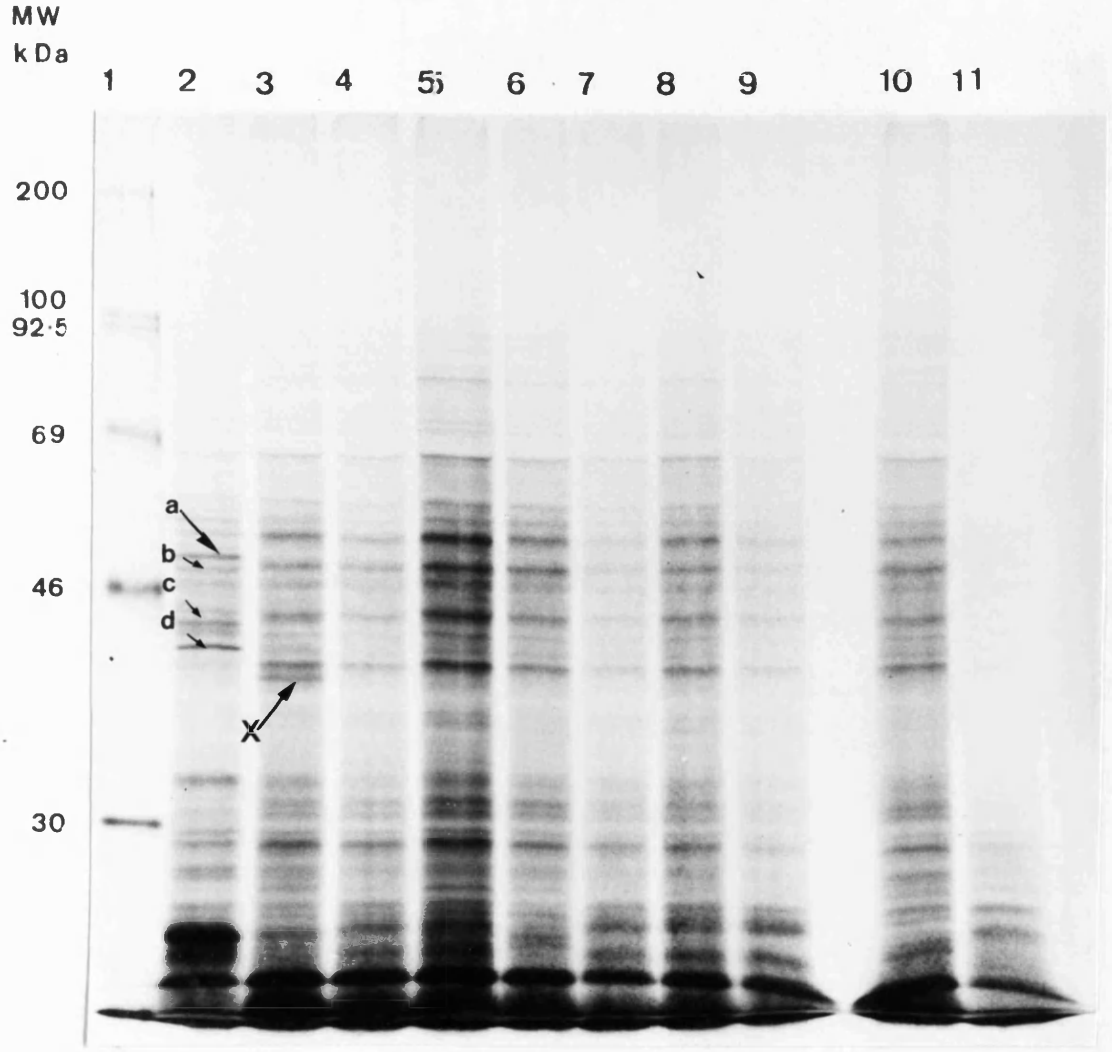

MW
kDa
1
2
3
4
5
6
7
8
9
10
11
200
100
92·5
69
46
30
a
b
c
d
X

Plate 27: Analysis of *in vitro* translated products by two dimensional gel electrophoresis (2D-PAGE).

300 ng of tobacco poly $(A)^+$ RNA obtained from 2d CIM (4d CIM) and 2d SIM (4d CIM) were translated *in vitro* using RRLS-N.90. 10 μl from each sample was subjected to 2D-PAGE followed by fluorography.

27A: 2d SIM (4d CIM)

27B: 2d CIM (4d CIM)

The polypeptides are not properly fractionated.

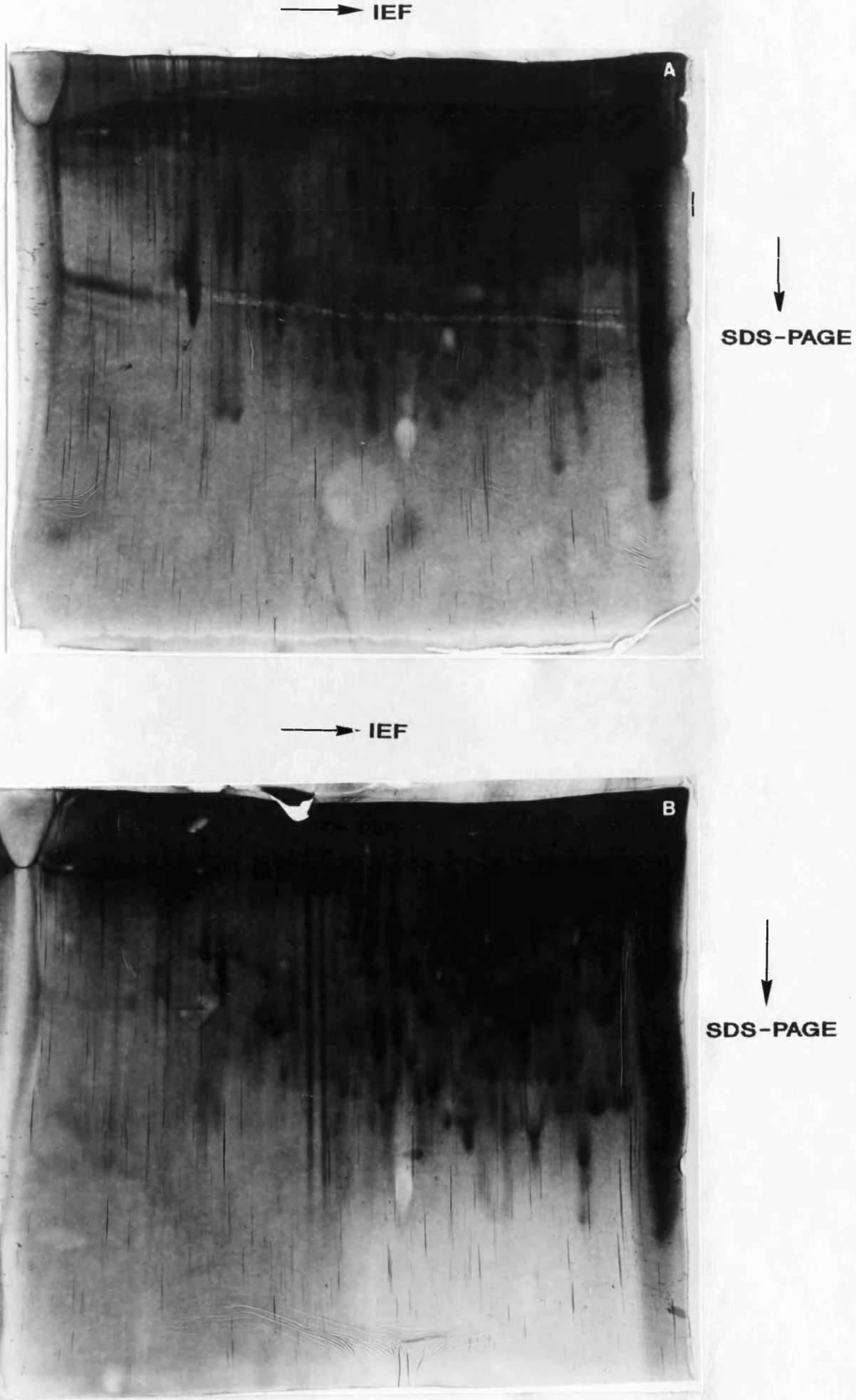
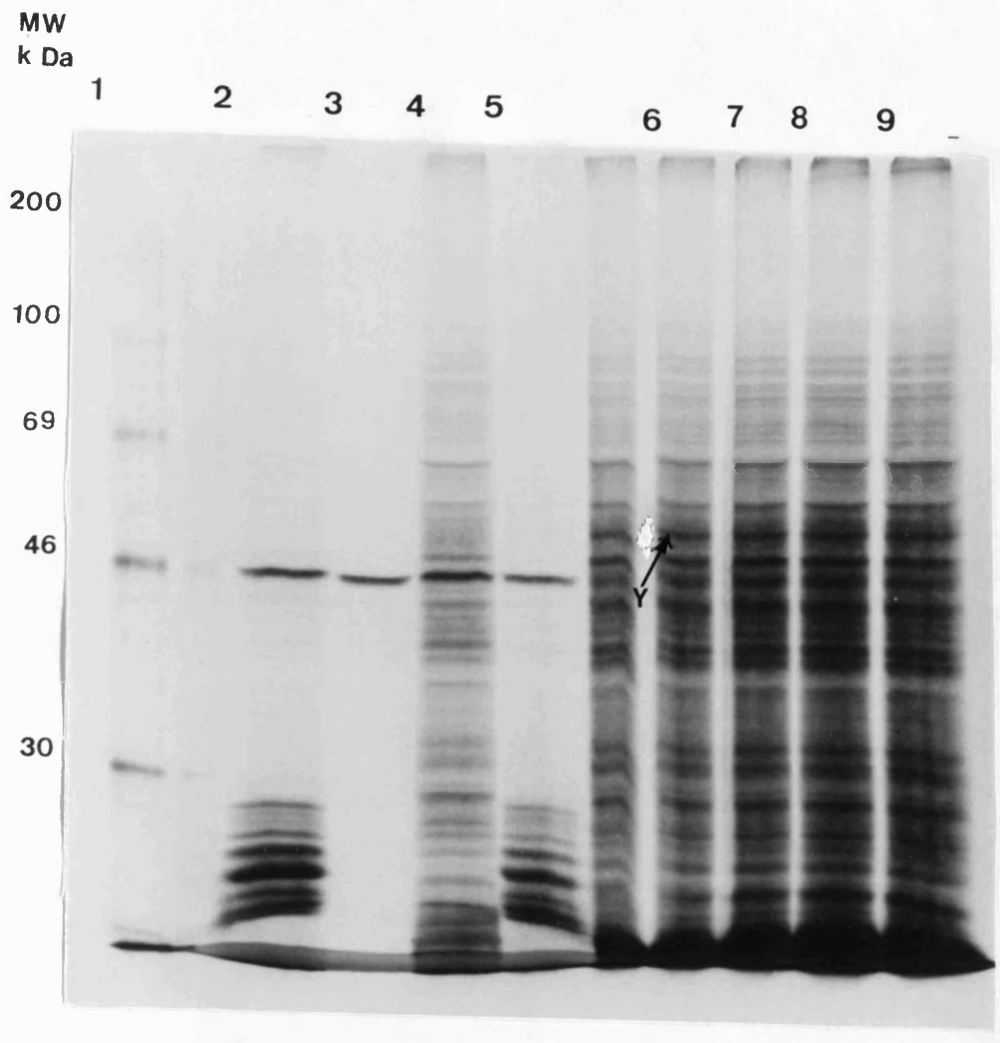

IEF
A
SDS-PAGE
IEF
B
SDS-PAGE

Plate 28: *In vitro* translation of poly(A)$^+$ RNA from second-stage CIM and SIM cultures.

300 ng of tobacco poly (A)$^+$ RNA isolated from 10-13 day second-stage CIM and SIM cultures were translated *in vitro* using RRLS-N.90. 1 μl from each sample was analysed by SDS-PAGE.

lane	sample
1	[^{14}C] molecular weight markers
2	10d CIM (4d CIM)
3	11d CIM (4d CIM)
4	12d CIM (4d CIM)
5	13d CIM (4d CIM)
6	10d SIM (4d CIM)
7	11d SIM (4d CIM)
8	12d SIM (4d CIM)
9	13d SIM (4d CIM)

Band Y (Mr approximately 53.1 kDa) is visible in SIM cultures and is not visible in 12d CIM (4d CIM) cultures.


MW
k Da
1
2
3
4
5
6
7
8
9
200
100
69
46
30
Y

it lacked band Y (Mr approximately 53.1 kDa) which was prominent in all SIM cultures. In addition, the intensity of the bands was higher in SIM cultures indicating better quality or higher translatability of poly $(A)^+$ RNA.

5.5 DISCUSSION

2d CIM (4d CIM) cultures had a translation profile different from day 0 samples (before culturing). Some bands that were apparent in day 0 samples seemed to disappear and some others appeared, when explants were cultured *in vitro* (Plate 26). Christianson and Warnick (1988) observed a change in translation profile in leaf explants of *Convolvulus arvensis* after two days on a root induction medium. They did not specify the type of changes. They concluded that this change was due to dedifferentiation which was completed after two days in culture. The change in translation profile observed in the experiment described in this chapter may also be related to the completion of the dedifferentiation process. In order to detect the time of disappearance and/or appearance of mRNAs, that were associated with dedifferentiation, it would be necessary to examine the *in vitro* translation products of day 0-4d CIM samples. Alternatively, some of the changes, such as appearance of some mRNAs observed in 2d CIM (4d CIM) may reflect the presence of mRNAs involved in cell division or in a differentiation process.

In this work the translation profiles of 2d CIM (4d CIM), which was considered as the control, showed an extra band (X, Mr approximately 39 kDa) which did not appear in 1-6 day SIM samples. Since band X is not detectable in 2d SIM (4d CIM) cultures, and since the only difference between these two media is in the type of hormone supplement, it seems likely that band X is directly or indirectly induced by the 2,4-D in CIM. 2,4-D induced mRNAs and proteins have been observed by other workers. For example, Van der Zaal *et al.* (1987) observed changes in the mRNA population when 2,4-D was added to cell suspension cultures grown in otherwise hormone free medium. Zurfluh and

Guilfoyle (1982 a,b) observed the appearance of a 15 kDa protein after 15 min of 2,4-D application. They also detected enhancement of 8 more translatable mRNAs after 30 min and 10 more after 60 min of 2,4-D application. Bevan and Northcote (1981b) observed the appearance of two polypeptides in soybean tissues (Mr 35 and 45 kDa), after a 2 h exposure to 2,4-D. The persistence of such mRNAs in cultures after six days in 2,4-D media has not been reported; to investigate whether band X was auxin induced, *in vitro* translations of mRNA from earlier stages of culture would have to be examined.

The absence of band X in SIM cultures could either be due to removal of the inducing agent, i.e., 2,4-D, or it could be due to a more active process involving repression by the cytokinin in SIM. Eichholz *et al.* (1983) observed inhibition of an auxin induced polypeptide (approximately 34 kDa) by cytokinin. However, Vanderhoef and Stahl (1975) found that cytokinin did not prevent auxin mediated mRNA accumulation.

No other qualitative differences were detected between 2d CIM (4d CIM) and SIM cultures or during the time course on SIM (Plate 26). It has been observed that cytokinin did not prevent auxin mediated mRNA accumulation, although it inhibited cell elongation (Vanderhoef and Stahl, 1975), suggesting that cytokinin could act by post-translational modification of proteins rather than by influencing transcription. Hence it is possible that one of the reasons for obtaining similar polypeptide profiles in 2d CIM (4d CIM) and SIM cultures (except the disappearance of the 39 kDa polypeptide, X, in 2d CIM (4d CIM) cultures) was that post-translational modifications of 2,4-D induced proteins in CIM cultures by BAP in SIM occurred. Teyssendier De la Serve *et al.* (1984) observed that there were no qualitative or quantitative differences between the *in vitro* translation products of mRNA isolated from cultures with high and low concentrations of BAP, although two different concentrations of BAP brought about two different morphogenetic effects (1.2.2). In the experiment reported here, where *in vitro* translations were carried out with mRNA isolated from 1-6

day SIM cultures, one dimensional SDS-PAGE similarly did not show any qualitative differences between them.

The lack of differences observed between the SIM and CIM cultures could also indicate that the proteins that change in SIM cultures are encoded by chloroplast genes rather than nuclear ones. It has been shown that cytokinins do influence chloroplast encoded proteins. Cytokinin dependent increase of ribulose 1,5 bisphosphate carboxylase (RUBISCO) in cucumber cotyledons was shown by Parthier *et al.* (1981, 1982). They used an *E. coli* system which selectively translated plastid mRNA and a wheat germ system which preferentially translated poly $(A)^+$ RNA to carry out *in vitro* translations of total RNA isolated from BAP-treated and untreated tissue. They found some quantitative differences but no qualitative differences between the two types of tissues. They observed an increased incorporation of radioactive methionine in treated tissue as compared to untreated tissue. They concluded that cytokinin controls both nuclear and chloroplast genomes in plants. In the experiment carried out here with SIM and CIM cultures, *in vitro* translation was carried out with poly $(A)^+$ RNA. The chloroplast mRNAs do not have poly (A) tails. Hence the translated products represented only the mRNA of cytoplasmic origin and would not have included chloroplast encoded proteins such as RUBISCO large subunit which might be expected to be differentially produced in CIM and SIM. However, some components of the photosynthetic apparatus are nuclear encoded, such as RUBISCO small subunit, yet no differences were observed in the translational profiles of CIM and SIM cultures. The explants after 2 days on CIM were still green and so it is possible that they were still producing mRNAs for photosynthetic enzymes. Therefore the differences in the amounts of these mRNAs between the two types of cultures may have been too small to show as differences in their translation products.

Finally, it is possible that the lack of observed differences in *in vitro* translation profiles was due to the limits of resolution of the one dimensional gel

electrophoresis system. It cannot be ruled out that in SIM cultures there were changes which were not detectable by the technique used. This may either be because the mRNAs involved in shoot induction were present in minute quantities and because *in vitro* translation allowed analysis of only the most abundant mRNA sequences (Theologis, 1986) they remained undetected, or because they co-migrated with other bands which obscured the differences. Analysis of polypeptides by 2D-PAGE provides more detailed information if the co-migrating polypeptides have different iso-electric points. However, since analysis of translated products by 2D-PAGE was not successful, such differences could not be detected. However, it was decided to proceed to cDNA cloning without spending more time perfecting the technique of 2D-PAGE.

When translated products were analysed by one dimensional SDS-PAGE, the highest intensity of bands was observed in translation products of poly $(A)^+$ RNA isolated from cultures grown for 2 days on SIM [2d SIM (4d CIM)]. Since an increase in translational capacity of mRNA has been observed concurrently with the changes of developmental stages during germination (Martin and Northcote, 1981), the observed increase in band intensity could represent increased translatability of mRNA.

In vitro translation of poly A^+ RNA isolated from older CIM cultures was not successful except 12d CIM (4d CIM) sample. Comparison of this sample with 10d SIM (4d CIM)-13d SIM (4d CIM)] samples showed the presence of band Y in SIM cultures (compare track 4 with tracks 6-9 in Plate 28). By this stage, i.e., after 10-13 days on second-stage medium, the SIM cultures showed large scale shoot formation, whereas there was no obvious organ formation in CIM cultures. Therefore band Y may correspond to a mRNA involved in shoot growth and development. Since shoot induction was apparently not synchronous it cannot be ruled out that some shoot induction processes were still occurring in a minority of cells in the explants. The identification of these shoot-induction specific mRNAs may be difficult due to

mRNA species related to shoot growth outnumbering them. Hence, the differences seen in Plate 28 were considered to be specific to shoot growth rather than shoot induction process.

It was seen earlier (section 3.3) that both total RNA and poly $(A)^+$ RNA accumulation and cell division (2.3.3) increased in 2d SIM (4d CIM) samples. *In vivo* labelling experiments showed increased synthesis of some polypeptides in 2d SIM (4d CIM) cultures. The translation profile of poly $(A)^+$ RNA isolated from 2d SIM (4d CIM) cultures showed higher intensities of bands, which could be due to greater translatabilty than the poly $(A)^+$ RNA isolated from rest of the cultures during the period of eight days on second-stage SIM. Therefore cultures grown for 2 days on second-stage SIM were chosen as a good stage from which to construct a complementary library.

6 CONSTRUCTION OF A COMPLEMENTARY DNA (cDNA) LIBRARY

6.1 INTRODUCTION

DNA complementary to mRNA can be synthesised and cloned into a suitable vector to provide a cDNA library. The advantage of cDNA clones over genomic DNA clones is that cDNA cloning generates a copy of the message, without the introns and therefore only represents the portion of the genome which is expressed. It is also possible to enrich mRNAs of interest before cDNA cloning, in order to reduce the size of the library.

The first step in constructing a cDNA library is to synthesise double stranded cDNA from mRNA. Once cDNA is synthesised from mRNA, it is inserted into either a plasmid or viral based vector and then the vector with the cDNA insert is introduced into *E. coli*, where replication takes place. This results in a library containing DNA copies of the mRNA population.

The objective was to identify and analyse developmentally significant mRNAs involved in the early stages of shoot regeneration. Experiments carried out earlier showed that shoot organogenesis was accompanied by rapid histological changes, increased total and poly $(A)^+$ RNA accumulation and synthesis of novel proteins. These changes were visible in cultures after two days on SIM. The translatability of poly $(A)^+$ RNA isolated from 2d SIM (4d CIM) cultures was found to be higher than the rest, during the time course of shoot regeneration. Therefore, it was decided to use poly $(A)^+$ RNA isolated from 2d SIM (4d CIM) cultures in constructing a cDNA library. The bacteriophage λ gt10 was used as a vector to clone the cDNA.

6.2 MATERIALS AND METHODS

6.2.1. Synthesis of double stranded cDNA

A commercially available cDNA synthesis system from Amersham International plc (cDNA synthesis system plus [RPN 1256]) was used to

synthesise cDNA. In this system hairpin loop formation and S1 nuclease digestion are avoided as S1 nuclease digestion is difficult to control and generally results in loss of 5' end. The first strand cDNA is primed with oligo (dT) or random primers and synthesised using AMV reverse transcriptase and a mixture of the four deoxynucleoside triphosphates (dNTPs). In second strand synthesis, RNase H is used to produce nicks and gaps in the mRNA strand of the mRNA/cDNA heteroduplex. *E. coli* DNA polymerase I is employed along with dNTPs to synthesise the second strand using nicked mRNA as a primer. Any 3' ends overhanging are removed by the 3'-5' exonuclease activity inherent in T4 DNA polymerase.

6.2.1.1 Synthesis of the first strand

For first strand synthesis the following reagents were added.

	µl
5 x first strand synthesis buffer	4
dNTPs (10 mM dATP, dGTP, dTTP and 5 mM dCTP)	2
sodium pyrophosphate solution	1
human placental ribonuclease inhibitor (20 units)	1
oligo (dT) primer (0.16 µg)	1
[α^{32}P] dCTP (5 µCi)	0.5
tobacco mRNA (1 µg)	1
water	8.5
	19.0

The contents were mixed gently and centrifuged for a few seconds in a microfuge. Then, reverse transcriptase (20 units) was added and incubated (42 °C, 40 min) after which the tube was kept on ice. An aliquot (2 µl) was removed into a tube containing water (20 µl) to estimate the percentage of radioactive incorporation and to analyse on a gel.

6.2.1.2 Synthesis of the second strand

The following components were added in the order given below.

	µl
first strand cDNA reaction mix	18.0
second strand synthesis reaction buffer	37.5
[α^{32}P] dCTP (50 µCi)	5.0
E.coli ribonuclease H (0.8 units)	1.0
E.coli DNA polymerase I (23 units)	6.5
water	31.0
	99.0

The contents of the tube were mixed gently and incubated at 12 °C (60 min) followed by a second incubation at 22 °C (60 min). The enzymatic reaction was stopped by incubating the tube at 70 °C (10 min). The tube was immediately placed on ice, T4 DNA polymerase (2 units) was added and incubated (37 °C, 10 min). The reaction was terminated by adding EDTA (4 µl, 0.25 M , pH 8). An aliquot (2 µl) was removed from the second strand cDNA mix for measurement of incorporation and size analysis on a gel.

6.2.1.3 Calculation of percentage incorporation into cDNA

The cDNA (2 µl) was added to water (20 µl) and mixed; two aliquots (2 µl) were spotted onto the centres of two discs (2.4 cm Whatman DE 81), designated as filter disc A and filter disc B. Disc B was washed six times with Na_2HPO_4 (0.5 M, pH 7.2, 5 min per wash), twice with water (1 min per wash) and twice with 95% ethanol (1 min per wash). Both filters A and B were dried thoroughly and the radioactivity was measured by Cerenkov counting (with no scintillant). The unwashed filter A measures the total radioactivity in the sample; filter B measures only the radioactivity incorporated into cDNA.

A sequential labelling procedure was followed according to the Amersham cDNA Synthesis plus manual. Here, the first strand was labelled with a low amount of isotope (5 µCi) and the second strand was labelled with a

higher amount (50 μCi). The calculations of radioactive incorporation for such a labelling reaction are given in Appendix C.

6.2.1.4 Alkaline gel electrophoresis of cDNA

The diluted sample of cDNA left after removing 4 μl to measure incorporation was vacuum dried and the pellet was dissolved in 5 μl of alkaline loading buffer (Appendix A). An agarose mini-gel (1.4%) was made in a solution of NaCl (50 mM) and EDTA (1 mM). The solidified gel was immersed in alkaline electrophoresis buffer (30 mM NaOH and 1 mM EDTA, 45 min), then the buffer was replaced, the samples were loaded and electrophoresis was carried out at a constant voltage (40 V, 3.5 h). The gel was placed on 5 layers of 3MM paper and dried between two glass plates (50 °C, 2 h). The gel was covered with Saran Wrap (Dow company) and exposed to Fuji RX X-ray film (4 h, room temperature).

6.2.1.5 Purification of double stranded (ds) cDNA

The ds cDNA in solution was extracted twice with an equal volume of phenol/chloroform (1:1), then once with an equal volume of chloroform. The unincorporated nucleotides were removed by precipitating ds cDNA with an equal volume of ammonium acetate (4 M) and 2 volumes of cold ethanol (-20 °C). The contents were chilled (15 min) on dry ice, brought to room temperature and centrifuged (10 min) in a microfuge. The supernatant was removed and the pellet was washed in ammonium acetate (50 μl of 2 M, room temperature) and ethanol (100 μl, -20°C). The pellet was washed again with cold ethanol (200 μl), dried in a vacuum desiccator (2-3 min) and resuspended in 10 μl of TE (pH 7.5).

6.2.2 cDNA cloning

Complementary DNA synthesised as above was cloned using the λ gt10 cloning system (Amersham International plc, cDNA cloning system λ gt10, code RPN 1257). Lambda gt10 has a unique *Eco*RI restriction site within the phage

repressor gene *C*I and cDNA is inserted into this site. The *C*I gene maintains the lysogenic nature of the phage whereby the bacteriophage integrates into the bacterial genome and is replicated with the host genome. In this event phage particles are not produced. When foreign DNA is inserted at *C*I, the *C*I gene is inactivated and the recombinant phage enters the lytic pathway whereby bacterial cell lysis occurs resulting in clear plaque formation.

The cloning kit from Amersham provided *E.coli* strains NM514 and L87. When λ gt10 enters L87 it follows the lysogenic pathway resulting in turbid plaque formation. The bacterial strain NM514 has a mutation conferring high frequency of lysogeny and λ gt10, when it enters, is forced to follow a lysogenic pathway so no plaques are formed. Recombinant phage when introduced into L87 gives clear plaques due to lysis of bacterial cells as opposed to the turbid plaques formed by parental λ gt10; only recombinant phage give clear plaques on NM514. Hence, the two strains of bacteria provide a biological selection system to obtain a high frequency of recombinants on NM514.

In carrying out cloning, the protocols given in the handbook from Amersham were followed. In all the enzymatic reactions the following general procedures were followed. The components were always added in the order given in the recipe. After combining all the components, the tube was vortexed very briefly and spun for a few seconds to bring the reaction mix together at the bottom of the tube. Then, the enzyme was added, mixed gently and incubated in a water bath at the prescribed temperature and time. Then the enzyme was inactivated by heating (70 °C, 10 min). A stepwise procedure was carried out starting with methylation of any *Eco*RI sites in the cDNA to be cloned.

6.2.2.1 Methylation of *Eco*RI restriction sites

Methylation of cDNA was carried out to protect possible internal *Eco*RI sites from restriction digestion.

	µl
cDNA solution	10
Eco RI methylase buffer	4
1×SAM	2
water	3
*Eco*RI methylase (20 units)	1
	20

The reaction was incubated (3 °C, 60 min). The methylated cDNA was then ligated to *Eco*RI linkers.

6.2.2.2 Addition of *Eco*RI linkers to cDNA

*Eco*RI linkers, phosphorylated at their 5' end were ligated to both ends of methylated cDNA. A reaction was then set up using the following reagents.

	µl
methylated cDNA	20
ligase buffer	3
*Eco*RI linker	2
water	3
T4 ligase (5 units)	2
	30

This ligation reaction was carried out (15 °C, 18 h) and an aliquot (2 µl) was withdrawn from the ligated mix to run on an agarose gel.

6.2.2.3 *Eco*RI digestion of linkered cDNA

In order to produce sticky ends in the linkered cDNA, digestion with *Eco*RI was performed. A reaction for restriction digestion with *Eco*RI was set up as given below and incubated (37 °C, 7 h).

	µl
linkered cDNA	28.0
Eco RI buffer	10.0
water	60.3
*Eco*RI enzyme (100 units)	1.7
	100.0

6.2.2.4 Separation of cDNA from excess linker molecules

The columns for separating excess linkers were provided with the cloning system. The column was equilibrated by passing 3 ml of STE buffer (Appendix A) twice. The sample of DNA (100 μl) followed by STE buffer (100 μl) were added onto the column and the eluate was collected in the tube labelled 1. Thereafter, 9 more aliquots (200 μl each) of STE buffer were passed through the column and the eluates of the aliquots were collected in tubes numbered 2-10 respectively. The radioactivity of each sample was measured by Cerenkov counting. The two fractions of cDNA with the highest cpm were pooled and subjected to ethanol precipitation. The pellet after drying was resuspended in STE buffer at a concentration less than 100 ng μl^{-1}. The amount of cDNA in the pellet was calculated (see Appendix C).

6.2.2.5 Ligation of cDNA to λ gt10 arms

The supplied λ gt10 was already digested with *Eco*RI, and the arms had been dephosphorylated to prevent self ligation. The following ligations were carried out.

a. Ligation of 0.5 μg of whole vector DNA. This is a control to test whether the biological selection of host cells is working and to test the efficiency of *in vitro* packaging.

b. Ligation of λ gt10 *Eco*RI phosphatased arms (1 μg). This monitors the background plating efficiency on the two kinds of host cells.

c. The *Eco*RI digested control DNA (125 ng) provided with the cloning system and arms (1 μg) to ensure that the ligation reaction has worked efficiently.

d. Experimental cDNA (125 ng) and arms (1 μg).

Ligations were carried out using T4 ligase (2.5 units) in a 10 μl reaction volume. The ligation mixes were incubated (15 ^{o}C, 17 h) and the DNA was precipitated with sodium acetate (1 μl of 3 M, pH 5.2), and absolute ethanol

(27 μl) by leaving on dry ice (15 min). The tubes were centrifuged (15 min) in a microfuge, and the pellets were dried in a vacuum desiccator (3 min) and redissolved in 2.5 μl of TE (pH 7.5) (Appendix A).

6.2.2.6 *In vitro* packaging of ligation mixtures

Packaging of the DNA into phage heads was performed *in vitro* using the extracts supplied with the cloning kit. The heads and the tails of bacteriophage which were stored at -70 °C in separate tubes were thawed just prior to commencement of the packaging reactions and left on ice. 10 μl of heads was added to the ligated DNA followed by immediate addition of 15 μl of tails. The contents were mixed gently and incubated (25 °C, 2 h). Finally, SM buffer (0.5 ml) and chloroform (10 μl) were added and mixed very gently. Packaged cDNA was stored in a refrigerator (4 °C) until needed.

6.2.2.7 Transfection of *E. coli*

a. Preparation of plating cells

Loopfuls from glycerol stocks of NM514 and L87 were plated on L-agar (Appendix B) plates and incubated (37 °C, overnight). A single colony was then inoculated into L-broth (10 ml, Appendix B) containing maltose (0.4%) and allowed to grow overnight in a shaking incubator (200 rpm, 37 °C). Each of these stationary cultures (1 ml) was inoculated into L-broth (50 ml) containing maltose (0.4%) and allowed to grow (200 rpm, 37 °C) until the optical density at 600 nm reached 0.5 (2.5×10^8 cells ml^{-1}). The cultures were cooled on ice and centrifuged (3000 rpm, 4 °C, 10 min). The pellet was resuspended in ice cold $MgSO_4$ (15 ml, 10 mM), shaken well and stored (4 °C).

b. Titration of λ gt10 recombinants

A series of dilutions of phage stocks were made starting 10^{-2} to10^{-7}. The following dilutions were selected to carry out transfection into L87 and NM514.

	L87	NM514
λ DNA	10^{-5} 10^{-6} 10^{-7}	10^{-3} 10^{-4} 10^{-5}
λ arms	10^{-2} 10^{-3} 10^{-4}	10^{-2} 10^{-3} 10^{-4}
control DNA	10^{-4} 10^{-5} 10^{-6}	10^{-3} 10^{-4} 10^{-5}
cDNA	10^{-4} 10^{-5} 10^{-6}	10^{-3} 10^{-4} 10^{-5}

Each of the above dilutions (100 μl) was mixed with bacterial cells (100 μl) and incubated (37 °C, 15 min). Molten top agarose (4 ml) was added to the cells and phage mix, swirled and poured onto 9 cm L-agar plates. The plates were left for the agarose to solidify (10 min, room temperature) and then incubated overnight (37 °C) for plaques to appear.

6.2.2.8 Isolation of DNA from lambda clones

A plaque was cored out using a sterile Pasteur pipette tip and introduced into an Eppendorf tube containing freshly prepared NM514 plating cells (0.5 ml). This was left for cells to adsorb phage (15 min, room temperature), before transferring to L-broth (5 ml) containing $CaCl_2$ (5 mM). The cultures were incubated in a shaking incubator (37 °C, 3.5-4.0 h) until a clear lysate with some cell debris was seen. A few drops of chloroform were added to the cultures which were shaken again (5 min). The supernatant obtained after centrifuging the contents in a bench centrifuge (3000 rpm,10 min) was stored at 4 °C. Similarly, phage lysate was prepared from L87 plaques (obtained by transfection with λ gt10 DNA), to use as the control.

To the phage lysate (4 ml), a solution (4 ml) containing polyethylene glycol (20%) and NaCl (2 M) was added and left on ice (1 h). The contents were centrifuged (3000 rpm, 20 min) in a bench centrifuge. The pellet was resuspended in L-broth (750 μl), DEAE cellulose (750 μl) (Appendix B) was added, mixed gently and centrifuged in a microfuge (5 min). The addition of cellulose and centrifugation was repeated until a clear supernatant was obtained. To the supernatant (750 μl), proteinase K (13 μl of 0.1 mg ml^{-1}) and SDS (32 μl of 10%) were added and incubated (5 min, room temperature). Then, potassium

acetate (130 μl of 3 M) was added, incubated (88 °C, 20 min), cooled on ice (10 min) and centrifuged in a microfuge (10 min). The supernatant (800 μl) was transferred to another tube, cold iso-propanol (800 μl) was added and DNA was precipitated at -70 °C (10 min). The tube was allowed to attain room temperature and centrifuged in a microfuge (10 min, room temperature). The pellet was dried and dissolved in 20 μl TE (pH 8).

6.2.2.9 Determination of presence and size of insert

In order to detect the presence and sizes of insert DNA, phage DNA was i) digested with *Eco*RI to release the insert and ii) double digested with *Bgl*II and *Hin* dIII to obtain a phage DNA fragment containing the insert. The fragment of DNA containing cDNA insert could be seen on a 0.8% agarose gel.

i) Digestion with *Eco*RI enzyme

Phage DNA (10 μl) was digested with *Eco*RI (6 units) in a 30 μl volume (see section 6.2.2.3). The DNA fragments were labelled by adding [α^{32}P] dATP (1 μCi) and DNA polymerase I-Klenow fragment (1 unit) followed by incubation (30 °C, 30 min). Wild type λ cut with *Hin*dIII was end labelled and used as molecular weight markers. The samples were electrophoresed on an agarose gel (1.5%).

ii) Double digestion with *Hin*dIII and *Bgl*I

Phage DNA (10 μl) was digested with *Hin* dIII (4 units) in a 18 μl volume. Then, NaCl (0.4 μl of 2.5 M) was added to increase the salt concentration to 50 mM (since *Bgl*II enzyme requires high salt buffer), before adding *Bgl*I (4 units). The reaction mix was incubated (37 °C, 1 h), BB loading buffer (2 μl) (Appendix A) was added, and the sample was electrophoresed on an agarose gel (0.8%). Wild type λ DNA digested with *Hin* dIII was used as molecular weight markers.

6.2.2.10 Amplification of the library

Prior to commencement of screening the library, amplification of the library was performed to enable the reuse of the cDNA library for screening. The cDNA library was plated on NM514 at a density of 3 x 10^4 pfu per 9 cm plate as described in section 6.2.2.7b. The plates were incubated (37 °C) until all the plaques merged. They were then overlaid with SM buffer (5 ml) and left overnight (4 °C) for the phage to disperse in the medium. The solution was transferred to a tube, washed with SM buffer (1 ml) and the washings were pooled. A drop of chloroform was added to the phage suspension before storage at 4 °C. The titre of this amplified library was measured as described earlier.

6.3 RESULTS

6.3.1 cDNA synthesis

Poly $(A)^+$ RNA isolated from cultures grown for 2 days on SIM [2d SIM (4d CIM)] was used to synthesise cDNA. In order to examine its integrity, the poly $(A)^+$ RNA (200 ng) isolated from 2d SIM (4d CIM) cultures was translated *in vitro* (in a 11 µl volume) and the translated mix (1 µl) was run on a gel as described in section 4.2.4. Bands of individual samples were clear with no smearing, indicating high quality of mRNA (Plate 29).

Poly $(A)^+$ RNA (1 µg) isolated from 2d SIM (4d CIM) cultures was used to synthesise ds cDNA. The amounts of cDNA synthesised was calculated. The methods of calculations are detailed in Appendix C.

6.3.1.1 Percentage incorporation during first strand synthesis

cpm on filter disc B = 1365

cpm on filter disc A = 53793

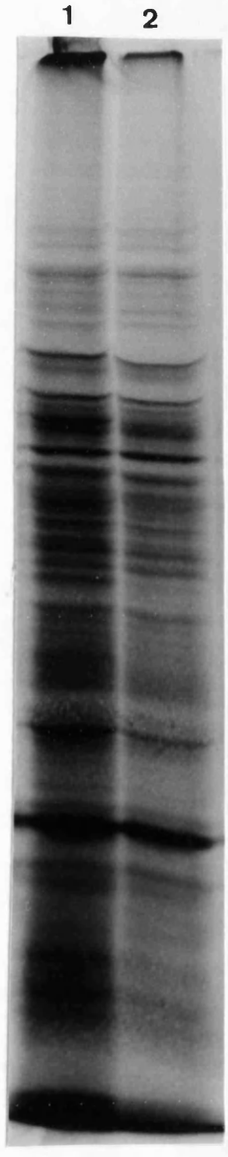
% of incorporation $= \frac{1365}{53793} \times 100$

$= 2.54\%$

Plate 29: Detection of quality of poly $(A)^+$ RNA.:

200 ng of poly $(A)^+$ RNA obtained from 2d SIM (4d CIM) cultures and 2d CIM (4d CIM) cultures were translated *in vitro* using RRLS-N.90 and 1 μl from each sample was subjected to SDS-PAGE and fluorography.

Lane 1 represents translated products of 2d CIM (4d CIM) and lane 2 represents that of 2d SIM (4d CIM).


1
2

6.3.1.2 Amount of first strand cDNA synthesised

$$= 2.54 \times 140 \text{ ng}$$

$$= 355.6 \text{ ng}$$

6.3.1.3. Percentage yield of first strand cDNA

$$= \frac{355.6}{1000} \times 100$$

$$= 35.56\%$$

6.3.1.4 Percentage incorporation during second strand synthesis

cpm on filter disc C = 99332

cpm on filter disc D = 3061

percentage incorporation $= \frac{3061 - 1365/5.2}{99332 - 1365/5.2} \times 100$

$$= 2.82\%$$

6.3.1.5 Amount of second strand synthesised

$$= 2.82 \times 140 \text{ ng}$$

$$= 394.8 \text{ ng}$$

6.3.1.6 Percentage yield of second strand cDNA

$$= \frac{394.8}{355.6} \times 100$$

$$= 111.0\%$$

Therefore, the percentage mRNA transcribed into the first strand cDNA is 35.56% and the percentage of first strand transcribed into the second strand cDNA was calculated to be 111.0%. The amount of double stranded cDNA obtained from 1 μg of poly $(A)^+$ RNA was around 750 ng.

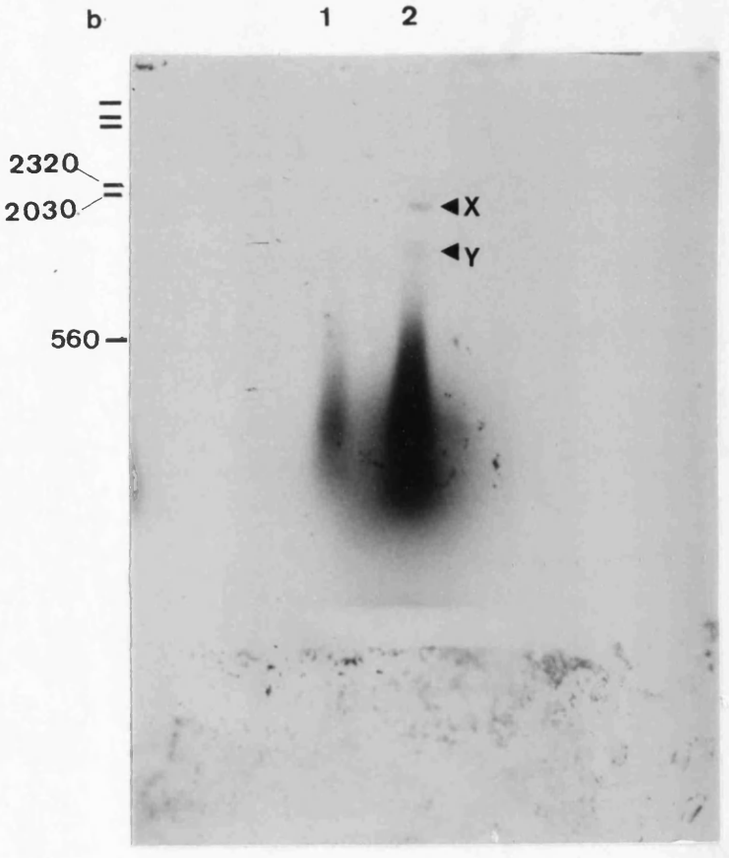
6.3.1.7 Alkaline agarose gel electrophoresis

Plate 30 shows the single and double stranded cDNA in lane 1 and lane 2 respectively. As seen in Plate 30 the majority of the cDNA was visible as a smear in the size range below 500 b. The bands labelled X and Y were approximately 2000 and 1200 b respectively. These bands may be artefacts as there was no smearing between the bands. Even if these high molecular weight

Plate 30: Size analysis of cDNA.

cDNA synthesised from poly $(A)^+$ RNA from 2d SIM(4d CIM) cultures was subjected to alkaline agarose gel electrophoresis.

Lane 1: single stranded cDNA; lane 2: double stranded cDNA. The fractions of cDNA X (approximately 2000 b) and Y (approximately 1200 b) are small in amount; majority of cDNA is less than 500 b in size.


b
1
2
2320
2030
560
X
Y

bands correspond to cDNA fractions, the amounts in them were insufficient for cloning. Hence it was decided to clone the cDNA without prior size fractionation.

6.3.2 cDNA cloning

6.3.2.1 Separation of *Eco*RI linkers

The radioactive incorporation of ten fractions (section 6.2.2.4) is given below.

fraction	cpm
1	12.0
2	122.0
3	237051.0
4	402036.0
5	34650.0
6	4818.0
7	6194.0
8	5726.0
9	1422.0
10	117.0

Since fractions 3 and 4 gave the highest radioactive incorporation these two fractions were chosen to proceed with the rest of the cloning procedure. The method of calculation of cDNA is given in Appendix C. In order to measure the incorporation of radioactive nucleotides 2 μl of the synthesis mix was diluted in 20 μl of water. Two microlitres of this was used to find the incorporation.

The incorporation of 2 μl diluted ds cDNA = 3061 cpm

The incorporation of 22 μl of diluted cDNA $= 3061 \times \frac{22}{2}$ cpm

$= 33671$ cpm

This value of 33671 cpm is equivalent to the incorporation of 2 μl of ds cDNA. Therefore, the incorporation of 98 μl of cDNA $= 33671 \times \frac{98}{2}$ cpm

$= 1649879$ cpm

The percentage of dCTP incorporated = 2.82%

Therefore, the incorporation into 1 ng of cDNA $= \frac{1649879}{2 \times 350 \times 4 \times 2.82/10}$

$= 2089.51$ cpm

The total cpm of fractions 3 & 4 = 639087 cpm

Therefore, the amount of cDNA in fractions 3 and 4 $= \frac{639087}{2089.51}$

$= 305.8$ ng

6.3.2.2 Transfection of *E.coli*

As described in section 6.2.2.5 the following ligations were made.

a. 0.5 μg of whole λ gt10

b. 1 μg of λ arms

c. 125 ng of *Eco*RI ended control DNA and λ arms

d. 125 ng of tobacco cDNA and λ arms

After *in vitro* packaging of these phage DNA, transfections into *E.coli* strains L87 and L514 were carried out. The results are shown in Table 5.

Treatment	L87 titre pfu /μg arms	NM514 titre pfu /μg arms
λ gt10	5.4×10^7	2.2×10^3
λ arms	2.19×10^3	2.2×10^4
control DNA	4.9×10^4	7.4×10^4
125 ng cDNA	3.21×10^4	7.8×10^4

Table 5- Total pfu obtained when L87 and NM514 were transfected with ligation mixes a, b, c and d.

The value obtained for the λ gt10 arms will indicate the background. The background on NM514 (2.2×10^4) was subtracted from total plaque forming units with cDNA or control DNA on NM514, to get the actual plaque forming units.

Hence,

The total recombinants for control DNA $= 5.2 \times 10^4$ ml^{-1}

The total recombinants for 125 ng of cDNA $= 5.6 \times 10^4$ ml^{-1}

Therefore, the total recombinants in 535 μl $= \frac{535 \times 5.6 \times 10^4}{1000}$ pfu

$= 29960$

The cloning efficiency /1 μg of cDNA $= \frac{29960 \times 1000}{125}$ pfu μg^{-1}

$= 2.4 \times 10^5$ pfu μg^{-1}

The library consisting of 29960 recombinants was obtained from 125 ng of cDNA. The titre of the amplified library was found to be 10^{11} pfu ml^{-1}.

6.3.2.3 Determination of presence and size of insert

In order to obtain an estimate of the average size of the inserts, DNA from 15 random clones along with λ gt10 (to use as the control) were purified, and 10 clones were digested with *Eco*RI and 5 clones with *Hin*dIII and *Bgl*II, and electrophoresed on 1.5% and 0.8% agarose gels respectively. Plate 31A represents the autoradiograph of the gel run with the *Eco* RI digests. As expected λ gt10 did not have an insert and two clones showed the presence of inserts of size less than 500 bp. The rest of the eight clones were uncut. Plate 31B represents the results of the double digestion of phage DNA with *Bgl*II and *Hin*dIII.

The size of the fragment released when λ gt10 is digested with *Bgl*II and *Hin*dIII is 1148 bp. This fragment will increase in size depending on the size of the insert. In order to obtain the actual size of the insert, the size of the λ fragment was deducted from the value of the fragment containing the insert. The sizes of fragments containing the inserts and the inserts alone are given in Table 6.

Plate 31: Analysis of sizes of inserts in λ gt10 recombinants.

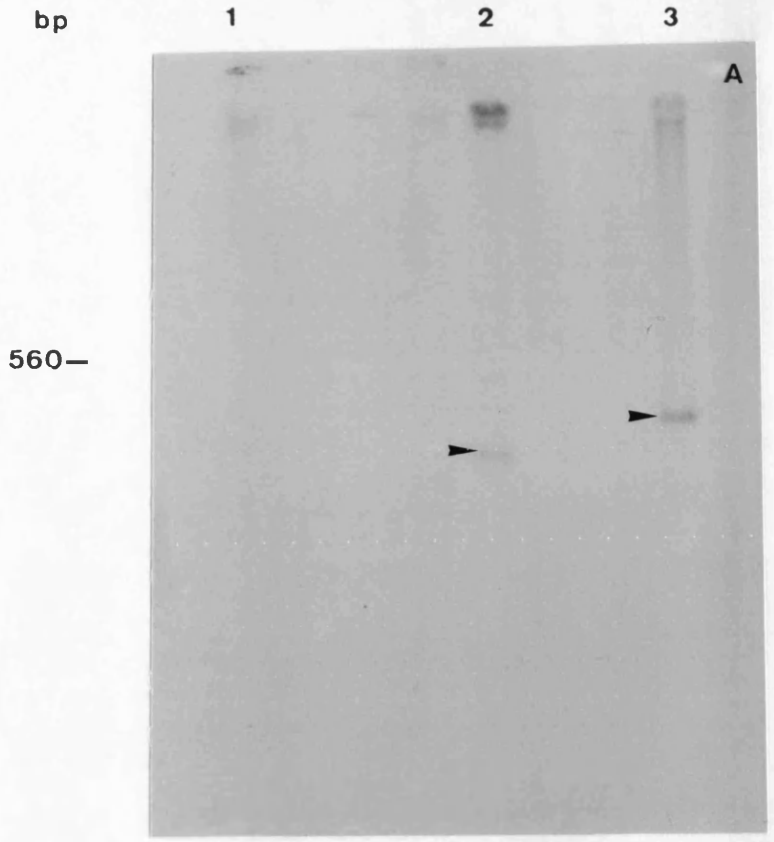
31A: DNA was digested with *Eco* RI, labelled with $\alpha[^{32}P]$ dATP and electrophoresed on a 1.5% agarose gel. The gel was dried and subjected to autoradiography.

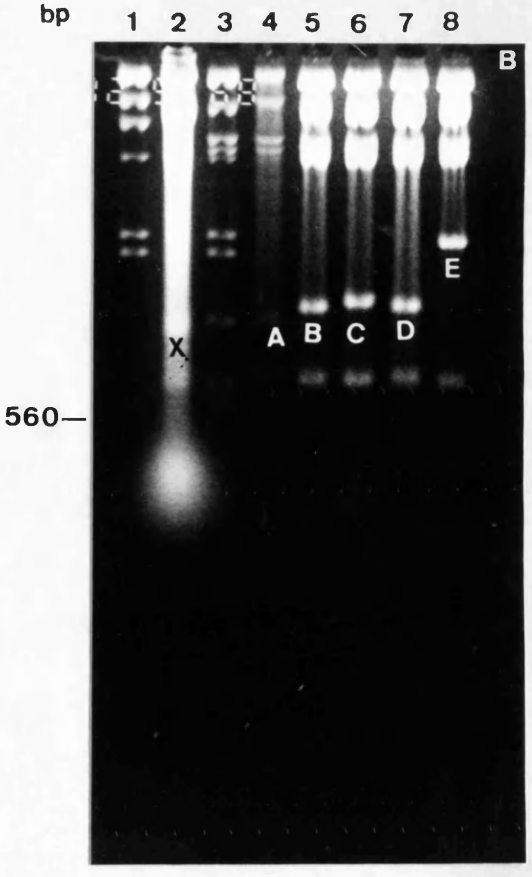
lane 1: λ gt10 (no insert); lanes 2 and 3: λ clones with inserts of size less than 500 bp (arrowed).

31B: DNA was digested first with *Bgl*II and then with *Hin*dIII and run on a 0.8% agarose gel. Wild type λ was digested with *Hin*dIII and *Bgl*II/*Hin*dIII to use as size markers. The gel was viewed on a UV transilluminator and photographed.

lane 1: wild type λ/*Hin*dIII; lane 2: λ gt10/*Bgl*II/*Hin*dIII; lane 3: wild type λ/*Bgl*II/*Hin*dIII; lanes 4-8: λ gt10 clones/*Bgl*II/*Hin*dIII.

Band X is the fragment of λ gt10 containing no insert. A, B, C, D and E are fragments of recombinants containing the inserts. The sizes of X, A, B, C, D and E were 1148, 1412, 1549, 1660, 1549 and 2454 bp respectively. The sizes of inserts in A, B, C, D and E were 264, 401, 512, 401 and 1306 bp respectively.


bp
1
2
3
A
560—


bp
1 2 3 4 5 6 7 8
B
E
A B C D
X
560—

sample	fragment (bp)	insert (bp)
λ gt10	1148	-
clone A	1412	264
clone B	1549	401
clone C	1660	512
clone D	1549	401
clone E	2454	1306

Table 6 - sizes of *Eco*RI fragments and sizes of inserts

6.4 DISCUSSION

The percentage of mRNA transcribed into first strand cDNA was about 35%. The percentage of first strand cDNA transcribed into second strand cDNA was 110%. This value should be greater than 90% but less than or equal to 100%. The higher value of 110% obtained may be due to experimental errors such as pipetting and inadequate washing of the filter disc B. Size analysis of cDNA showed that the majority was less than 500 b in size. One of the most important considerations in obtaining long and full length cDNA is that the mRNA used must be of a high quality. The integrity of the mRNA to be used in cDNA synthesis was determined by translating mRNA into proteins *in vitro* and analysing them by SDS-PAGE. The method used for cDNA synthesis was based on priming first strand cDNA with oligo (dT) primers. This method is reported to yield a high percentage of full length cDNA (Kimmel and Berger, 1987). In this experiment, the majority of cDNA synthesised was less than 500 b. Since mRNA previously tested proved to be intact one possible reason for obtaining small fragments of cDNA could be the degradation of the mRNA during synthesis of the first strand cDNA. The Amersham cDNA kit may not have been free of ribonucleases as it had been already used twice before it was used in this experiment. Ribonucleases being ubiquitous would have easily

contaminated the reagents unless strict precautions were taken by the users of this kit.

Reverse transcriptase is a key enzyme in cDNA synthesis, which has two functions; polymerase and RNase H activities. In the first strand synthesis it is the polymerase activity that is exploited. However, RNase H can deadenylate the 3' end of mRNA and it can also produce small fragments which will prevent in yielding full length cDNA. The fidelity of the reverse transcriptase used was not tested prior to its use in this experiment. Since the main use of the library was to isolate clones of interest, cDNA of small sizes would not be a limiting factor. Hence a library was constructed with unfractionated cDNA.

According to Amersham, the cloning efficiency using their kit should be greater than 1×10^6 pfu μg cDNA^{-1}. The cloning efficiency obtained for tobacco cDNA was four fold less than this value. The titre of intact λ gt10 DNA was 5.4×10^7 indicating the high efficiency of the packaging reaction. If the ligation had worked successfully, reactions c and d in section 6.3.2.2. should have given higher values than those obtained. The control DNA (*Eco*RI ended) provided with the cloning system gave approximately the same number of recombinants as the cDNA. This indicated that all the steps in the cloning procedure had worked successfully except the ligation step. Since nearly 30000 recombinants were produced, it was decided to carry out further investigations with this library.

Out of the 10 random clones digested with *Eco* RI, only two clones showed presence of inserts. When the digestions of these 10 clones along with the control λ gt10 were carried out, the cocktail made was sufficient only for 8 digestions as the required amount of water had not been added by mistake, in making the cocktail. The rest of three samples were digested individually. The eight clones digested with the cocktail remained uncut, probably due to the high concentration of enzyme and salt in the reaction mix. The fact that the two arms cannot be seen in these 8 samples on the autoradiograph proved that the DNA

was not cut. Five clones that were subjected to *Hin*dIII/ *Bgl*I digestion showed presence of inserts in all five clones. Four of the clones were in the range of 260-500 bp while the other was about 1300 bp. The reason for obtaining inserts of small size may be due to small fragments of cDNA preferentially ligating with the λ gt10 arms, leaving behind the longer fragments which were small in quantity as compared to small fragments. All the clones tested had inserts in them. Therefore, despite the fact that the number of recombinants obtained was considerably lower than expected, it was decided to carry out differential screening with this library.

7 IDENTIFICATION OF SIM SPECIFIC CLONES

7.1 INTRODUCTION

A cDNA library contains a large number of recombinants, only a few of which may represent the mRNAs of interest. One of the methods to identify the desired clones is by differential screening. This method involves probing replicas of the library with cDNA to mRNA isolated from the time-point which was used to make the library and from some other differential state, in which genes of interest are either not expressed or are expressed at a significantly lower level.

Genes specific for various developmental stages in plants have been identified by constructing cDNA libraries and screening them with various relevent probes. They include, germination (Martin *et al.*, 1984); flowering (Gasser *et al.*, 1988); fruit ripening (Grierson *et al.*, 1986) and somatic embryogenesis (Choi and Sung, 1984). Goldberg *et al.* (1986) identified various organ specific genes in tobacco by constructing cDNA libraries from the mRNA isolated from various organs and carrying out differential hybridisation. Thus the technique of differential hybridisation of cDNA libraries has made it possible to identify clones corresponding to developmentally important mRNAs.

Messenger RNA isolated from leaf explants on 2d SIM (4d CIM) was selected to construct the cDNA library, therefore mRNA isolated from leaf explants on 2d CIM(4d CIM) was selected as the candidate for differential hybridisation. It was therefore aimed to synthesise single stranded cDNA from both 2d SIM(4d CIM) and 2d CIM(4d CIM) mRNA and carry out differential screening of the cDNA library. Complementary DNA clones which give a positive signal with 2d SIM (4d CIM), but not with 2d CIM (4d CIM) can then be identified. These clones can then be used to measure the levels of the corresponding mRNAs in *in vitro* cultures.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of plaque lifts

An aliquot of the amplified library was diluted with SM buffer to contain approximately 10^4 pfu. This was plated on L-agar plates using top agarose (section 6.2.2.7b). After 7 h of incubation at 37 °C, when the plaques just appeared as pin pricks, the plates were transferred to 4 °C for at least 1 h.

The plaque lifts were made with Hybond-N 82 mm circles (Amersham plc.) as described below. The filter was placed on the plate with gloved hands, taking care to exclude air bubbles, and three asymmetric pricks were made with a sterile hypodermic needle. The first filter was left for 30 sec, after which it was peeled off with a pair of blunt forceps. Three more replica filters were made from each master plate but leaving the filters 30 sec longer each time. The DNA on the filters was denatured by leaving the filters, DNA side up for 5 min on Whatman 3 MM filter paper, soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The filters were then transferred to neutralising solution (0.5 M Tris/HCl, pH 7, 1.5 M NaCl) and left for 5 min before rinsing with 2 x SSC. The filters were air dried on 3 MM filter paper, wrapped in plastic film (Saran Wrap, Dow company) and exposed to UV (transilluminator, UV products), with the DNA side down (3 min).

7.2.2 Differential hybridisation of the filters

7.2.2.1 Synthesis of single stranded cDNA probe

Amersham's cDNA synthesis system (code No: RPN 1257) was used with the following modifications.

1. Instead of the deoxynucleotide triphosphate mix in the Amersham system, 200 μM each of cold dATP, dGTP and dTTP was used.

2. Instead of 5 μCi of [α^{32}P] dCTP in a 20 μl reaction mix, 50 μCi of [α^{32}P] dCTP was used.

3. Subsequent to the initial incubation (42 °C), cold dCTP (20 μM) was added and incubation continued (30 min).

A reaction was set up with the following reagents added in the order given below.

	μl
5 x first strand synthesis buffer	4.0
sodium pyrophosphate solution	1.0
human placental ribonuclease	1.0
10 mM dATP, dGTP, dTTP (0.4 μl of each)	1.2
[α^{32}P] CTP (50 μCi)	5.0
mRNA (1 μg)	1.0
water	5.8
	19.0

The contents in the tube were mixed and centrifuged in a microfuge briefly to bring the contents to the bottom. Then, reverse transcriptase [20 units (1 μl)] was added, mixed gently and incubated in a water bath (42 °C, 40 min). Cold dCTP was added (20 μM) and the incubation was continued (30 min) (Gasser *et al.*, 1989). The reaction was stopped with EDTA (0.1 volume, 0.1 M). The solution was brought up to 100 μl by adding STE buffer (Appendix A) and the unincorporated nucleotides were separated using chromatography through Sephadex G50 as described below.

7.2.2.2 Chromatography through Sephadex G50

30 g of Sephadex G50 (Sigma) was slowly added to 250 ml of TE, pH8 (Appendix A) to disperse it. The mixture was left overnight at room temperature. The supernatant was decanted and replaced with an equal volume of the same buffer and stored at 4 °C. The bottom of a sterile 1 ml syringe was plugged with sterile glass wool, filled with the resin and subjected to centrifugation (4000 rpm, Sorvall-RC 34, 10 min) repeatedly until a column of 0.9 ml was obtained. 100 μl of STE was added to the column and centrifugation was continued until an equal volume of eluate was obtained

(Maniatis *et al.*, 1982). Single stranded cDNA (in a volume of 100 μl in STE) was applied onto the column and centrifuged (Sorvall-SS 34 rotor) at 4000 rpm for 10 min. The eluate contained cDNA leaving behind the unincorporated nucleotides in the column.

7.2.2.3 Hybridisation of plaque lifts

The radioactive cDNA was ethanol precipitated (section 6.2.1.5) and the pellet was dissolved in 200 μl of water. Then, NaOH (60 μl of 1.0 N) was added and incubated at room temperature for 10 min. The alkali was then neutralised with HCl (60 μl of 1.0 N) and SSPE (60 μl of 20 x) (Gasser *et al.*, 1989). The filters were prehybridised overnight in 10 ml of prehybridisation solution (2 x SSPE (Appendix B), 4 x Denhardt's solution (Appendix B), 0.1% SDS and 10 μg ml^{-1} poly (A), 100 μg ml^{-1} denatured salmon sperm DNA) at 68 °C. The prehybridisation solution was discarded and hybridisation solution (5 ml) of the same composition as the prehybridisation solution but with the probe, was added and hybridisation was carried out (48 h at 68 °C). The hybridisations were carried out in glass cylinders fitted to a rotating wheel in an oven (Hybaid). After hybridisation the following series of washes was performed.

solution	time	temperature
1 x SSPE, 0.1% SDS	30 min	room temperature
0.3 x SSPE, 0.1%SDS	20 min	room temperature
0.3 x SSPE, 0.1%SDS	20 min.	room temperature
0.3 x SSPE, 0.1%SDS	20 min	68 °C

The needle pricks in the filters were marked with radioactive ink with low counts (approximately 20 cpm), covered with Saran Wrap and subjected to autoradiography at -70 °C, ranging from a few hours to 4 days (depending on the intensity of radioactive labelling). The duplicate autoradiographs were compared and the plaques which appeared only on filters hybridised with SIM

cDNA were picked out and stored in SM (Appendix B) (500 μl, 4 °C). A secondary screening was carried out by replating the plaques at a density of about 500 plaques per plate. The plaques which showed positive hybridisation with 2d SIM (4d CIM) cDNA were stored at -4 °C. These were further purified by plating at a density below 100 plaques per plate and repeating the hybridisations as earlier.

7.2.3 Subcloning of inserts into a plasmid vector

The plasmid vector pUC18 was chosen to subclone the inserts. Amongst other properties, this vector will allow bulk isolation of insert DNA. As the first step, purification of recombinant phage DNA was carried out.

7.2.3.1 Purification of lambda recombinant DNA

Phage lysate was prepared as described in section 6.2.2.8. Lambda DNA was purified from 50 ml of lysate using a Qiagen 100 pack (Hybaid). The pack contained the following reagents.

Buffer L1- RNase A (20 mg ml^{-1}), DNase (6 mg ml^{-1}) in 10 mM EDTA, 100 mM Tris HCl, 300 mM sodium chloride (NaCl), bovine serum albumin (0.2 mg ml^{-1}), pH 7.5
Buffer L2- 30% polyethylene glycol (PEG 6000), 3 M NaCl
Buffer L3- 100 mM Tris HCl, 100 mM NaCl, 20 mM EDTA
Buffer L4- 4% sodium dodecylsulphate (SDS)
Buffer L5- 2.556 M potassium acetate
Buffer QB- 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0
Buffer QC- 1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0
Buffer QF-1.2 M NaCl, 50 mM MOPS, 15 % ethanol, pH 8.0.

To 50 ml of lysate, 100 μl of buffer L1 was added and incubated (37 °C, 30 min). L2 buffer (10 ml, ice cold) was then added, mixed gently and incubated on ice (60 min). The contents were centrifuged (1200 rpm, 10 min, 4 °C) and the supernatant discarded. The pellet was resuspended in L3 buffer (3 ml) and then L4 buffer (3 ml) was added. After mixing the contents very

gently, the tube was incubated (70 °C, 20 min) and cooled on ice. L5 buffer (3 ml) was finally added, mixed and centrifuged (12000 rpm, 30 min, 4 °C). The supernatants containing DNA were further purified. The columns supplied along with the Qiagen 100 pack were used to purify the phage DNA. The columns were equilibrated with QB (2 ml) and the supernatants were added on to them. The columns were washed twice with QC (4 ml) and the bound DNA was eluted with QF (4 ml per column). The DNA was precipitated with iso-propanol (0.8 volumes, at room temperature). The contents were centrifuged (12000 rpm, 30 min, room temperature). The pellet was washed with 70% ethanol dried (3 min) in a freeze drier and redissolved in TE, pH 8 (20 μl) (Appendix A).

7.2.3.2 Subcloning procedure

Plasmid pUC18 was kindly given by Dr. S. Dymock, University of Bath. The vector was linearised with *Eco*RI enzyme (see section 6.2.2.3). An aliquot of the digestion mix was run on an agarose gel to observe whether the plasmid was effectively cut (section 3.2.1.3b). Lambda clones with inserts were digested with *Eco*RI and ligated to cut pUC18 plasmids. Ligations were carried out as described in section 6.2.2.5.

a. Transformation of Escherichia coli

Escherichia coli strain JM 101 was used with the standard chromogenic assay. The procedure described in section 6.2.2.7a was followed to obtain a pellet of JM 101 cells. The pellet was resuspended in 10 ml of 0.1 M calcium chloride, left on ice for 30 min to make cells competent, and centrifuged. The cells were used immediately or stored under liquid nitrogen in calcium chloride (0.1 M) with glycerol [15% (v/v)].

The ligated DNA mix (from section 7.2.3.2) was added to a cold Eppendorf tube containing competent cells (100 μl) and kept on ice (30 min), then transferred to a water bath (42 °C, 3 min). The contents were added to L-

broth (1 ml) prewarmed to 37 °C and incubated in a shaking incubator (30-60 min) to allow time for the expression of antibiotic resistance.

b. Screening for transformants

L-agar plates containing ampicillin (100 μg ml^{-1}) were made in 9 cm Petri dishes. An aqueous solution of isopropyl-thiogalactoside (IPTG) (100 mM) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) (2%) in dimethyl formamide were prepared and stored at -20 °C. The plates were dried and IPTG (50 μl of 100 mM) and X-gal (20 μl of 2%) were spread on each plate and left for 30 min. Aliquots of transformed *E.coli* cells (100 μl) were plated neat and at 10^{-1} and 10^{-2} dilutions. The plates were incubated 37 °C, overnight). In order to enhance the blue colour of the non-recombinants, the plates were placed in the refrigerator (4 °C for 4 h).

c. Determination of the sizes of inserts

Plasmid mini-preps (Maniatis *et al.*, 1982) were used to isolate plasmid DNA. The plasmid DNA was digested with *Eco*RI and run on an agarose gel (0.7%). Lambda DNA digested with *Dra* I was used as molecular weight markers, to determine the sizes of inserts.

7.2.4 Probing RNA with insert DNA

In order to investigate the levels of mRNAs in *in vitro* cultures, corresponding to cDNA sequences that are specific to shoot regenerating cultures, hybridisation of RNA on a slot blot and a Northern blot was carried out, using these cDNAs as probes.

7.2.4.1 Preparation of DNA to be labelled

Plasmid DNA was digested to completion with *Eco*RI and run on a 0.6% low melting point agarose (Sigma agarose, type vii) gel. The sections of the gel containing inserts were cut out and their weights were recorded. Assuming 1 g of agarose was equivalent to 1 ml, the concentration of DNA was calculated.

Prior to labelling, insert DNA was denatured by boiling in a water bath (7 min) and then transferred to another water bath at 37 °C (10 min).

7.2.4.2 Oligolabelling

The method developed by Feinberg and Vogelstein (1984) was used. The oligolabelling buffer was made by combining solutions A, B and C, in the ratio of 2:5:3. Compositions of A, B and C are given below.

A - 625 μl Tris-HCl 2 M (pH 8.0); 25 μl of magnesium chloride ($MgCl_2$) 5 M; 350 μl of water; 18 μl β mercaptoethanol; 5 μl dATP; 5 μl dGTP; 5 μl of dTTP (each dissolved in Tris-HCl 3 mM, EDTA 0.2 mM (pH 7.0) at a concentration of 0.1 M).

B - HEPES 2 M, titrated to pH 6.6 with NaOH.

C - Hexadeoxyribonucleotides in Tris-HCl 3 mM, EDTA 0.2 mM (pH 7.0) at 90 OD units ml^{-1}. The following components were added in the order given below.

	μl
water to make a volume of 15 μl	x
oligolabelling buffer	3
[a]bovine serum albumin (10 mg ml^{-1})	0.6
probe DNA, volume to give 10 ng	y
[b][α^{32}P] dCTP	1
[c]Klenow fragment of DNA polymerase	0.6
	15.0

[a] - enzyme grade BSA from Boehringer Mannheim, [b] - 10 μCi μl^{-1} (Amersham plc.), [c] - 1 unit μl^{-1} (Northumbria Biologicals Ltd).

7.2.4.3 Preparation of RNA slot blots

A commercially available slot blot apparatus (Bio Rad) was used to bind RNA on to Hybond-N membranes (Amersham plc.). The apparatus was assembled according to the instructions given in the manufacturer's manual. Hybond-N membranes were cut to the specified size (9 x 12 cm) and pre-wetted

in 10 x SSC. The membrane was placed in the apparatus and a slight vacuum was applied to enable flow of liquid through the apparatus. The flow was adjusted leaving sufficient time for the RNA to bind onto the membrane (100 μl min^{-1}). The RNA was denatured (65 °C, 5 min) in three volumes of the following solution.

	μl
formamide	500
formaldehyde (37% solution)	162
10 x MOPS buffer (Appendix A)	100

The denatured RNA was chilled on ice and an equal volume of 20 x SSC was added. Then the samples (200 μl) were loaded on to the apparatus and allowed to drain through the membrane by applying vacuum appropriately. The membrane was washed twice with 500 μl of 10 x SSC per slot and subjected to UV cross-linking by placing RNA side down on a UV-transilluminator (3 min).

7.2.4.4 RNA for Northern analysis

For Northern analysis, RNA was electrophoresed on a denaturing agarose gel and blotted onto Hybond-N membrane. The RNA on the membrane was probed with labelled insert DNA. Prior to electrophoresis, RNA was denatured by incubating (65 °C, 5 min) in the following solution:

	μl
RNA (final volume)	6.0
formamide	12.5
[a]10 x MOPS buffer	2.5
formaldehyde (37%)	4.0

a-see Appendix A.

The samples were chilled on ice, then glycerol [2.5 μl, 50% (v/v)] containing bromophenol blue (0.1 μg ml^{-1}) was added and the samples were run on an agarose gel (1.2%) prepared by adding the following.

agarose	1.2 g
10 x MOPS buffer	10.0 ml
formaldehyde (37%)	17.0 ml
water	73.0 ml.

The gel was electrophoresed in running buffer (1 x MOPS) at 100 V for 4 h. RNA was blotted onto the Hybond N by capillary blot according to the procedure recommended by Amersham. The RNA on the membrane was fixed by UV-cross linking (3 min on a UV transilluminator, with the RNA side facing the light).

7.2.4.5 Hybridisation of RNA with insert DNA

The protocol outlined by Amersham was followed to carry out the hybridisations. The prehybridisation solution was prepared by mixing the following.

5 x SSPE
5 x Denhardt's solution
0.5% (w/v) SDS
50% formamide

After adding 25 ml of the above solution to the membrane, 0.5 ml of salmon sperm DNA (1 mg ml^{-1}), denatured by boiling (5 min) in a water bath was added and incubated (42 °C, 4 h). The prehybridisation solution was replaced by hybridisaton solution (5 ml) of the same composition with the addition of the denatured probe, and incubated (42 °C, 12-24 h). The filters were washed twice in the following solutions (250 ml).

solution	time	temp.
2 x SSPE, 0.1% SDS	10 min	room temp.
2 x SSPE, 0.1% SDS	10 min	room temp.
1 x SSPE, 0.1% SDS	15 min	65 °C
0.1 x SSPE, 0.1% SDS	10 min	65 °C

The filter was then wrapped in Saran Wrap and exposed to Fuji X-ray film in a cassette with intensifying screens (-80 °C) for autoradiography.

7.2.4.6 Removal of probe and re-use of blots

The radio-labelled probe bound to the membrane was removed by pouring a boiling solution of 0.1% SDS on to the membrane and leaving until room temperature was attained. The radioactivity was monitored with a counter and if radioactivity was detectable the process of probe removal was repeated.

7.3 RESULTS

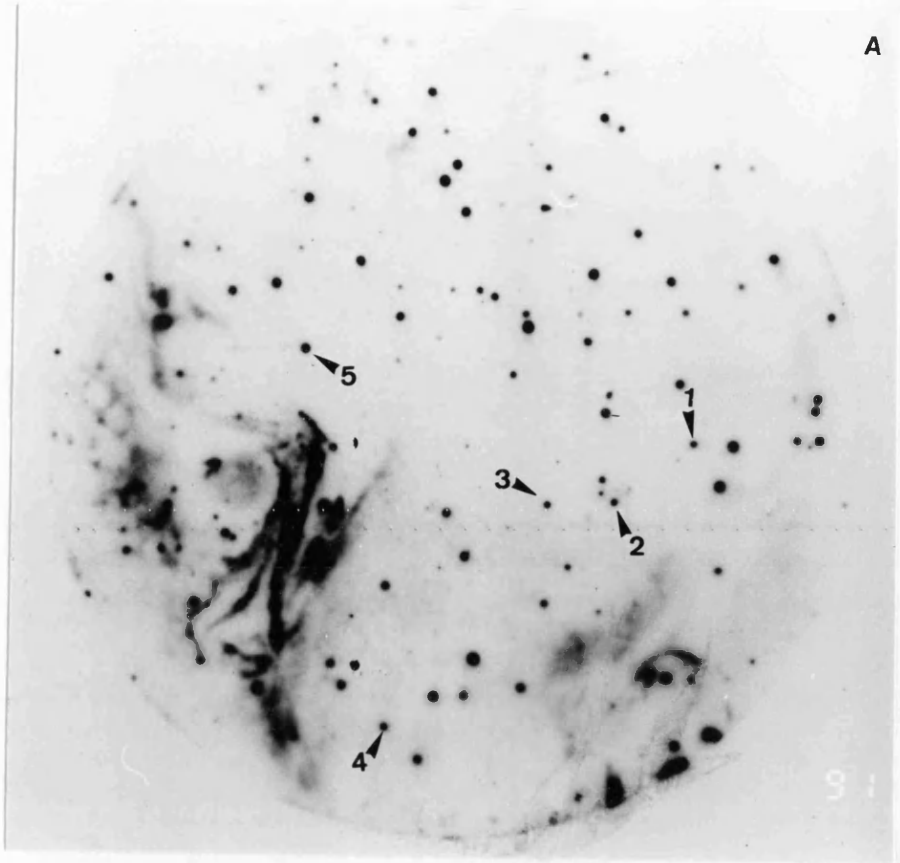
7.3.1 Differential hybridisation

The cDNA library was plated at a density of 10^4 pfu per 13 cm Petri dish and two replica filters were made as described in Materials and Methods. These were then probed with single stranded cDNA synthesised from mRNA isolated from explants grown for 2 days on second-stage CIM and SIM, according to the method described in section 7.2.2.1. This method yielded cDNA with specific activity greater than 10^8 cpm μg^{-1}. The comparison of the autoradiographs of filters hybridised with cDNA prepared from mRNA from 2d SIM (4d CIM) and 2d CIM (4d CIM) tissues showed 14 plaques which appeared to be specific to SIM cultures. On secondary screening, only five of these phages showed positive hybridisation with cDNA synthesised from 2d SIM (4d CIM) mRNA. (Plate 32). These were further purified by plating less than 100 plaques per Petri dish and repeating the process of hybridisation (Plate 33). The clones were designated as λSIM3, λSIM5, λSIM7, λSIM9 and λSIM11. To verify the presence and sizes of inserts in the five λ clones, DNA was isolated, digested with *Eco*RI and subjected to electrophoresis (1.5% agarose gel, 2.5 V cm^{-1} for 3 h) by Dr J.R. Beeching (Plate 34). The sizes of cDNA inserts in λSIM3, λSIM5, λSIM7, λSIM9 and λSIM11 were 298, 281, 281, 230 and 163 bp respectively.

Plate 32: Rescreening of SIM clones

The clones which appeared to be present only on filters hybridised with cDNA from 2d SIM (4d CIM) poly $(A)^+$ RNA were further purified by plating approximately 500 pfu per 13 cm plate and hybridising with single stranded cDNA (ss cDNA) synthesised from poly $(A)^+$ RNA from 2d SIM (4d CIM) and 2d CIM (4d CIM) cultures. The filters were washed as described in section 7.2.2.3 and subjected to autoradiography. 'A' corresponds to the hybridisation of plaques with ss cDNA derived from 2d SIM (4d CIM) poly $(A)^+$ RNA and 'B' corresponds to the hybridsation of plaques with ss cDNA derived from 2d CIM (4d CIM) poly $(A)^+$ RNA.

Some of the plaques which appeared only on A are marked by arrows and numbers and the respective plaque positions are circled and numbered in B.


A
5
1
3
2
4

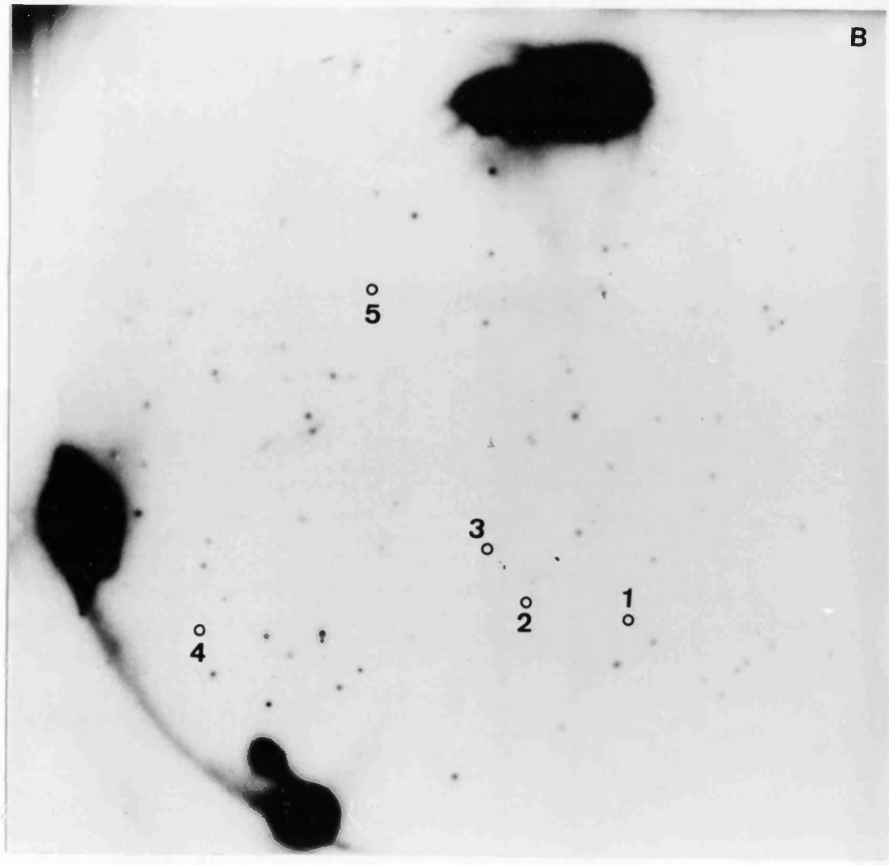
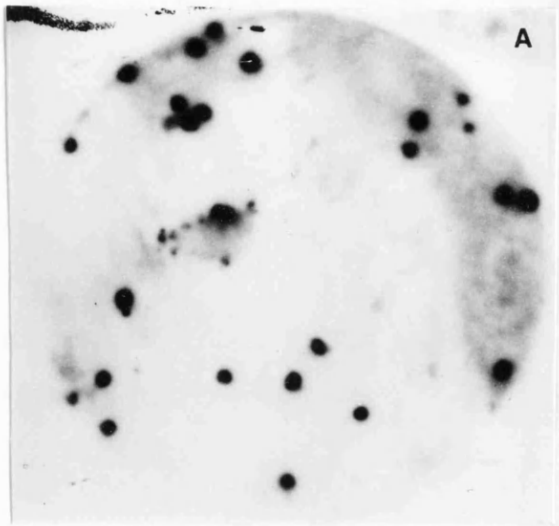

B
5
3
1
2
4

Plate 33: Purification of one of the SIM clones (LSIM3).

The clones after secondary screening were further purified by plating at approximately 100 plaques per 9 cm plate and hybridising with ss cDNA derived from 2d SIM (4d CIM) poly $(A)^+$ RNA and 2d CIM (4d CIM) poly $(A)^+$ RNA. The filters were washed as described in section 7.2.2.3 and subjected to autoradiography.

A: hybridisation of clone LSIM3 with ss cDNA derived from 2d SIM (4d CIM) poly $(A)^+$ RNA.
B: no hybridisation occurred with ss cDNA derived from 2d CIM (4d CIM) poly $(A)^+$ RNA.


A

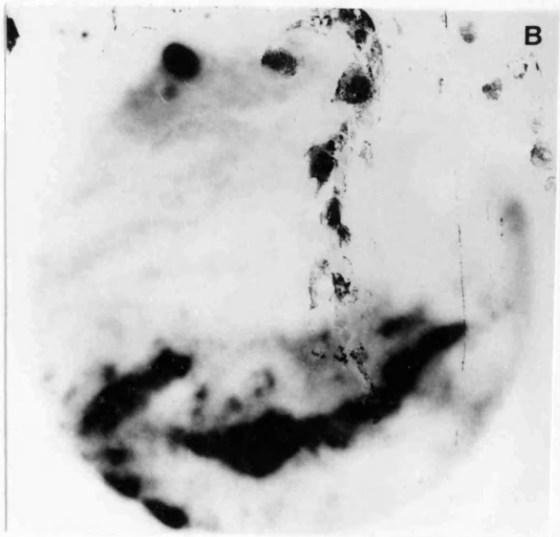
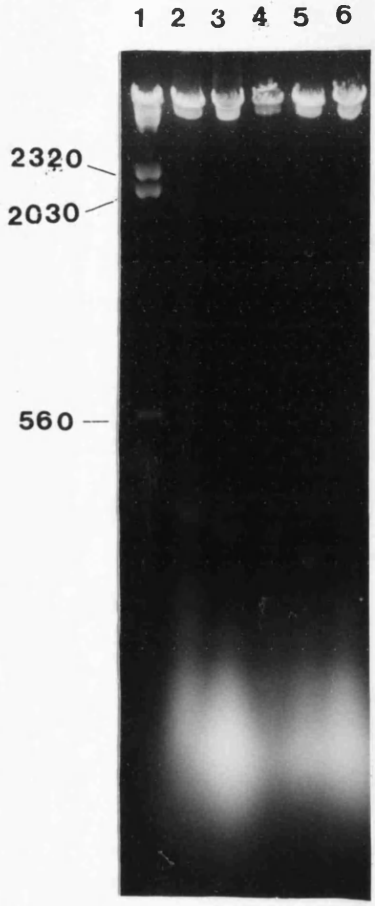

B

Plate 34: Determination of presence and sizes of inserts in λ clones.

Recombinant λ DNA was isolated from SIM specific clones, digested with *Eco* RI and λ wild type DNA digested with *Hin*dIII (molecular weight markers) were subjected to electrophoresis on a 1.5% agarose gel. The sizes of inserts were calculated by extrapolating the standard curve obtained by plotting the sizes of the molecular weight markers against the distance travelled by DNA fragments.

lane: 1-size markers; lanes 2-6: cDNA inserts 3, 5, 7 9 and 11, of sizes 298, 281, 281, 230 and 163 bp respectively.


1 2 3 4 5 6
2320
2030
560

7.3.2 Subcloning of inserts into plasmid vector

DNA was purified from the lambda phage clones, cut with *Eco*RI and the inserts were sub-cloned into *Eco*RI-cut pUC18 using *E. coli* JM 101 as host.

7.3.3 Purification of insert DNA from plasmids

Plate 35 shows the *Eco* R1 digests of recombinant plasmids. The presence of five inserts is evident. The sizes of inserts 3, 5, 7, 9 and 11 were 316, 288, 288, 251 and 346 bp respectively. The low melting point agarose gel run with *Eco*RI digests of clones, showed only three inserts 7, 9 and 11, the sizes of which were 290. 224 and 316 bp respectively (Plate 36). The inserts were designated as SIM7, SIM9 and SIM11. These three inserts were used as probes to find the corresponding sequences in mRNAs in shoot organogenic and callus tissues.

It must be borne in mind that the sizes of inserts given here may not be accurate, as the graphs representing molecular weight markers were extrapolated to calculate the sizes of inserts. However, three different gels yielded approximately similar sizes for the inserts, so the values can be considered as reliable estimates of the true sizes.

7.3.4 Levels of SIM specific mRNA during culture

7.3.4.1 Probing RNA on a slot blot

Since shoots were visible after 8 days on SIM, any changes in mRNAs pertaining to shoot organogenesis would be expected to occur during the first 8 days on SIM. Hence RNA isolated from 0-11 days from second stage CIM and SIM cultures were probed with inserts to measure mRNA levels corresponding to cDNA inserts. 10 μg of total RNA per slot was loaded in three replicates from each RNA sample, as described in section 7.2.4.3. The membrane was hybridised initially with the cDNA SIM7. The results obtained are shown in Plate 37; there was hardly any difference in intensity of hybridisation of SIM7 to RNA samples isolated through the time course.

Plate 35 (top): Analysis of sizes of inserts.

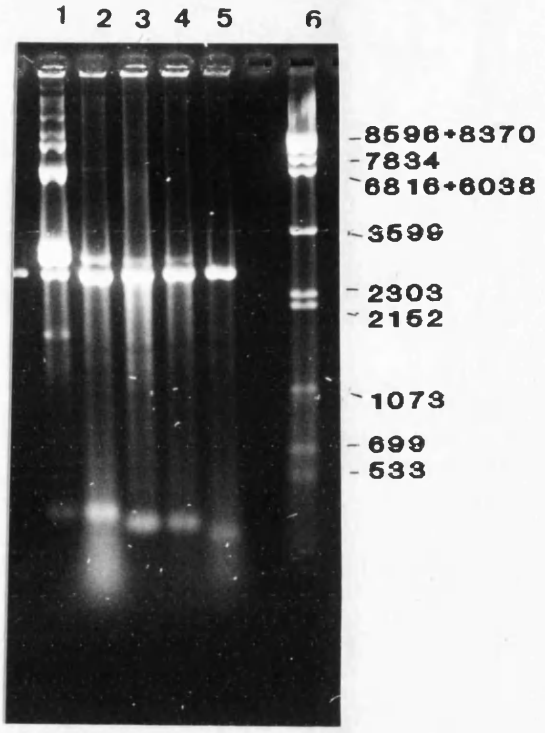
Recombinant pUC18 clones digested with *Eco*RI and λ wild type digested with *Dra*I were run on a 0.8% agarose gel. The sizes of inserts were calculated by extrapolating the standard curve obtained by plotting the sizes of the molecular weight markers against the distance travelled by DNA fragments.

lanes 1-5: The sizes of cDNA inserts 11, 3, 5, 7 and 9 were 346, 316, 288, 288 and 251 bp respectively; lane 6: size markers.

Plate 36 (bottom): cDNA inserts on a 0.6% low melting point agarose gel.

Recombinant pUC18 DNA digested with *Eco*RI and λ wild type DNA digested with *Hin* dIII (molecular weight markers) were electrophoresed on a low melting point agarose gel. The sizes of inserts were calculated by extrapolating the standard curve obtained by plotting the sizes of the molecular weight markers against the distance travelled by DNA fragments.

lane 1: size markers; lanes 2-6: cDNA inserts 3, 5, 7, 9 and 11. Only inserts 7, 9, and 11 of sizes 290, 224 and 316 bp respectively were present.


1 2 3 4 5 6
8596+8370
7834
6816+6038
3599
2303
2152
1073
699
533

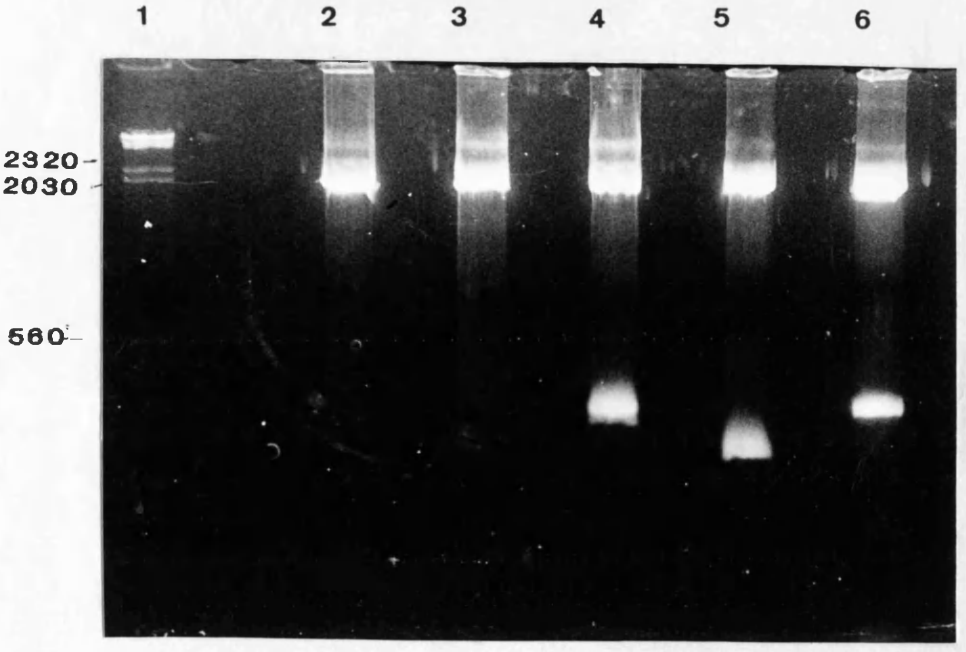
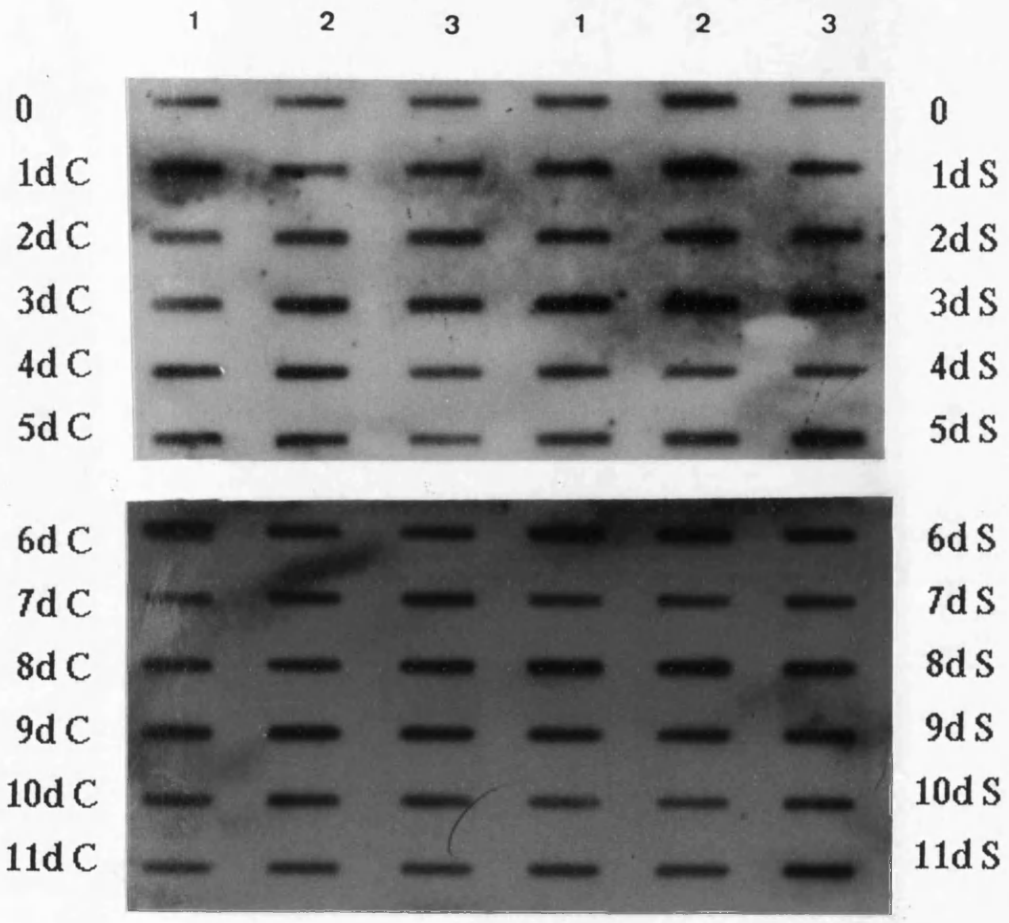

1 2 3 4 5 6
2320
2030
560

Plate 37: Measurement of mRNA levels corresponding to SIM7.

cDNA insert 7 (SIM7) was oligolabelled as described in section 7.2.4.2 and hybridised with RNAs (10 μg per slot; 3 replicates per sample) on slot blots. The filters were washed as described in section 7.2.4.5 and subjected to autoradiography.

slot	sample	slot	sample
0	4d CIM	0	4d CIM
1d C	1d CIM (4d CIM)	1d S	1d SIM (4d CIM)
2d C	2d CIM (4d CIM)	2d S	2d SIM (4d CIM)
3d C	3d CIM (4d CIM)	3d S	3d SIM (4d CIM)
4d C	4d CIM (4d CIM)	4d S	4d SIM (4d CIM)
5d C	5d CIM (4d CIM)	5d S	5d SIM (4d CIM)
6d C	6d CIM (4d CIM)	6d S	6d SIM (4d CIM)
7d C	7d CIM (4d CIM)	7d S	7d SIM (4d CIM)
8d C	8d CIM (4d CIM)	8d S	8d SIM (4d CIM)
9d C	9d CIM (4d CIM)	9d S	9d SIM (4d CIM)
10d C	10d CIM (4d CIM)	10d S	10d SIM (4d CIM)
11d C	11d CIM (4d CIM)	11d S	11d SIM (4d CIM)

The intensities of all the bands are similar.


1
2
3
1
2
3
0
1d C
2d C
3d C
4d C
5d C
0
1d S
2d S
3d S
4d S
5d S
6d C
7d C
8d C
9d C
10d C
11d C
6d S
7d S
8d S
9d S
10d S
11d S

The probe was stripped from the membrane and then the membrane was reprobed with SIM9. Plate 38 represents the autoradiograph of the membrane. It was seen that the mRNA corresponding to SIM9 was more abundant during the first three days after transfer to SIM as compared to CIM. The degree of intensity of the band corresponding to 6d CIM (4d CIM) was similar to that of 1d SIM (4d CIM) RNA. An increased level of mRNA SIM9 was seen again in 11d SIM (4d CIM) cultures.

The probe was stripped and the membrane was hybridised with SIM11. The results are given in Plate 39. The intensities of all the bands were relatively low as compared to those obtained by hybridising with either SIM7 or SIM9, even though the membrane hybridised with SIM 11 was subjected to a longer exposure.

7.3.4.2 RNA on a Northern blot

10 μg aliquots of total RNA isolated from 1-5 day second-stage SIM and CIM cultures were run on a denaturing gel and blotted onto Hybond-N as described in section 7.2.4.4. The RNA on the filter was probed with oligolabelled SIM9. Plate 40 represents the results of this Northern analysis. SIM9 showed hybridisation to RNA isolated from days 1-4 days SIM [1d SIM (4d CIM)-4d SIM (4d CIM)] cultures and day 5 second-stage CIM cultures [5d CIM (4d CIM)]. The maximum hybridisation was seen with RNA from 3d SIM (4d CIM) cultures.

7.4 DISCUSSION

Plasmids carrying putative SIM specific clones when digested with *Eco* RI and run on an agarose gel showed the presence of inserts in all five plasmids (Plate 35). This DNA was isolated from mini-preps. When the same plasmids isolated from a larger volume of cells were digested and run on a low melting point agarose gel only three inserts were present. The plasmids all appear to have digested completely. Therefore it is possible that the inserts from clones 3

Plate 38: Measurement of mRNA levels corresponding to SIM9.

The slot blot that was hybridised with SIM7 was deprobed and used to hybridise with SIM9. cDNA insert 9 (SIM9) was oligolabelled as described in section 7.2.4.2 and hybridised with the slot blot. The filters were washed as described in section 7.2.4.5 and subjected to autoradiography.

slot	sample	slot	sample
0	4d CIM	0	4d CIM
1d C	1d CIM (4d CIM)	1d S	1d SIM (4d CIM)
2d C	2d CIM (4d CIM)	2d S	2d SIM (4d CIM)
3d C	3d CIM (4d CIM)	3d S	3d SIM (4d CIM)
4d C	4d CIM (4d CIM)	4d S	4d SIM (4d CIM)
5d C	5d CIM (4d CIM)	5d S	5d SIM (4d CIM)
6d C	6d CIM (4d CIM)	6d S	6d SIM (4d CIM)
7d C	7d CIM (4d CIM)	7d S	7d SIM (4d CIM)
8d C	8d CIM (4d CIM)	8d S	8d SIM (4d CIM)
9d C	9d CIM (4d CIM)	9d S	9d SIM (4d CIM)
10d C	10d CIM (4d CIM)	10d S	10d SIM (4d CIM)
11d C	11d CIM (4d CIM)	11d S	11d SIM (4d CIM)

Increase in levels of mRNAs corresponding to SIM9 is seen in 1d SIM (4d CIM), 2d SIM (4d CIM), 3d SIM (4d CIM), 11d SIM (4d CIM) and 5d CIM (4d CIM) samples (labelled as 1d S, 2d S, 3d S, 11d S and 5d C respectively).

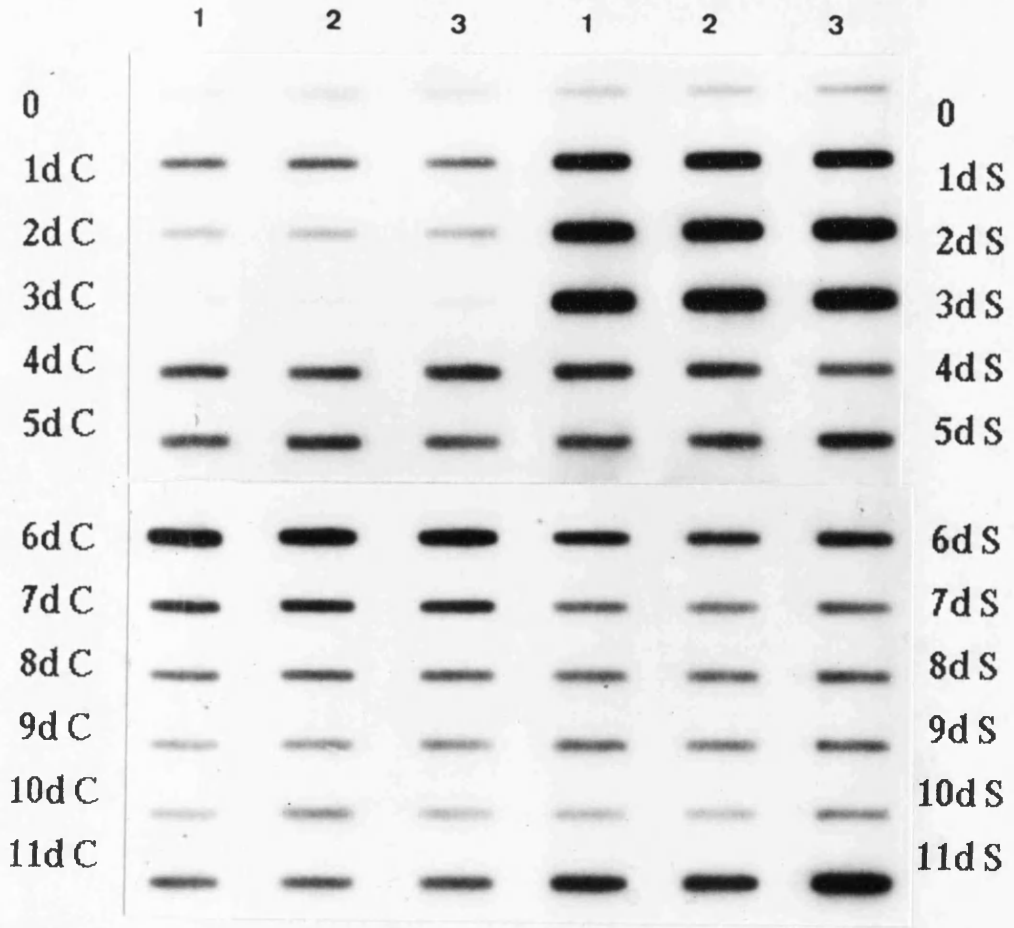

1
2
3
1
2
3
0
1d C
2d C
3d C
4d C
5d C
6d C
7d C
8d C
9d C
10d C
11d C
0
1d S
2d S
3d S
4d S
5d S
6d S
7d S
8d S
9d S
10d S
11d S

Plate 39: Measurement of mRNA levels corresponding to SIM11.

The slot blot that was hybridised with SIM9 was deprobed and used to hybridise with SIM11. cDNA insert 11 (SIM11) was oligolabelled as described in section 7.2.4.2 and hybridised with the slot blot. The filters were washed as described in section 7.2.4.5 and subjected to autoradiography.

slot	sample	slot	sample
0	4d CIM	0	4d CIM
1d C	1d CIM (4d CIM)	1d S	1d SIM (4d CIM)
2d C	2d CIM (4d CIM)	2d S	2d SIM (4d CIM)
3d C	3d CIM (4d CIM)	3d S	3d SIM (4d CIM)
4d C	4d CIM (4d CIM)	4d S	4d SIM (4d CIM)
5d C	5d CIM (4d CIM)	5d S	5d SIM (4d CIM)
6d C	6d CIM (4d CIM)	6d S	6d SIM (4d CIM)
7d C	7d CIM (4d CIM)	7d S	7d SIM (4d CIM)
8d C	8d CIM (4d CIM)	8d S	8d SIM (4d CIM)
9d C	9d CIM (4d CIM)	9d S	9d SIM (4d CIM)
10d C	10d CIM(4d CIM)	10d S	10d SIM (4d CIM)
11d C	11d CIM(4d CIM)	11d S	11d SIM (4d CIM)

The intensity of hybridisation was very low.

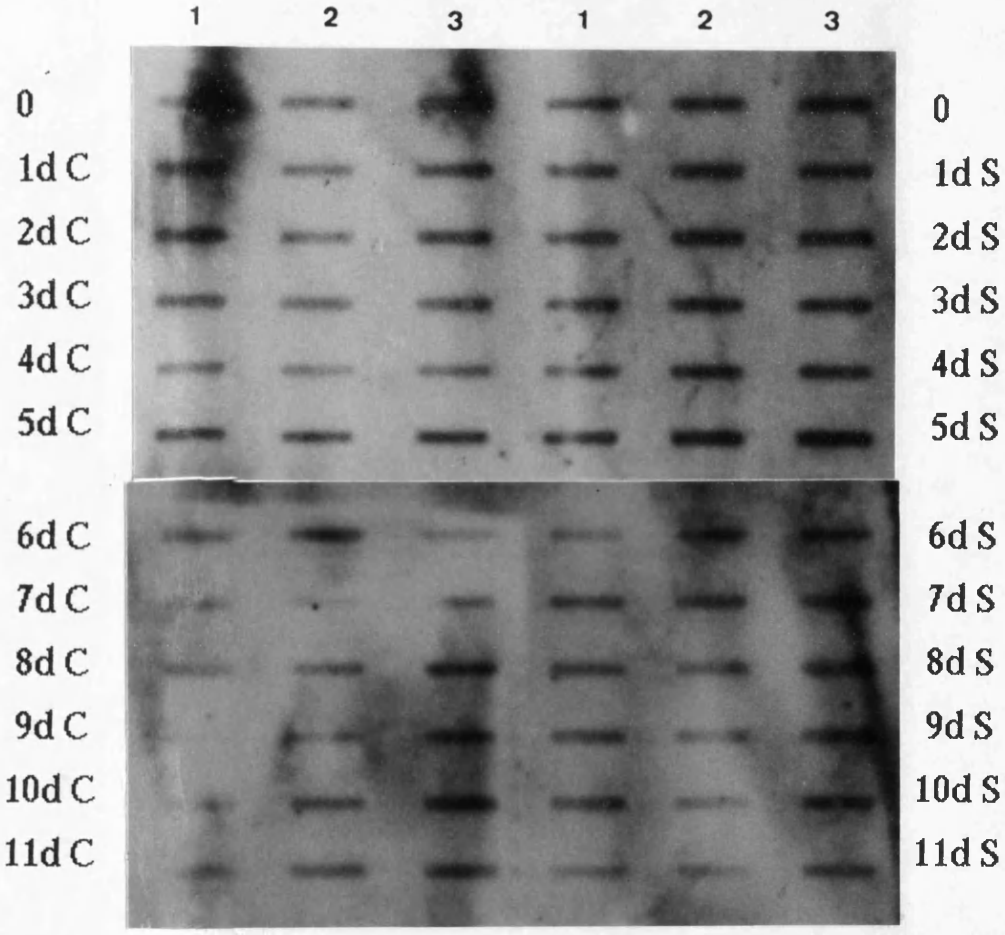
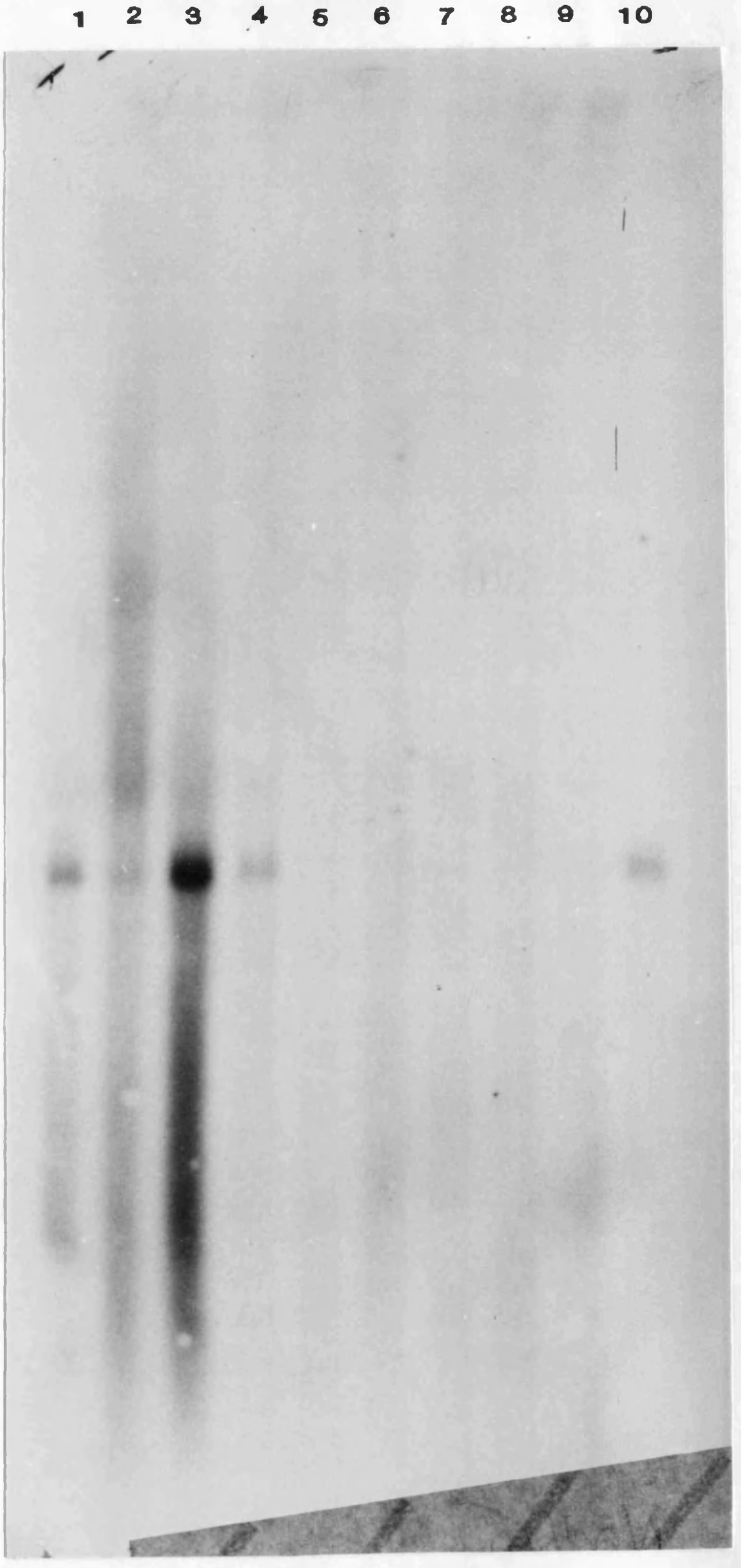

1
2
3
1
2
3
0
1d C
2d C
3d C
4d C
5d C
6d C
7d C
8d C
9d C
10d C
11d C
0
1d S
2d S
3d S
4d S
5d S
6d S
7d S
8d S
9d S
10d S
11d S

Plate 40: Measurement of mRNA levels corresponding to SIM9 by Northern analysis.

Total RNA was denatured and run on a 1% agarose gel under denaturing conditions and blotted onto the Hybond N filter (section 7.2.4.4). cDNA insert 9 (SIM9) was oligolabelled as described in section 7.2.4.2 and hybridised with the Northern blot. The filters were washed as described in section 7.2.4.5 and subjected to autoradiography.

lane	sample
1	1d SIM (4d CIM)
2	2d SIM (4d CIM)
3	3d SIM (4d CIM)
4	4d SIM (4d CIM)
5	5d SIM (4d CIM)
6	1d CIM (4d CIM)
7	2d CIM (4d CIM)
8	3d CIM (4d CIM)
9	4d CIM (4d CIM)
10	5d CIM (4d CIM)

Increase in levels of mRNA corresponding to SIM9 is seen in 1d SIM (4d CIM), 2d SIM (4d CIM), 3d SIM (4d CIM) and 5d CIM (4d CIM) samples (labelled as 1, 2, 3 and 10 respectively).


1 2 3 4 5 6 7 8 9 10

and 5 were lost due to instability during subculturing between the two plasmid isolations. It was decided to use the three inserts instead of repeating the insert DNA isolation and digestion, as time available for the completion of the project was limited.

Hybridisation of RNA on the slot blot with SIM7 did not show any difference between SIM and CIM cultures. This may indicate that mRNA corresponding to SIM7 is present at equal levels in both types of cultures. This contrasts with the results of the differential screening which showed that ss cDNA synthesised from 2d CIM (4d CIM) poly $(A)^+$ RNA did not appear to hybridise with λSIM7. Therefore, probably the differential screening was unreliable in this case. However, as a control sample was not included along with experimental RNA, to check for non-specific binding under the experimental conditions used, possible hybridisation of SIM7 non-specifically to RNA from CIM cultures cannot be excluded.

Reprobing of the same membrane with SIM9 showed an increase in the level of the corresponding mRNA during the first three days on SIM. The Northern blot (Plate 40) also showed a peak at day 3. In the Northern blot, although there was a slight background smear, which would have been due to degradation of RNA, SIM9 gave a single band on the gel, suggesting that it was hybridising to a single species of mRNA. The size of the hybridising mRNA cannot be determined because the molecular weight markers were not visible on the gel. It would be necessary to repeat the experiment to determine the size of the band.

The lack of hybridisation to some tracks of the Northern blot probably reflects a much reduced level of the mRNA corresponding to SIM9 in those samples. However, the possibility that RNA degradation had occurred cannot be excluded. Since subsequent hybridisation of the same Northern blot with a chitinase cDNA clone (Appendix D) showed that all tracks contained

undegraded RNA, it is suggestive that the observed variation in mRNA corresponding to SIM9 during the time course of the experiment was genuine.

To quantify the amounts of mRNA in different samples at different times, the Northern and slot blotting experiments would have to be repeated using known amounts of control RNA as standards and by using preflashed autoradiography film to ensure that the response of the film was directly proportional to the amount of radioactivity present.

There is a discrepancy between the Northern and slot blots regarding the time when the SIM9 mRNA level peaks in CIM cultures. In the slot blot, the levels on days 4 and 5 are roughly equal to that in SIM cultures of the same age, and by day 6 the level has reached that on day 1 on SIM. In the Northern blot, though, no hybridisation to RNA is visible in the day 4 CIM track, even though there is a signal on day 5. One possible explanation is that the RNA preparations used for the Northern blot were not the same as those used for slot blotting. It is possible that the RNA extraction from callus was variable as callus cultures are known to show great variation in morphological, cytological and biochemical status (Tran Thanh Van, 1981). Hence it may be possible to get such variation of a particular mRNA at different stage in culture. Since a 6d CIM (4d CIM) RNA sample was not included in the Northern blot it cannot be certain whether the particular message is absent in 6d CIM (4d CIM) samples. The bands appearing on a Northern blot are more reliable as the RNA species are separated and therefore give a more accurate picture of mRNAs than slot blots, and the autoradiographic signal is the measure of the concentration of the specific RNA, provided the film is preflashed.

The slot blot membrane, after removal of SIM9 and reprobing with SIM11, showed a very low intensity of hybridisation. The gel in Plate 34 showed the sizes of λSIM11 was 163 bp. However, Plates 35 and 36 showed that the size of SIM11 subclone (in pUC18) was around 300 bp. This suggests that during subcloning an extraneous piece of DNA was ligated to the cut pUC18

DNA. This would explain the observation that there was only faint hybridisation of SIM11 to the RNA slot blot, since there would have been little or no homology between the probe and the RNA on the blot.

Out of the three inserts SIM7, SIM9 and SIM11 only SIM9 showed varying levels of mRNA during the time course according to the slot blotting. The level of SIM9 mRNA in SIM cultures were highest in 3d SIM (4d CIM) cultures. It is not exclusively present in SIM cultures as a somewhat lower level of SIM9 mRNA was evident in 6d CIM (4d CIM) cultures and maybe in 5d CIM (4d CIM) cultures.

It is therefore possible that this sequence could be associated with any of the early changes brought about at the gene level by SIM conditions. The results suggest that the mRNA corresponding to SIM9 is not associated with dedifferentiation as this process would have been completed during the first-stage culture on CIM (Christianson and Warnick, 1988).

Histological studies revealed rapid cell division and tracheid formation in 4d SIM (4d CIM) cultures (section 2.3.3). Hence mRNAs pertaining to these processes would be expected to be present prior to such changes. The appearance of tracheids was also seen in callus cultures but at a later stage than in SIM cultures, so the later peak in SIM9 in CIM cultures could be related to the later appearance of mRNA levels involved in these processes in CIM cultures. The different timing of tracheid formation in the two culture types could be due to the differing hormone regimes. It has been shown that a high cytokinin/auxin ratio (1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA) was required to bring about xylogenesis and cell division and a low cytokinin/auxin ratio (0.001 mg l^{-1} BAP and 0.1 mg l^{-1} NAA) was functional in bringing about only cell division in *Zinnia* mesophyll cell cultures (Fukuda and Komamine, 1982). They reported that cell wall bound peroxidase activity was highest at 72 h (just before lignification) and 84 h (during active lignification) and that phenylalanine ammonia lyase activity was highest between 72-96 h in cultures with tracheary element differentiation.

Subsequently they reported that the synthesis of some RNA and proteins between 24-60 h was correlated to xylogenesis (Fukuda and Komamine, 1983). There are some reports of tissue cultures where xylogenesis occurred only in the presence of auxin without any cytokinin in the medium, although most *in vitro* systems have a dual requirement for auxin and cytokinin in the initiation of xylogenesis. Hence, most of these systems which showed xylogenesis only in the presence of auxin may have contained endogenous cytokinin. For example, although exogenously applied auxin caused xylogenesis in *Helianthus tuberoses* the tuber contained endogenous cytokinin (Roberts, 1988). It has been also found that carrot cells synthesised cytokinin during culture (Linstedt and Reinert, 1975). Xylogenesis in tobacco pith tissues in the complete absence of cytokinin was assumed to be due to the occurrence of cytokinin biosynthesis in prolonged cultures (Fosket, 1980). Tucker *et al*. (1986) suggested that the cytokinin dependent determination event of tracheary element formation in lettuce pith occurred earlier than the same process involving auxin. Therefore, it is a possibility that this mRNA (SIM9) is involved in xylem differentiation which occurred early in the presence of BAP in SIM and somewhat later in CIM cultures due to combination of endogenous cytokinin with depleted levels of 2,4-D during culture.

An increase in metabolic activities during organogenesis has been observed by Thorpe (1980). Therefore SIM9 may correspond to a mRNA which produces an enzyme involved in such high metabolic activity during rapid cell division and xylem tissue differentiation under SIM conditions and to a lesser extent under CIM conditions.

An increased level of SIM9 was also seen in 5d CIM (4d CIM) cultures. If SIM9 was unique to shoot organogenesis, presence of some mRNAs corresponding to SIM9 in CIM cultures would indicate that shoot production occurred in CIM cultures. Both histological studies and visual observations

proved the absence of any shoot formation in CIM cultures, ruling out the possibility that SIM9 was unique to shoot organogenesis.

Prior to any organogenic event meristematic cells must be present. It is possible that SIM9 may represent a mRNA involved in meristematic cell formation in both culture types. The possibility of existence of meristematic cells common to root or shoot organogenesis and also embryogenesis has been reported (Tran Thanh Van, 1981). There are some examples where cells were undetermined initially, and therefore can develop into either a shoot or a root. For example, cultured *Selaginella* root tips can develop into shoots, if auxin was withheld and *Nasturium* meristems which develop into shoots can be made to form roots by the application of auxin (Lyndon, 1990). In this situation it is also possible that the mRNA corresponding to SIM9 produces a protein common to organogenesis: root or shoot. In order to decide whether such an explanation is plausible, the timing of meristem formation in the cultures must be analysed. Since the appearance of tunica like tissue was seen in 4 day old SIM cultures (section 2.3.3), meristematic tissue formation must have occurred much earlier. Meada and Thorpe (1979) reported the appearance of meristematic tissues determined to form shoots that were in association with patches of tracheids in tobacco callus cultures. Gronroos and von Arnold (1985) observed the presence of meristematic tissues next to tracheid nests and tracheid nests were considered to be a prerequisite for root formation in *Pinus*. Since nodules of tracheids were seen in 4 day SIM cultures, meristematic tissue formation may have occurred prior to this. In CIM cultures, organogenesis seemed to occur much later, so the associated mRNA accumulation would be later. The incidence of shoot formation in SIM cultures was much higher than endogenous root formation under CIM condition. Therefore a higher level of expression of SIM9 can be expected in SIM cultures than in CIM cultures.

Other work suggests that root and shoot organogenesis do not start from a common primordium. Results obtained by Christianson and Warnick (1985)

with *Convolvulus* showed that determination of meristematic tissues to form either shoots or roots occurred as a one-step process rather than via an undefined primordium; in most cases the determination took place within 1-3 days in the presence of the organ inducing hormone. Since histological studies of tobacco leaf explants during shoot organogenesis showed a close resemblance to the observations made by Christianson and Warnick (1988) during *in vitro* shoot formation in *Convolvulus* , it is likely that the developmental pathway is similar at the cellular and gene level. This suggests that it is unlikely that such meristematic tissues capable of producing either shoot or root occur in tobacco explants, and argues against the hypothesis that the mRNA corresponding to SIM9 represents a message that appears during formation of meristematic tissues.

The percentage of cells involved in organogenesis cannot be estimated unless the whole series of histological sections is taken at very short intervals during the entire period of organogenesis. Even then the cells capable of receiving the organogenic stimulus (meristemoids) cannot be identified easily with the staining procedure adopted. Meins (1986) reported that meristemoids are rare (one in ten thousand cells). This gives an idea of the abundance of any mRNA which may be responsible for the initial stages in the shoot induction process. The changes occurring after receiving the stimulus of organogenesis may be so rapid that a particular mRNA involved in the process of shoot induction (as a 'defined state') may be present only for a very short period. This would make the detection of these mRNAs very difficult. If a sequence of events are taking place in the group of cells involved in organogenesis, where each neighbouring cell has a different function, then the mRNA population in each cell may also differ accordingly. Identification of such variation would be extremely diffcult as the changes may be so minute that they remain undetectable.

The method of differential screening used in this study may not be efficient enough to ascertain changes during shoot induction process at the gene

level. It has been estimated that mRNA representing 0.1% of total mRNA can be detected by +/- differential screening (Sargent, 1987). A better method of detecting rare messages (approximately 0.01% of total mRNA) is the construction of a subtraction library or screening the library made out of entire population of poly $(A)^+$ RNA with subtraction cDNA. In this procedure, cDNA from one type of tissue (A) is hybridised with mRNA of the related tissue (B) where some mRNAs are absent or present at very low levels. The sequences common to both A and B are removed by passing through a hydroxyapatite column and the unhybridised cDNA which is enriched for messages specific to A is used to construct a library or to use as probes to screen the library made out of total poly $(A)^+$ RNA. However, such a technique may not be suitable when the cDNAs are short because when the cDNA is of a small size the annealing of cDNA to some mRNAs may not be sufficient to hold the products on the hydroxyapatite column. This would yield cDNAs that were not properly subtracted. Since the cDNA obtained (Chapter 6) was mostly around 500 bp it might not have been suitable to use these cDNAs to construct a subtraction library.

In the alternative method of +/- differential hybridisation, it is essential to maximise the abundance of mRNA sequences by selecting suitable culture conditions, comparing the developmental stage with maximum availability of particular mRNAs with a related tissue in which such a developmental stage is absent (Sargent, 1987). In this study, the tissue culture systems established showed high incidence of shoot formation, and no shoot formation in the control tissue. Even so, for the reasons given above, it is unlikely that shoot specific messages pertaining to the shoot induction event could be detected using the method adopted in this experiment. Therefore it is very likely that SIM9 corresponds to a mRNA that is associated with one of the predominant hormone-induced events occurring in SIM cultures, e.g., xylogenesis. For instance, the protein coded by this mRNA could be an enzyme associated with

lignification, such as phenylalanine ammonia lyase, peroxidases or an enzyme involved in the biosynthesis of wall components. The possibility of this mRNA coding for an enzyme involved in any other process common to CIM and SIM cultures cannot be excluded.

8 CONCLUSION

In order to study gene expression during regeneration of shoots from tobacco tissue cultures, two culture systems were established; one which formed only shoots with very little callus (SIM) and the control where only callus was formed with endogenous root primordia in some cultures (CIM). In order to prevent the wound responsive genes masking the shoot-regenerating genes, a two stage culture system was used, where the leaf explants were cultured on first-stage CIM followed by transfer either to second-stage SIM (for shoot organogenesis) or to second-stage CIM as a control. The reason for transferring the control leaf pieces to fresh CIM of the same composition after a first-stage on CIM was to minimise any possible differences due to subculture, since Bevan and Northcote (1981a) showed that subculture itself could cause an increase in polysome levels and induction of mRNAs.

Developmental measurements of explants grown on the two culture systems showed a greater increase in fresh weight per explant in SIM cultures than in CIM cultures over the same period. Explants transferred from first-stage CIM to second-stage SIM turned greener and shoot primordia were visible in 8 day SIM cultures. Cytokinin is known to have a stimulatory effect on chloroplast development (Parthier *et al.*, 1981; Bracale, 1988) and therefore the BAP in SIM would have enhanced chlorophyll and plastid formation in SIM cultures. Explants transferred from first-stage CIM to second-stage CIM continued to produce callus at the cut edges and the central region of the explant turned reddish brown with some pale green patches. This discoloration may be due to the disruptive effect of 2,4-D on plastid structure (Kaminek *et al.*, 1981) or may be due to enhanced ethylene formation caused by the 2,4-D resulting in a response similar to senescence.

Explants after 2 days on the first-stage CIM showed cell enlargement. The period of cell enlargement is regarded as the period where cells accumulate RNA, proteins and polysaccharides for subsequent cell division and change in

morphology in *in vitro* cultures (Fukuda and Komamine, 1985). Transfer of explants to SIM resulted in active cell division and differentiation and patches of tracheids appeared in 4 day old SIM cultures. Shoot apex formation was evident with the development of a tunica like structure in 4 day SIM cultures and rapid emergence of shoot primordia was seen in 8 day SIM cultures. CIM cultures showed callus composed of large vacuolated cells and some cultures showed the appearance of root initials after 8 days on second-stage CIM. Endogenous root primordia were seen in some of the 12 day old CIM cultures. To summarise, all the SIM cultures produced shoots, and CIM cultures produced mainly callus with occasional endogenous root primordia formation. This was in accordance with the basic findings of Skoog and Miller (1957): a high auxin/cytokinin ratio enhanced callus and/or root production and high cytokinin/auxin ratio favoured shoot production. Since the two systems selected produced contrasting morphological phenomena, it was considered to be appropriate to look at the changes in mRNA population in the two types of cultures.

RNA isolation was carried out with first-stage CIM, second-stage CIM and second-stage SIM cultures (chapter 3). Total RNA accumulation was found to be greater in second-stage SIM cultures than second-stage CIM cultures. There was also a slight increase in poly $(A)^+$ RNA per gram of tissue in second-stage SIM cultures compared with second-stage CIM cultures. It has been reported that cytokinins increase RNA synthesis, especially ribosomal RNA, and polysome formation (Neumann and Khokhlova, 1981). Therefore the increased accumulation of total RNA may be mainly due to increased synthesis of rRNA influenced by BAP in SIM. Tan and Liang (1991) reported a decrease in rRNA (especially 5S species) and tRNA and an increase in RNase activity in tobacco callus cultures by day 10 in callus induction medium containing 2,4-D (2 mg l^{-1}) and BAP (0.5 mg l^{-1}). They suggested that this decrease was due to initiation of senescence similar to the process occurring in detached leaves. Therefore the

decrease in RNA observed in second-stage CIM may be due to a process similar to senescence. The slight increase in poly $(A)^+$ RNA may be related to the increased rate of cell division and the rapid anatomical changes that occurred in SIM cultures; this is consistent with the observation that there is high metabolic activity accompanied by increased production of enzymes in shoot-regenerating cultures (Thorpe, 1980).

In vivo labelling of proteins of day 0 (before culturing) samples showed qualitative and quantitative differences when compared to 2d NHM, 2d CIM or 2d SIM *in vitro* cultures. These changes may be related to the process of dedifferentiation (Christianson and Warnick, 1988) including activation of wound response genes as an initial response to injury of the plant material. Qualitative and quantitative changes in the mRNA population between day 0 and 2d CIM (4d CIM) samples may also be due to the completion of the dedifferentiation process under *in vitro* conditions. There were no qualitative differences between *in vitro* translated products of one to eight day [1d SIM (4d CIM) - 8d SIM (4d CIM)] cultures. Similar results have been obtained by Christianson and Warnick (1988) in association with root organogenesis in *Convolvulus*. They observed changes in protein profile within 2 days in culture, which thereafter remained the same for 14 days, before detecting changes which could have been due to formation of roots. They concluded that the dedifferentiation was completed during the first two days in culture.

Transfer of explants from first-stage CIM to second-stage SIM resulted in a change in levels of some proteins. Cytokinins singly or in combination with other growth substances (mainly auxins) modify a wide range of developmental or physiological events including cell division, organogenesis and plastid differentiation. Therefore some of the proteins detected as differences in SIM and CIM cultures may be related to events taking place in some of the above mentioned biological processes under the influence of the BAP in SIM. Fosket and Tepfer (1978) suggested that cytokinin regulates events at G2 or transition

from G2 to mitosis in the cell cycle. They observed the appearance of four polypeptides within 24 h of cytokinin application, some of which were detectable as early as 3 h. In my experiment, differences were observed after one day on SIM, and it is possible that some of these could be related to the cell cycle as the explants on SIM showed rapid cell division resulting in small nucleated cells as opposed to large vacuolated cells in CIM cultures. Various laboratories (for example, Reynolds 1989, 1990; Renaudin *et al*, 1991) have analysed the proteins synthesised during *in vitro* shoot formation. In many instances they have analysed the proteins after organogenesis has been initiated. The difficulty in detecting differences prior to the organogenesis process, could be a result of the small number of cells responding to inductive stimulus for organogenesis. In this study, although differences in the protein complement were observable after one day on SIM, it is not possible to state whether the changes were part of the shoot induction process, without knowing the time taken for the shoot induction process to be initiated. *In vitro* translation of 1d SIM (4d CIM) mRNA did not show any change in protein profile as compared with the control, indicating either that new mRNAs were absent or, more probably, that they were present at such a low level that they remained undetectable. It is possible that the initial event in shoot induction occurs in only a small number of cells, setting off a cascade of events in neighbouring cells, which eventually results in shoot formation. Each stage might only involve small changes in mRNAs in a few cells so that no one major 'shoot induction' event would be detectable in RNA isolated from the material as a whole. The fact that shoot formation is non-synchronous would make it even more difficult to detect associated changes in mRNA, as any stage of development in a particular part of the tissue may be masked by changes in other parts.

Cytokinin-induced plastid and chlorophyll formation has been reported by many workers (see review by Parthier, 1979). Therefore it is possible that some of the proteins detected in *in vivo* labelling experiments include plastid

proteins (for example, large subunit of ribulose bisphosphate carboxylase) or some photosynthetic enzymes. The mRNAs for these plastid proteins do not possess poly (A) tails and therefore will be excluded during poly $(A)^+$ RNA isolation. Therefore *in vitro* translations of poly $(A)^+$ RNA will not produce polypeptides corresponding to plastid mRNAs. This may be one reason for not detecting any changes between *in vitro* translation profiles of SIM cultures and the control. According to Parthier (1979), cytokinins exhibit relatively low stimulation of the cytoplasmic enzymes and marked stimulation of plastid enxyme formation. Therefore although poly $(A)^+$ RNA does include mRNAs for small subunit of ribulose bisphosphate carboxylase, the differences in amounts between SIM and the control may not have been sufficient to show on SDS-PAGE. *In vivo* labelling studies showed increased synthesis of a Mr 52.5 kDa band protein in SIM cultures. Ellis and Judd (1987) reported the RUBISCO large subunit in *Pinus ponderosa* as of Mr 52 kDa and McFaddon (1986) reported the RUBISCO large subunit in tobacco as 55 kDa approximately. It is not possible to be certain whether the Mr 52.5 kDa protein in my results, is in fact RUBISCO large subunit because the size is similar to what is found in *Pinus* rather than what is found in tobacco. One of the proteins that was visible in first-stage CIM and second-stage CIM cultures upto eight days was not visible in SIM cultures. This protein of Mr 27.5 may be only present in callus cultures as evident from the work of Renaudin *et al.* (1991), where a protein of Mr 27.9 was found to be present only in callus cultures.

Differential hybridisation carried out with the cDNA library, followed by slot blot and Northern analysis identified SIM9 as abundant during 1-3 days of SIM cultures and 5 (by Northern analysis) and 6 days (by slot blot analysis) in CIM cultures. This sequence may correspond to mRNA involved in active cell division, meristematic tissue formation, cell differentiation such as tracheid formation or any other metabolic activity occurring early in SIM cultures and somewhat later in CIM cultures. Some effects of 2,4-D upon growth, structure

and biochemical responses of plant cells are apparently very similar to those caused by cytokinins; for example cell division, tracheid formation, tumour induction, fasciation etc. (Kaminek *et al.*, 1981). It is not known, however, whether some of these effects are due to increased levels of endogenous cytokinins as 2,4-D is known to induce cytokinin autonomy in tobacco cells. Nadakavukaren and McCracken (1977) reported that 2,4-D accumulated in plastids, which had a disruptive effect on their structure, thereby preventing utilisation of endogenous cytokinin associated with plastid development resulting in accumulation of cytokinin. Therefore the increased levels of SIM9 in CIM cultures may be due to an increase in the level of endogenous cytokinin which may have induced some process that is occurring in SIM cultures. The late initiation of roots may be due to 2,4-D depletion or an increase in the level of cytokinin with time reaching the right balance of auxin/cytokinin ratio for root organogenesis. These organogenic cultures producing roots and shoots probably have many mRNAs in common, as suggested by Lyndon (1990), who notes that most of the cell components at initial stages of development are similar in both tissue types. Therefore SIM9 could be related to an enzyme involved in an event common to both culture types SIM and CIM. It is important to identify SIM9 and to observe the localisation of the protein by carrying out *in situ* hybridisation as it was more abundant and appeared early in SIM cultures.

REFERENCES

Abeles F.B. (1973). *Ethylene in Plant Biology*. Academic Press, New York.

Akiyoshi D.E., Klee H., Amasino R.M., Nestler E.W. and Gordon M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA* 81, 5594-5598.

Ananiev E.D., Karagyozov L.K. and Karanov E.N. (1987). Effect of cytokinins on ribosomal RNA gene expression in excised cotyledons of *Cucurbita pepo*.L. *Planta* 170, 370-378.

Armstrong D.J., Murai N., Taller B.J. and Skoog F. (1976). Incorporation of cytokinin ^{6}N benzyladenine into tobacco callus transfer ribonucleic acid and ribosomal ribonucleic acid preparations. *Plant Physiol.* 57, 15-22.

Barckhausen R. (1978). Ultrastructural Changes in Wounded Plant Storage Tissue Cells. In: *Biochemistry of Wounded Plant Tissues*. (ed.) G. Kahl. Walter de Gruyter and Co., Berlin. pp 1-42.

Baulcombe D.C. and Key J.L. (1980). Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyl. *J. Biol. Chem*.255, 8907-8913.

Baulcombe D.C., Kroner P. A. and Key J.L. (1981). Auxin and gene regulation. In: *Levels of genetic control in development*. (eds) Subtleny S. and Abbott U.K. Liss, New York. pp 83-97.

Beaudoin-Eagan L.D. and Thorpe T.A. (1985). Tyrosine and Phenylalanine Ammonia Lyase Activities during Shoot Initiation in Tobacco Callus Cultures. *Plant Physiol.* 78, 438-441.

Beerhues L., Witte B., Hahlbrock K. and Kombrink E. (1990). Structure and expression of chitinase and 1,3-beta glucanase genes in potato. *Supplement to Plant Physiology* 93(1), 154.

Bevan M. and Northcote D.H.(1979). The loss of morphogenetic potential and induction of phenyl ammonia lyase in suspension cultures of *Phaseolus vulgaris*. *J. Cell Sci.* 39, 339-353.

Bevan M. and Northcote D.H. (1981a). Subculture induced protein synthesis in tissue cultures of *Glycine max* and *Phaseolus vulgaris*. *Planta* 152, 24-31.

Bevan M. and Northcote D.H. (1981b). Some rapid effects of synthetic auxins on mRNA levels in cultured plant cells. *Planta* 152, 32-35.

Borkird C., Choi J.H. and Sung Z.R. (1986). Effect of 2,4-Dichlorophenoxy acetic acid on the Expression of Embryogenic Program in carrot. *Plant Physiol.* 81, 1143-1146.

Bracale, M., Longo, G.P., Rossi G. and Longo C.P. (1988). Early changes in morphology and polypeptide pattern of plastids from watermelon cotyledons induced by benzyladenine or light are very similar. *Physiol. Plant.* 72, 94-100.

Broglie K.E., Gaynor J.J. and Broglie R.M. (1986). Ethylene regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. *Proc. Natl. Acad.Sci. USA.* 83, 6820-6824.

Broglie K.E., Biddle P., Cressman R. and Broglie R. (1989). Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. *Plant Cell* 1, 599-607.

Burrows W.J., Skoog F. and Leonard N.J. (1971). Isolation and Identification of Cytokinins Located in the Transfer Ribonucleic Acid of Tobacco Callus Grown in the Presence of 6-Benzylaminopurine. *Biochemistry* 10(12), 2189-2194.

Chambers, J.A.A and Russo V.E.A. (1986). Isolating RNA is easy and fun. *Neurosp. Newsl.* 33, 23-24.

Chen, C. (1989). Cytokinin modulated Macromolecular Synthesis and Gene Expression. In: *Plant Biotechnology*. (eds.) Shain-dow Kung and C.J. Arntzen. pp 245-254.

Chen M.H., Wang P.J. and Maeda E. (1987). Somatic embryogenesis and plant regeneration in *Carica papaya* L. Tissue culture derived from root explants. *Plant Cell Reports* 6, 348-351.

Choi J.H. and Sung Z.R. (1984). Two dimensional gel analysis of carrot somatic embryonic proteins. *Plant. Mol. Biol. Rep.* 2. 19-25.

Choi J.H., Liu L.S., Borkird C. and Sung Z.R. (1987). Cloning of genes developmentally regulated during plant embryogenesis. *Proc. Natl. Acad. Sci. USA* 84, 1906-1910.

Chory J., Voytas D.F., Olszewski N.E. and Ausubel F.M. (1987). Gibberellin-Induced Changes in the Populations of Translatable mRNAs and Accumulated Polypeptides in Dwarfs of Maize and Pea. *Plant Physiol.* 83, 15-23.

Christianson M.L. and Warnick D.A. (1985). Temporal Requirement for Phytohormone Balance in the Control of Organogenesis *in vitro*. *Dev. Biol.* 112, 494-497.

Christianson M.L. and Warnick D.A. (1988). Organogenesis *in Vitro* as a Developmental Process. *Hort. Sci.* 23(3), 515-519.

Christofferson R.E. and Laties G.G. (1982). Ethylene regulation of gene expression in carrots. *Proc. Natl. Acad. Sci. USA*. 79, 4060-4063.

Cleland, R. (1973). Auxin-induced hydrogen ion excretion from *Avena* coleoptiles. *Proc. Natl. Acad. Sci. USA* 70, 3092-3093.

Clemens M.J. (1986a). Purification of eukaryotic messenger RNA. In: *Transcription and Translation - A Practical Approach*. (eds.) B.D.Hames and S.J.Higgins. IRL Press, Oxford. pp 211-230.

Clemens, M.J. (1986b). Translation of eukaryotic messenger RNA in cell free extracts. In: *Transcription and Translation - A Practical Approac*. (eds.) B.D.Hames and S.J.Higgins. IRL Press, Oxford. pp 231-270.

Crowell D., Kadleck A., Manorama J. and Amasino R. (1990). Cytokinin induced complementary DNA clones from soybean. *Supplement to Plant Physiology* 93(1), Abstract 9.

Cyr R.J., Bustos M.M., Guiltinan M.J. and Fosket D.E. (1987). Developmental modulation of tubulin protein and mRNA levels during somatic embryogenesis in cultured carrot cells. *Planta* 171, 365-376.

Datta K., Parker H., Averyhart-Fullard V., Schmidt A. and Marcus A. (1987). Gene expression in the soybean seed axis during germination and early seedling growth. *Planta* 170, 209-216.

Dommes J. and Northcote D.H. (1985). The action of exogenous abscisic and gibberellic acids on gene expression in germinating castor beans. *Planta* 165, 513-521.

Dure L. III, Greenway S.C. and Galau G.A. (1981). Developmental biochemistry of cotton seed embryogenesis and germination - changing messenger ribonucleic acid populations as shown by *in vitro* and *in vivo* protein synthesis. *Biochemistry*. 20, 4162-4168.

Eichholz R., Harper J., Felix G. and Meins F.Jr. (1983). Evidence for an abundant 33,000 dalton polypeptide regulated by cytokinins in cultured tobacco tissues. *Planta* 158, 410-415.

Ellis D.D. and Judd R.C. (1987). SDS-PAGE analysis of bud-forming cotyledons of *Pinus ponderosa*. *Plant Cell, Tissue and Organ Culture* 11, 57-65.

Feierabend J. (1981). Influence of Cytokinin on Plastid Biogenesis in Rye leaves. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 241-289.

Feinberg A. and Vogelstein B. (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6-13.

Finkelstein R., Estelle E., Martinez-Zapater J. and Somerville C. (1988). *Arabidopsis* a Tool for the Identification of genes Involved in Plant Development. In: *Temporal and Spatial Regulation of Plant Genes*. (eds.) D.P.S. Verma and R.B. Goldberg. pp 1-25.

Flores S. and Tobin E.M. (1986). Benzyl adenine modulation of the expression of two genes for nuclear encoded chloroplast proteins in *Lemna gibba* : Apparent post transcriptional regulation. *Planta* 168, 340-349.

Fosket, D.E. (1980). Hormonal control of morphogenesis in cultured tissues. In: *Plant Growth Substances*. (ed.) F. Skoog. Springer Berlin, Heidelberg, New York. 362-369.

Fosket, D.E. and Tepfer D.A. (1978). Hormonal regulation of growth in cultured plant cells. *In Vitro* 14(1), 63-73.

Fosket, D.E., Morejohn L.C. and Westerling K.E. (1981). Control of growth by cytokinin: An Examination of Tubulin Synthesis During Cytokinin-Induced Growth in Cultured Cells of Paul's Scarlet Rose. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 194-211.

Fox, J.E. (1966). Incorporation of a Kinin, ^{6}N-Benzyladenine into soluble RNA. *Plant Physiol.* 41, 75-82.

Fox, J.E. and Gregerson E. (1982). Variation in a cytokinin binding protein among several cereal crop plants. In: Plant Growth Substances. (ed.) P.E.Wareing. Academic Press. pp 207-214.

Fukuda H. and Komamine A. (1982). Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Planta* 155, 423-430.

Fukuda H. and Komamine A. (1983). Changes in the Synthesis of RNA and Protein during Tracheary Element Differentiation in Single Cells Isolated from the Mesophyll of *Zinnia elegans*. *Plant and Cell Physiol.* 24(4), 603-614.

Fukuda H. and Komamine A. (1985). Cytodifferentiation. In: *Cell Culture and Somatic Cell Genetics of Plants*. Academic Press, New York. London. vol. 2, pp. 149-212

Gasser C.S., Smith A.G., Budelier K.A., Hinchee M.A., McCormick S., Horsch R.B., Shah D.M. and Fraley R.T. (1988). In: *Temporal and spatial regulation of plant genes* . (eds.) Verma D.P.S. and Goldberg R.B. Springer Verlag, New York. pp 84-96.

Gasser C.S., Budelier K.A., Smith A.G., Shah D.M. and Fraley R.T. (1989). Isolation of Tissue-Specific cDNAs from Tomato Pistils. *The Plant Cell*1, 15-24.

Gefter N.L and Russell R.L. (1969). Role of modifications in tyrosine transfer-RNA: a modified base affecting ribosome binding. *J. Mol. Biol.* 39, 145-157.

Goldberg, R.B. (1986). Regulation of Plant Gene Expression. *Phil. Trans. R. Soc. Lond.* B314, 343-353.

Goldsworthy, A. (1988). Growth control of plant tissue cultures. In *Biotechnology in Agriculture*. (ed.) Mizrahi A.V. 35-52.

Gronroos R and Von Arnold S. (1985) Initiation and development of wound tissue and roots on hypocotyl cuttings of *Pinus sylvestris in vitro*. *Physiol. Plant* 64, 393-401.

Grierson, D. (1985). Gene expression in ripening tomato fruit. *CRC Critical Rev. in Plant Sci.* 3, 113-132.

Grierson D. and Covey S.N. (1985). In: *Plant Molecular Biology*. Chapman and Hall, New York.

Grierson D., Maunders M.J., Slater A., Ray J., Bird C.R., Schuch W., Holdsworth M.J., Tucker J. A. and Knapp J. E. (1986). Gene expression during tomato ripening. *Phil. Trans. Roy. Soc. Lond.* B314, 399-410.

Grosset J., Yves M., Chartier Y., Kauffmann S., Legrand M. and Fritig B. (1990). Tobacco mesophyll cells synthesise 1-3 beta glucanase, chitinase and osmotins during *in vitro* cultures. *Plant Physiol.* 92(2), 520-527.

Habaguchi, K. (1977). Alterations in polyphenol oxidase activity during organ redifferentiation in carrot calluses cultured *in vitro*. *Plant Cell Physiol.* 18, 181-189.

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflanzenzellen. Sitzungsberichte der Akademie der Wissenschaften in Wien. *Mathematischenaturwissenschaftliche Klasse*. 111, 69-92.

Hagen, G. (1987). The control of gene expression by auxin. In: Plant Hormones and their Role in Plant Growth and Development. Davies P.J. (ed.) Martinus Nijhoff, Dordrecht, 149-163.

Harada J.J., Dehisle A.J., Boden C.S. and Crouch M.L. (1989). Unusual sequence of an abscisic acid-inducible mRNA which accumulates late in *Brassica napus* seed development. *Plant Mol.Biol.* 12, 395-401.

Hasegawa P.M., Yasuda T. and Cheng T.Y. (1979). Effect of auxin and cytokinin on newly synthesised proteins of cultured Douglas fir cotyledons. *Physiol. Plant.* 46, 211-217.

Henshaw G.G., O'Hara J.J. and Webb K.J. (1982). Morphogenetic studies in plant tissue cultures. In: Differentiation *in vitro*. (eds.) M.M.Yeoman and D.E.S.Truman. Cambridge University Press. 231-251.

Hong J.C., Nagao R.T. and Key J.L. (1987). Characterization and sequence analysis of a developmentally regulated putative cell wall gene isolated from soybean. *J. Biol. Chem.* 262, 8367-8376.

Huxter T.J., Thorpe T.A. and Reid D.M. (1981). Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. *Physiol. Plant.* 53, 319-326.

Inohara N., Shimomura S., Fukur T. and Futai M. (1989). Auxin binding protein located in the endoplasmic reticulum of maize shoots. Molecular cloning and complete primary structure. *Proc. Natl. Acad. Sci. USA*. 86, 3564-3568.

Inze D., Follin A., Van Lizsebettens M., Simoens C., Genetello C., Van Montagu M. and Schell J. (1984). Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. *Mol.Gen. Genet.* 194, 265-274.

Jacobson, A. (1987). Purification and fractionation of poly $(A)^+$ RNA. *Methods in Enzymology* 152, 254-261.

Jacobson, H.J. (1981). Soluble auxin binding proteins in Pea. *Cell Biol. Int. Rep.* 5, 768.

Jayabaskaran, C. (1990). Isolation and characterization of a cytokinin-binding protein from cucumber cotyledons. *Plant Growth Regulation* 9, 9-17.

Johnson-Flanagan A. M. and Singh J. (1987). Alteration of Gene Expression during the Induction of Freezing Tolerance in *Brassica napus* Suspension Cultures. *Plant Physiol.* 85, 699-705.

Jouanneau J.P., Teyssendier De La Serve B. and Peaud-Lenoel C. (1984). Frequency of ^{6}N benzyl adenine incorporation into major RNA fractions of tobacco cell suspensions grown at stimulatory or cytostatic cytokinin concentration. *Plant Physiol.* 74, 663-668.

Kaminek M., Hadakova V. and Lustinek J. (1981). Plastids and the origin of cytokinin autonomy in tobacco tissue cultures. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J. Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 241-251.

Key, J.L. (1969). Hormones and nucleic acid metabolism. *Annu. Rev. Plant Physiol.* 20, 449-473.

Key J.L. and Ingle J. (1969). Effects of auxin on RNA metabolism: RNA metabolism in response to auxin. In: *Biochemistry and Physiology of Plant Growth Substances* (eds.) F. Wightman and G. Setterfield, Runge Press, Ottawa. pp. 711-722.

Kim E.S. and Kim S.S. (1985). Isoperoxidases and translatable isoperoxidases messenger RNA species in shoots and non shoot forming tobacco callus. *Korean Biochem. J.* 18(3), 259-265.

Kim J.J. and Kim S.S. (1987). *In vitro* translation and characterisation of peroxidase messenger RNA from tobacco callus. *Korean Biochem. J.* 20(3), 293-300.

Kimmel A.R. and Berger S.L. (1987). Preparation of cDNA and the Generation of cDNA Libraries: Overview. *Methods in Enzymology* 152, 307-316.

Kinoshita I., Katagiri K. and Tsuji H. (1979). Effects of benzyladenine and light on changes in various RNA species in etiolated cucumber cotyledons. *Plant Cell Physiol.* 20, 707-713.

Klambt, D. (1990). A view about the function of auxin-binding proteins at plasma membranes. *Plant Mol. Biol.* 14, 1045-1050.

Kochba J., Spiegal-Roy P., Neumann H. and Saad S. (1978). Stimulation of embryogenesis in citrus ovular callus by ABA, ethephon, CCC and Alar and its suppression by GA_3. *Z. Pflanzenphysiol.* 89, 427-432.

Kulaeva, O.N. (1981). Cytokinin Action on Transcription and Translation in Plants. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 218-222.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Liebermann, M. (1979). Biosynthesis and action of ethylene. *Annu. Rev. Plant. Physiol.* 30, 533-591.

Lin L.S. and Ho T.H.D. (1986). Mode of action of abscisic acid in barley aleurone layers and induction of new proteins by abscisic acid. *Plant Physiol.* 82, 289-297.

Lincoln J.L., Cordes S., Read E. and Fischer R.L. (1987). Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. *Proc. Natl. Acad. Sci. USA*. 84, 2793-2797.

Linstedt D. and Reinert J. (1975). Occurrence and properties of a cytokinin in tissue cultures of *Daucus carota*. *Naturwissenchaften* 62, 238-239.

Liu L.S., Borkird C. and Sung Z.R. (1985). Molecular cloning of cDNA complementary to poly $(A)^+$ mRNA differentially expressed during embryogenesis. In: Terzi M., Pitto L. and Sung Z.R. (eds.). *Somatic embryogenesis*. Rome IPRA. pp. 70-76.

Logemann J., Schell J. and Willmitzer L. (1987). Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163, 16-20.

Lyndon, R.F. (1990). *Plant Development: The cellular basis*. (ed.) R. F. Lyndon. Unwin Hyman. Boston, Sydney,Wellington. 203-231.

Mader M., Munch P. and Bopp M. (1975). Regulation und bedeutung der Peroxidase-Musteranderungen in sprossdifferenzierenden kalluskulturen von *Nicotiana tabacum* L. *Planta* 123, 257-265.

Maeda E. and Thorpe T.A. (1979). Shoot histogenesis in tobacco callus cultures. *In Vitro* 15 (6), 415-424.

Maheshwaran G. and Williams E.G. (1985). Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. *Ann. Bot.* 56, 619-630.

Maniatis T., Fritsch E.F. and Sambrook J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. p 545.

Marcu K. and Dudock. B. (1974). *Nucleic Acids Reseach.* 1, 1385-1397.

Marriot K.M. and Northcote D.H. (1975). The induction of enzyme activity in the endosperm of germinating castor bean seeds. *Biochem. J.* **148**, 139-144.

Marriot K.M. and Northcote D.H. (1977). The influence of abscisic acid, adenosine 3'5' cyclic phosphate and gibberellic acid on the induction of isocitrate lyase activity in the endosperm of germinating castor bean seeds. *J. Exp. Bot.* **28(102)**, 219-224.

Martin C. and Northcote D.H. (1981). Qualitative and Quantitative Changes in mRNA of Castor Beans During the Initial Stages of Germination. *Planta* **151**, 189-197.

Martin C. and Northcote D.H. (1982 a). The action of exogenous gibberellic acid on protein and mRNA in germinating castor bean seeds. *Planta* **154**, 168-173.

Martin C. and Northcote D.H. (1982 b). The action of exogenous gibberellic acid on isocitrate lyase mRNA in germinating castor bean seeds. *Planta* **154**, 174-183.

Martin C. and Northcote D.H. (1983). The action of exogenous gibberellic acid polysome formation of mRNA in germinating castor bean seeds. *Planta* **158**, 16-27.

Martin C., Beeching J.R. and Northcote D.H. (1984). Changes in levels of transcripts in endosperms of castor beans treated with exogenous gibberellic acid. *Planta* **162**, 68-76.

Masuda, Y. and Kamisaka S. (1969). Rapid stimulation of RNA biosynthesis by auxin. *Plant. Cell. Physiol.* 10, 79-86.

McClure B.A., Hagen G., Brown C.S., Gee M.A. and Guilfoyle T.J. (1989). Transcription, organisation and sequence of an auxin regulated gene cluster in soybean. *The Plant Cell* 1, 229-239.

McFadden B.A., Torres-Ruiz J., Daniell H and Sarojini G. (1986). Interaction functional relations and evolution of large and small subunits in Rubisco from Prokaryota and Eukaryota. *Phil. Trans. R. Soc. lond.* **B313**, 347-358.

McGlassen, W.B. (1985). Ethylene and fruit ripening. *Hort. Sci.* **20**, 51-54.

Meins, Y. (1986). Determination and morphogenetic competence in plant tissue culture. In: *Plant cell technology*. (ed.). M.M.Yeoman. pp. 7-25.

Meins F.Jr., Foster R. and Lutz J. (1982). Quantitative studies of bud initiation in cultured tobacco tissue. *Planta* 155, 473- 477.

Meins F.Jr., Neuhaus J., Jeefe K., Hinz U. and Meyer A. (1990). Developmental regulation of β 1,3-glucanase and chitinase in tobacco. *Plant Physiol.* 79, 10.

Memelink J., Schilperoort R.A. and Hoge J.H.C. (1988). Changes in the tissue specific prevalence of translatable mRNAs in transgenic tobacco shoots containing the DNA cytokinin gene. *Plant Mol. Biol.* 11, 625-631.

Mettler, I.J. (1988). A Simple and Rapid Method for Minipreparation of DNA from Tissue Cultured Plant Cells. *Plant Mol. Biol. Rep.* 5(3), 346-349.

Meyer Y., Aspart L. and Chartier Y. (1984). Auxin induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated *in vitro*. *Plant Physiol.* 75, 1034-1039.

Minocha S.C. and Halperin W. (1974). Hormones and Metabolites which control Tracheid Differentiation, with or without Concomitant Effects on Growth, in Cultured Tuber Tissue of *Helianthus tuberosus* L. *Planta* 116, 319-331.

Murashige, T. (1961). Analysis of the Inhibition of Organ Formation in Tobacco Tissue Culture by Gibberellins. *Physiol. Plant.* 17, 636-643.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473-479.

Nadakavukaren M.J. and Mckracken D.A. (1977). Effect of 2,4-dichlorophenoxyacetic acid on the structure and function of developing chloroplasts. *Planta* 137, 65-69.

Nelson, N. (1944). A photometric adoption of the Somoygi method for the determination of glucose. *J. Biol. Chem.* 153, 375-380.

Neumann D. and Khokhlova W.A. (1981). Studies on the Action of Cytokinin and Light in RNA synthesis of Pumpkin Cotyledons. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C.Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 267-274.

O'Farrell, P.H. (1975). High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021.

Ougham, H.J. (1987). Gene expression during leaf development in *Lolium tementulentum*. *Physiol. Planta.* 70, 479-484.

Parthier, B. (1979) The Role of Phytohormones (Cytokinins) in Chloroplast Development. *Biochem. Physiol. Pflanzen*, 174, 173-214.

Parthier B., Lerbs S. and Klyachko (1981). Plastogenesis and Cytokinin Action. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 275-286.

Parthier B., Lehmann J., Lerbs S., Lerbs W. and Wollgiehn R. (1982). Cytokinin control in the synthesis of plastid proteins: ribulose-1,5-bisphosphate carboxylase. In: *Plant Growth Substances*. (ed.) P.F. Wareing. Academic Press. 225-236.

Pelham H.R.B. and Jackson R.J. (1976). An efficient mRNA dependent translation system from reticulocyte lysate. *Eur. J. Biochem*. 67(4), 247-256.

Phillips, R. (1987). Effects of Sequential Exposure to Auxin and Cytokinin on Xylogenesis in Cultured Explants of Jerusalem Artichoke (*Helianthus tuberosus* L. *Annals of Botany* 59, 245-250.

Prescott A. and Martin C. (1987). A rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant Mol. Biol. Rep*. 4(4) 219-224.

Rajyalakshmi K., Grover A., Maheshwari N., Tyagi A.K. and Maheshwari S.C. (1991). High frequency regeneration of plantlets from the leaf-bases via somatic embryogenesis and comparison of polypeptide profiles from morphogenic and non-morphogenic calli in wheat *(Triticum aestivum) Physiol. Plant.* 82, 617-623.

Reddy A.S.N., Jena P.K., Mukherjee S.K. and Poovaiah B.W. (1990). Molecular cloning of cDNAs for auxin induced mRNA and developmental expression of auxin inducible genes. *Plant Mol. Biol*. 14, 643-653.

Reinert, J. C. (1959). Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen in Gewebekulturen aus Kanetten. *Planta* 53, 318-333.

Renaudin J.P., Tournaire C. and Teyssendier de la Serve B. (1991). Quantitative analysis of protein changes during meristem initiation and bud development in protoplast-derived *Petunia hybrida* callus. *Physiol. Plant.* 82, 48-56.

Reynolds, T.L. (1989). Changes in RNA, Protein, and Translatable messenger RNA Synthesis and Accumulation During Adventive Organogenesis in Somatic Tissue Cultures of *Solanum carolinense*. *Plant Sci*. 65, 77-85.

Reynolds, T.L. (1990). A Two Dimensional Electrophoretic Analysis of Protein Synthesis and Accumulation during Adventitious Shoot Formation in Somatic Tissue Cultures of *Solanum carolinense* L. *J. Plant .Physiol*. 136, 213-218.

Roberts, L.W. (1988). Hormonal aspects of vascular differentiation. In: Vascular differentiation and plant growth regulators. (eds.) L.W. Roberts, B. Gahan and R. Aloni. Springer-Verlag. p. 24.

Rosbash M and Ford, P.J. (1974). Polyadenylic acid containing RNA in *Xenopus laevis* oocytes. *J. Mol. Biol*. 85, 87-101.

Ry Meeks-Wagner, D., Dennis, S.D., Tran Thanh Van, K., and Peacock, W.J. (1989). Tobacco Genes Expressed during *in Vitro* Floral Initiation and Their Expression during Normal Plant Development. *The Plant Cell* 1, 25-35.

Sachs, T. (1978). Patterned differentiation in plants. *Differentiation* 11, 65-73.

Sammons D.W., Adams L.D. and Nishizawa E.E. (1981). Ultrasensitive silver based colour staining of polypeptides in polyacrylamide gels. *Electrophoresis*. 2, 135-141.

Sargent, T.D. (1987) Isolation of differentially expressed genes. *Methods in enzymology* 152, 423-432.

Sauerbrey E., Grossmann K. and Jung J. (1987). Is ethylene involved in the regulation of growth of cell suspension cultures. *J.Plant Physiol.* 127, 471-479.

Schröder, G., Waffenschmidt S., Weiler E.W. and Schröder J. (1984). The T-region of Ti plasmid codes for an enzyme synthesising indole-3-acetic acid. *Eur. J. Biochem.* 138, 387-391.

Shepard, J.F. (1980). Abscisic Acid Enhanced Shoot Initiation in Protoplast Derived Calli of Potato. *Plant. Sci. Lett.* 18, 327-333.

Shimomura S., Inohara N., Fukui T. and Futai M. (1988). Different properties of two types of auxin-binding sites in membranes from maize coleoptiles. *Planta* 175, 558-566.

Shinshi H., Mohnen D. and Meins F.(Jr). (1987). Regulation of a plant pathogenesis related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proc. Natl. Acad. Sci. USA* 84, 89-93.

Skoog F. and Miller C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultured *in vitro Symp. Soc. Exp. Biol.* 11, 118.

Slater, R.J. (1986). The Estimation of mRNA Content by Poly (U) Hybridisation. In: *Methods in molecular biology, volume 2, Nucleic acids*. (ed.) Walker J.M. Hamana Press, Clifton. pp 125-130.

Sponsel, V. (1987). Gibberellin biosynthesis and metabolism. In: *Plant Hormones and Their Role in Plant Growth and Development.* (ed.) Davies P.J. Dordrecht: Martinus Nijhoff Publishers. pp 43-75.

Stabel P., Eriksson T and Engström P. (1990). Changes in protein synthesis upon cytokinin-mediated adventitious bud production and during seedling development in Norway spruce, *Picea abies*. *Plant Physiol.* 92, 1174-1183.

Steward F.C., Mapes M.O. and Smith J. (1958). Growth and organised development of cultured cells. Growth and division of freely suspended cells. *Am. J. Bot.* 45, 693-703.

Steward F.C., Mapes M.O., Kent A.E. and Holsten R.D. (1964). Growth and development of cultured plant cells. *Plant Sci.* **163**, 20-27.

Street, H.E. (1977). Embryogenesis and chemically induced organogenesis. In: *Plant Cell and Tissue Culture : Principles and Applications* (eds.) W.R. Sharp, P.O. Larson, E.F. Paddock and V. Raghavan. Ohio State University Press, Columbus, Ohio. 123-153.

Sung Z.R. and Okimoto R. (1981). Embryonic proteins in somatic embryos of carrots. *Proc. Natl. Acad. Sci. USA* 78, 3683-3687.

Sung Z.R. and Okimoto R. (1983). Coordinate gene expression during somatic embryogenesis in carrots. *Proc. Natl. Acad. Sci. USA* 80, 2661-2665.

Szweykowska, A., E. Gwozdz and Spychala M. (1981). The Cytokinin Control of Protein Synthesis in Plants. In: *Metabolism and Molecular Activities of Cytokinins*. (eds.) J.Guern and C. Peaud-Lenoel. Springer-Verlag, Berlin, Heidelberg, New York. pp 213-217.

Tan B.C. and Liang H.G. (1989). Changes in Polyribosome Population During the Growth and Senescence of Tobacco Callus. *Acta Phytophysiologia Sinica* 15(2), 199-204.

Tan B.C. and Liang H.G. (1991). Changes in metabolism of nucleic acids during the growth and senescence of tobacco callus. *Physiol. Plant.* 81, 121-126.

Teyssendier De La Serve B. and Jouanneau J.P. (1979). Preferential incorporation of an exogenous cytokinin ^{6}N benzyladenine into 18S and 25S ribosomal RNA of tobacco cells in suspension culture. *Biochimie* 61, 913-922.

Teyssendier De La Serve B., Jouanneau J.P. and Peaud-Lenoel C. (1982). Covalent link of the ^{6}N (2-isopentyl) adenine nucleotide to the rRNA of cytokinin requiring and cytokinin autonomous tobacco cells. *J. Plant Growth Regulation* 1, 25-35.

Teyssendier De La Serve B., Jouanneau J.P. and Peaud-Lenoel C. (1984). Incorporation of ^{6}N Benzyladenine into messenger polyadenylated RNA of tobacco *Nicotiana tabacum* cells incubated at stimulatory or cytostatic cytokinin concentration. *Plant Physiol.* 74(3), 669-674.

Teyssendier De La Serve B., Axelos M. and Peaud-Lenoel C. (1985). Cytokinins modulate the expression of genes encoding the protein of the light harvesting chlorophyll A-B complex. *Plant Mol. Biol.* 5(3), 155-164.

Theologis, A. (1986). Rapid gene regulation by auxin. *Annu. Rev. Plant Physiol* 37, 407-438.

Theologis A. and Ray P.M. (1982a). Early auxin regulated polyadenylated sequences in pea stem tissue culture. *Proc. Natl. Acad. Sci. USA* 79, 418-421.

Theologis A. and Ray P.M. (1982b). Changes in messenger RNAs under the influence of auxins. In: *Plant Growth Substances*. (ed.) P.F. Wareing. Academic Press. 42-57.

Theologis A., Huynh T.V. and Davies R.W. (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *J. Mol. Biol.* 183, 53-68.

Thimann, K.V. (1969). In: *The Physiology of Plant Growth and Development*. (ed.) M.B.Wilkins. McGraw Hill London.

Thomas T. and Wilde D. (1985). Analysis of gene expression in carrot somatic embryos. In: M. Terzi, L. Pitto and Z.R. Sung (eds.) *Somatic Embryogenesis*. Rome IPRA. 77-85.

Thomas T. and Wilde D. (1987). Analysis of carrot embryo gene expression program. Plant Tissue and Cell Culture. Alan R. Liss Inc. pp. 83-93.

Thorpe, T.A. (1978). Physiological and biochemical aspects of organogenesis *in vitro* In: Frontiers of Plant Tissue Culture. (ed.) T.A. Thorpe. Univ. of Calgary, Calgary. Alberta. pp. 49-58.

Thorpe, T.A. (1980). Organogenesis *in vitro*: Structural, Physiological and Biochemical Aspects. *Int. Rev. Cyt.* Supplement 2a , 71-111.

Thorpe T.A. and Meier D.D. (1973). Effects of gibberellic acid and abscisic acid on shoot formation in tobacco callus cultures. *Physiol. Plant* . **29**, 121-124.

Thorpe T.A. and Murashige T. (1968). Starch accumulation in shoot-forming tobacco callus cultures. *Science* **160**, 421-422.

Thorpe T.A. and Murashige T. (1970). Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Can. J. Bot.* 48, 277-285.

Tisserat B. and Murashige T. (1977). Probable identity of substances in citrus that repress asexual embryogenesis. *In vitro* 13, 785-789.

Tran Thanh Van, K.M. (1981). Control of morphogenesis in *in vitro* cultures. *Annu. Rev. Plant Physiol.* **32**, 291-311.

Trewavas, A.J. (1968). Effect of IAA on RNA and protein synthesis. *Arch. Biochem. Biophys.* 123, 324-325.

Trewavas, A.J. (1983). Nitrate as a plant hormone. *Brit. Plant. Growth. Regul. Group. Monogr.* **9**, 97-110.

Tucker W.Q., Wilson J.W. and Gresshoff P.M. (1986). Determination of Tracheary Element Differentiation in Lettuce Pith Explants. *Ann. Bot.* 57, 675-679.

Tukey H.B., Went F.W. and Van Overbeck J. (1954). Nomenclature of chemical plant regulators. *Plant. Physiol.* **29**, 307-308.

Vanderhoef L.N. and C. A. Stahl. (1975). Separation of two responses to auxin by means of cytokinin inhibition. *Proc. Natl. Acad. Sci. USA* 72, 1822-1825.

Van der Zaal E.J., Memelink J., Mennes A.M., Quint A. and Libbenga K.R. (1987). Auxin induced mRNA species in tobacco cell cultures. *Plant Mol. Biol.* 10, 145-157.

Vasil I.K. and Vasil V. (1972). Totipotency and Embryogenesis in Plant Tissue Cultures. *In Vitro* 8, 117-127.

Walbot, V. (1978). Control mechanisms for plant embryogeny. In: *Dormancy and Developmental Arrest. Experimental Analysis in Plants and Animals*. (ed). M.E.Clutter. Academic.Press, New York 114- 166.

Walker J.C. and Key J.L. (1982). Isolation of cloned cDNAs to auxin-responsive poly $(A)^+$ RNAs of elongating soybean hypocotyl. *Proc. Natl. Acad. Sci. USA* 79, 7185-7189.

Walker J.C., Legocka J., Edelman L. and Key J.L. (1985). An analysis of growth interactions and gene expression during auxin induced cell elongation using clones of complementary DNA to auxin responsive messenger RNAs. *Plant Physiol.* 77, 847-850.

Wareing P.G. and Phillips I.D.J. (1983). Abscisic acid in bud dormancy and apical dominance. In: *Abscisic acid*. (ed.) F.T. Addicott. New York, Praeger Publishers. pp. 301-330.

Wasilewska L.D., Bralczyk J. and Szczegielniak J. (1987). The Role of Gibberellin in Regulation of Dwarf Plants Development. *Plant Sci.* 53, 11-19.

Webb K.J., Osifo E. and Henshaw G.G. (1983). Root regeneration from leaflet discs of six cultivars of potato. *Plant Sci. Lett.* 30, 1-8.

White, P.R. (1939). Controlled differentiation in a plant tissue culture. *Bull. Torrey Bot. Club* 66, 507-513.

Wolter K.E. and Skoog F. (1966). Nutritional requirements of *Fraxinus* tissue cultures. *Amer. J.Bot.* 53, 263-269.

Yamaguchi T. and Nakajima T. (1984). Effect of abscisic acid on adventitious bud formation in cultured tissues derived from root tubers of sweet potato and from stem tubers of potato. *3rd Int. Cong. Plant Tissue and Cell Culture.* Leicester England. Abstract 65.

Yang S.F. (1985). Biosynthesis and action of ethylene. *Hort. Sci.* 20, 41-45.

Yasuda T., Hasegawa P.M. and Cheng T.Y. (1980). Analysis of newly synthesised proteins during differentiation of cultured Douglas fir cotyledons. *Physiol. Plant* 48, 83-87.

Yoshii H, and Imaseki H. (1982). Regulation of Auxin induced Ethylene Biosynthesis. Repression of Inductive formation of 1-Aminocyclopropane-1-carboxylate Synthase by Ethylene. *Plant. Cell. Physiol.* **23(4)**, 639-649.

Zurfluh L.L. and Guilfoyle T.J. (1980). Auxin-induced changes in the patterns of protein synthesis in soybean hypocotyl. *Proc. Natl. Acad. Sci. USA* **77**, 357-361.

Zurfluh L.L. and Guilfoyle T.J. (1981). Auxin-induced nucleic acid and protein synthesis in the soybean hypocotyl. 39th Symp. Soc. Dev. Biol. New York. Liss, 99-118.

Zurfluh L.L. and Guilfoyle T.J. (1982a). Auxin-induced changes in the population of translatable messenger RNA in elongating maize coleoptile sections. *Planta* **156**, 525-527.

Zurfluh L.L. and Guilfoyle T.J. (1982b). Auxin-induced changes in the population of translatable messenger RNA in elongating sections of soybean hypocotyl. *Plant Physiol.* **69**, 332-337.

Zurfluh L.L. and Guilfoyle T.J. (1982c). Auxin- and ethylene-induced changes in the population of translatable messenger RNA in basal sections and intact soybean hypocotyl. *Plant Physiol.* **69**, 338-340.

Appendix-A

RNA Extraction Buffer-D

Tris.HCl	50 mM
LiCl	150 mM
EDTA	5 mM
SDS	5 %
pH 9.0	

Oligo dT Chromatography

Loading Buffer

NaCl	0.5 M
EDTA	1.0 mM
pH 7.5	

Elution Buffer

EDTA	1.0 mM
SDS	0.05 %
Tris.HCl	10.0 mM
pH 7.5	

Agarose electrophoresis

5 × TBE per litre

Tris.HCl	54.0 g
Boric acid	27.5 g
EDTA (0.5M)	20.0 ml
pH 8.0	

BB loading buffer

Bromophenol blue	0.25 %
Xylene Cyanol	0.25 %
Ficoll (in water)	0.15 %

10 × MOPS buffer

3-N-(morpholino) propanesulfonic acid	0.2 M
Sodium acetate	0.05 M
EDTA	0.01 M
pH 5.5-7.0	

Appendix A

Protein analysis

Sample Buffer A	
Water	4.0 ml
Tris (0.5M, pH 6.8)	1.0 ml
Glycerol	0.8 ml
SDS (10%)	1.6 ml
2-mercaptoethanol	0.4 ml
Bromophenol blue (0.05%)	0.2 ml
Sample Buffer B	
SDS	2 %
Gycerol	20 %
Bromophenol blue	0.001 %
Tris HCl (pH 6.8)	60 mM
2-mercaptoethanol	70 mM
5 x Gel running buffer per litre	
Tris HCl	15 g
Glycine	72 g
SDS	5 g

10 x Alkaline agarose gel buffer

NaCl	500 mM
EDTA	10 mM

10 x Alkaline electrophoresis buffer

NaOH	300 mM
EDTA	10 mM

Alkaline loading buffer

NaOH	50 mM
EDTA	1 mM
Ficoll (type 400 Pharmacia)	2.5 %
bromocresol green	0.025 %
(or bromophenol blue)	

TE buffer

Tris.HCl	10 mM
EDTA (pH 8.0 or 7.5)	1 mM

STE buffer

NaCl	100 mM
Tris.HCl	10 mM
EDTA	1 mM

Appendix B

SDS-PAGE

30% Acrylamide/Bis-acrylamide stock solution (100 ml)

Acrylamide	29.2 g
N'N' Bismethelene acrylamide	0.8 g

10% resolving gel (40 ml)

Water	16.2 ml
Tris.HCl (1.5M, pH 8.8)	10.0 ml
SDS(10%)	0.4 ml
30% acrylamide stock	13.2 ml
Ammonium persulphate	0.2 ml
TEMED	0.02 ml

4% stacking gel (10 ml)

Water	6.1 ml
Tris.HCl (0.5M, pH 6.8)	2.5 ml
SDS (10%)	0.1 ml
30% acrylamide stock	1.3 ml
Ammonium per sulphate	0.05 ml
TEMED	0.01 ml

20 × SSC (one litre)

NaCl	3 M
Tri-sodium citrate	0.3 M
pH 7.0	

20 × SSPE

NaCl	3.6 M
Sodium phosphate	0.2 M
EDTA (pH 7.7)	0.02 M

SM (one litre)

NaCl	5.8 g
$MgSO_4$	2.0 g
1M Tris.HCl (pH 7.5)	5.0 ml
2% gelatin	5.0 ml

Appendix B

Denhardt's solution × 100

BSA (w/v)	2.0 %
Ficoll (Pharmacia) (w/v)	2.0 %
PVP (Polyvinylpyrolidone)(w/v)	2.0 %

L-broth (one litre)

Bactotryptone	10.0 g
Bacto-yeast extract	5.0 g
NaCl	10.0 g

pH 7.0

L-agar-same composition as L-broth but with Bacto-agar (15 g l^{-1})

Top agar/agarose (100 ml)

Bactotryptone	1.0 g
Bacto-yeast extract	0.5 g
NaCl	0.5 g
$MgSO_4$	0.25 g
Bacto-agar/agarose	1.0 g

pH 7.0

Preparation of DEAE cellulose

100 g of DEAE cellulose -DE52 (Whatman) was stirred in 0.05 N HCl until the pH reached below 4.5. Concentrated NaOH was added until pH reached 7.5, replaced the supernatant with 2 volumes of L-broth after washing the resin twice with L-broth.

Appendix C

RNA extraction methods

Amounts of RNA per gram of tobacco tissue obtained by methods A, B, C and D (section 3.2.2).

method	RNA g^{-1} of tissue	$A_{260:280}$ nm
A	293.30	1.86
B	120.00	1.62
C	404.34	1.51
D	893.24	1.86

2 μg of RNA samples obtained by each of the above methods were electrophoresed on 0.7% agarose gels. Presence of DNA was observed in all the samples. The lower values ($A_{260:280}$ nm <1.8) indicated that samples obtained by methods B and C would have been contaminated with proteins. RNA isolated by method D (section 3.3.2) was subjected to LiCl precipitation until samples run on an agarose gel showed no bands corresponding to DNA (Fig 1a, 1b and 1c).

A sample of RNA (2 μg) run on a denaturing gel (section 2.1.1.5) showed bands corresponding to ribosomal units, indicating integrity of RNA (Plate 2). The band X corresponds to DNA in the sample which was not subjected to a third LiCl precipitation.

Removal of polysaccharides (section 3.2.2.2) at different steps during the isolation procedure showed no difference in the yield of RNA by method D. When the amount of tissue was less than 2 g the polysaccharide pellet retained no detectable amount of RNA as measured by UV spectrophotometer at 260 nm. However, when higher amounts of tissue were used to isolate RNA, about 80 μg of RNA was recovered.

Appendix C

Plate 1 (top): Detection of purity of RNA:

RNA obtained by method D was electrophoresed on a 0.7% agarose gel; (2 μg of RNA per lane);

1A: RNA after the first LiCl precipitation; Band X (DNA), RNA (arrowed).

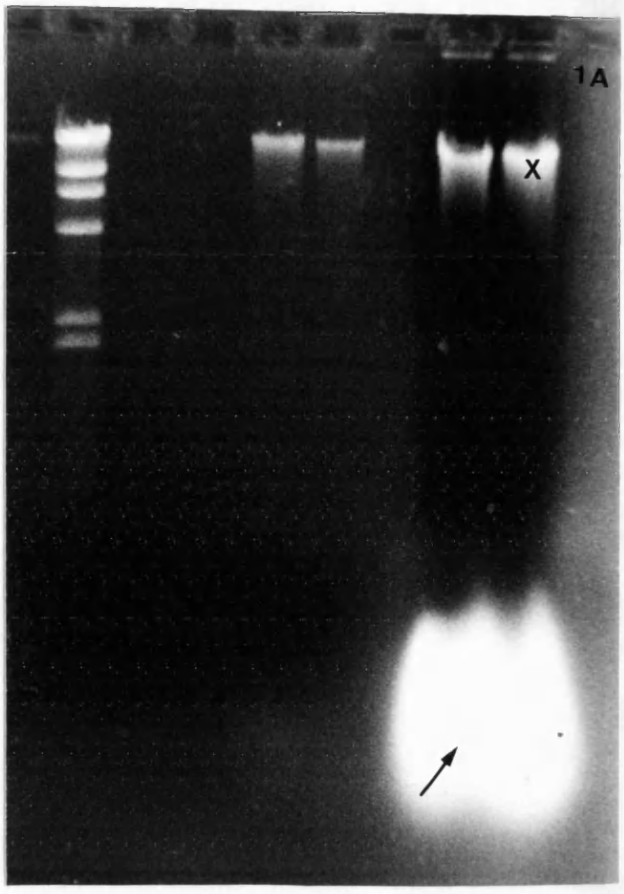
1B: RNA after the second LiCl precipitation; Band X (DNA), RNA (arrowed).

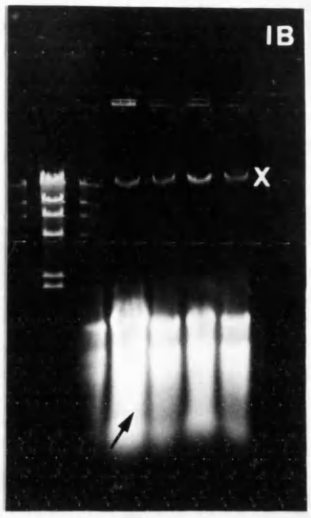
1C: RNA after the third LiCl precipitation; RNA (arrowed).

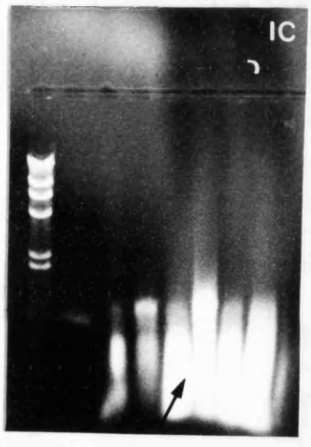
Plate 2 (bottom): Detection of integrity of RNA.

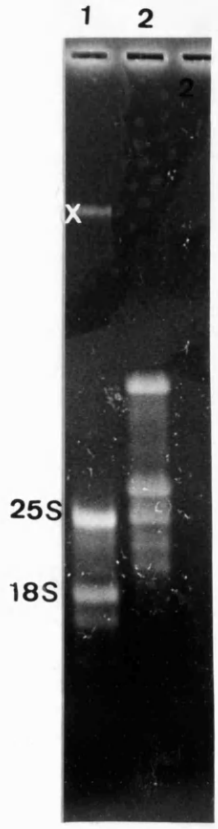
RNA isolated by method D (2 μg per lane) after the second LICl precipitation (3.2.1.4) was electrophoresed on a 1.1% agarose gel under denaturing conditions; presence of intact ribosomal units (25S and 18S) indicated the integrity of RNA.

Lane 1: native RNA; lane 2: denatured RNA; band X corresponds to DNA in the sample.


1A
X


IB
X


IC


1
2
2
X
25S
18S

Appendix C

Poly (U) hybridisation

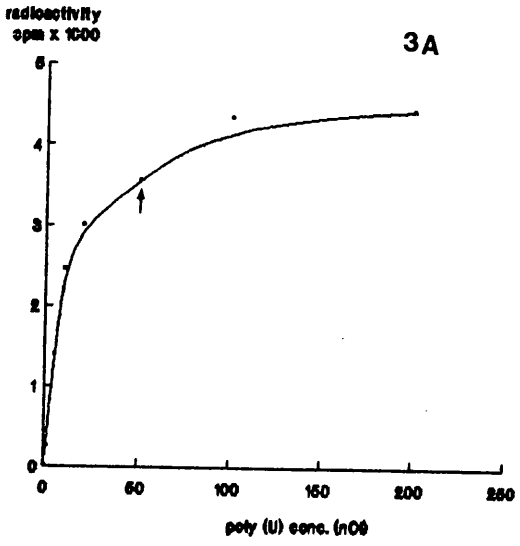
Fig 3A shows the hybridisation curve for 0.01 μg of commercial poly (A) with increasing amounts (0-200 nCi) of [^{3}H] poly (U) (1.63 mCi mg^{-1}). The point of saturation was considered to have occurred at 50 nCi (=0.03 μg). Although this may not represent complete saturation, Slater (1986) used a similar point on the graph and also reported that three times the theoretical value of [^{3}H] poly (U) is sufficient for 'saturation' of poly (A). He also stated that a lesser amount could be used for most of the reactions in calculating poly (A) tail in various samples.

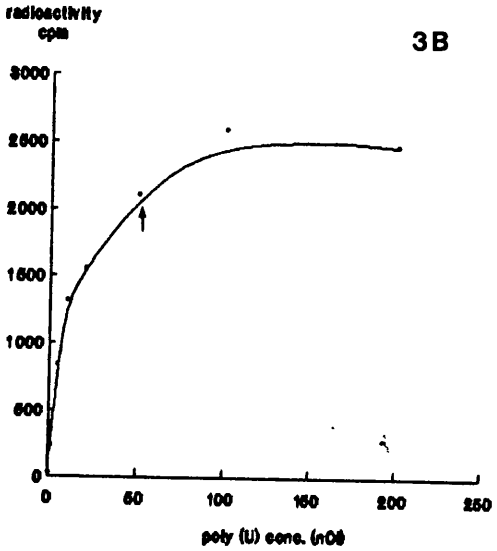
Fig. 3B shows the hybridisation curve for 5 μg of total RNA with increasing amounts of [^{3}H] poly (U) (0-200 nCi). The point of saturation was considered to be at 50 nCi (=0.03μg).

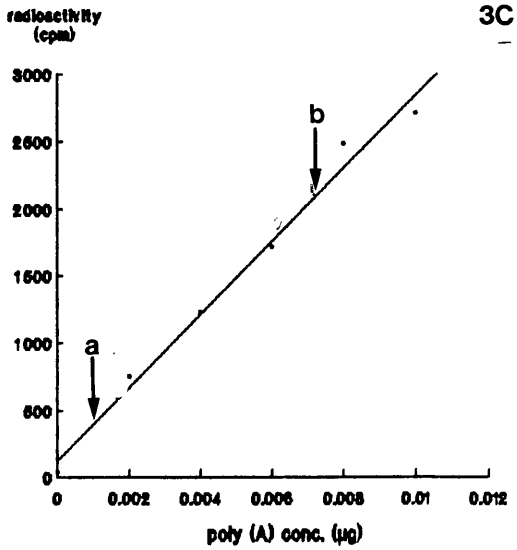
Fig. 3C shows the standard curve obtained after hybridisation of 50 nCi of [^{3}H] poly (U) (this value was obtained fron Figs. 3A and 3B) with increasing amounts of commercial poly (A). The cpm obtained for experimental samples (for example a and b) were read against this standard curve.

sample	cpm	poly (A), μg
a (tobacco poly (A)$^+$ RNA)	410.50	0.0016
b (5 x sample a)	2140.52	0.0062

'a' is a sample of tobacco poly (A)$^+$ RNA and 'b' is five-fold concentration of sample 'a'. The corresponding concentrations of poly (A) in samples a and b were 0.0016 and 0.0062 μg respectively.


radioactivity
cpm x 1000
3A
5
4
3
2
1
0
0
50
100
150
200
250
poly (U) conc. (nO$


radioactivity
cpm
3B
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
poly (U) conc. (nO$


radioactivity
(cpm)
3C
3000
2500
2000
1500
1000
500
0
a
b
0
0.002
0.004
0.006
0.008
0.01
0.012
poly (A) conc. (µg)

Appendix C

cDNA synthesis

Calculation of radioactive incorporation

C-the total amount of radioactivity

D-cpm incorporated into nucleic acid

Since the first strand mix was 1/5.2 the volume of the second strand mix, A and B is divided by 5.2.

	first strand	second strand
total input of radioactivity	A/5.2	C
radioactivity in nucleic acid	B/5.2	D

Therefore, percentage incorporation=(D-B/5.2) (C-B/5.2) × 100% during second strand synthesis.

Calculation of amount of cDNA synthesised

The following calculation is applied for the 20 μl of first strand and 100 μl of the second strand cDNA synthesis reaction mixes.

Let, % of labelled α [^{32}P] dCTP incorporated =X%

Amount of unlabelled dCTP in the reaction mix =10 nmoles

Assume the amount of unlabelled nucleotides incorporated =X% of 10 nmoles

=X/10 nmoles

Total amount of dNTP incorporated =4X/10 nmoles

Assuming the molecular weight of dNMP (1 mole) is 350 g,

then, the weight of cDNA synthesised = 350 × 4X/10 ng

=140 x X ng

If, the weight of mRNA used is Z ng,

then, the % yield of cDNA = 140/Z × X × 100%

Yield of first strand cDNA $= \frac{\text{amount of first strand}}{\text{amount of input mRNA}} \times 100$

yield of second strand cDNA $= \frac{\text{amount of second strand}}{\text{amount of first strand}} \times 100$

Appendix C

Calculation of amount of cDNA in the sample after separation of linkers

Let, the amount of label in cDNA be B.

The percentage of labelled dCTP incorporated in double stranded cDNA	=X%
Amount of unlabelled dCTP in the second strand mix (Y= 10 nmoles, when using the Amersham kit)	=Y nmoles
Amount of dCMP incorporated	= X/Y nmoles
Therefore, total amount of all four deoxynucleotides incorporated	=4 × X/Y nmoles
The weight of one nmole of dNMP	=350 ng
Therefore, the weight of second strand	=350 × 4X/Y ng
Therefore, cpm corresponding to 1 ng of cDNA	$=\frac{\text{B cpm}}{350 \times 4X/Y}$
	=C

let, the cpm in the fractions after separation of excess linkers be F,

Then, the amount of cDNA corresponding to F cpm = F/C

Appendix D

The Northern blot from section 7.2.4.4 which had been previously hybridised with SIM9 was used to determine mRNA levels corresponding to a chitinase cDNA insert from bean. Chitinase cDNA insert was oligolabelled as described in section 7.2.4.2 and hybridised with the Northern blot. The filters were washed twice in the following solutions and subjected to autoradiography.

solution	time	temp.
2 x SSPE, 0.1% SDS	10 min	room temp.
2 x SSPE, 0.1% SDS	10 min	room temp.
1 x SSPE, 0.1% SDS	15 min	65 °C

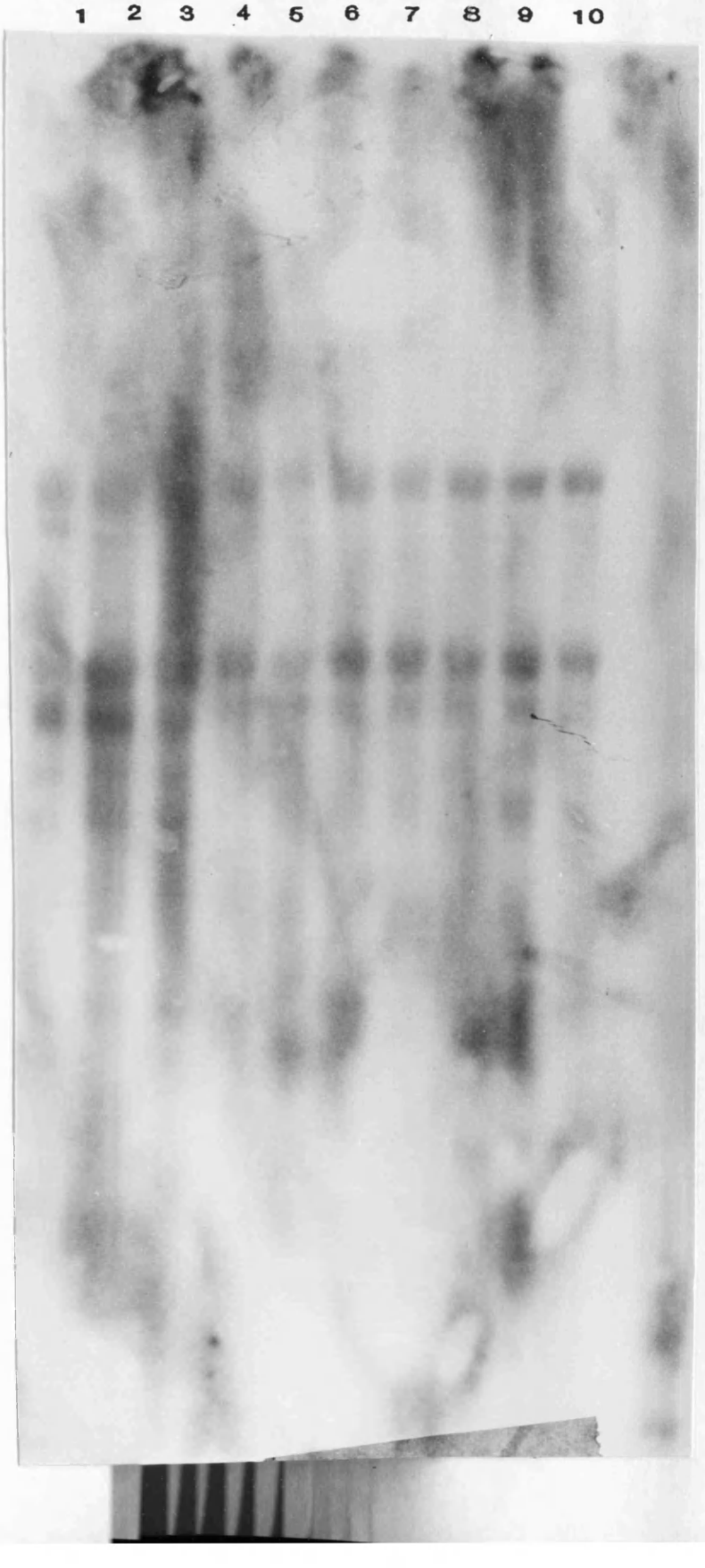
The results showed hybridisation to multiple bands (Plate 4). Since bands are present in all the tracks, it can be certain that RNA was not degraded in any of the tracks in the Northern blot.

Appendix D

Plate 4: Measurement of mRNA levels corresponding to chitinase cDNA by Northern analysis.

lane	sample
1	1d SIM (4d CIM)
2	2d SIM (4d CIM)
3	3d SIM (4d CIM)
4	4d SIM (4d CIM)
5	5d SIM (4d CIM)
6	1d CIM (4d CIM)
7	2d CIM (4d CIM)
8	3d CIM (4d CIM)
9	4d CIM (4d CIM)
10	5d CIM (4d CIM)

Multiple bands appeared in all the lanes.


1 2 3 4 5 6 7 8 9 10