Construction and screening of a pea root cDNA library

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CONSTRUCTION AND SCREENING OF A PEA ROOT cDNA LIBRARY

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ABSTRACT

A cDNA library was synthesized using poly(A)$^+$ RNA purified from the total RNA from roots of pea (*Pisum sativum* L.). Ten representative clones encoding abundant root proteins were isolated from the library after screening using colony hybridization method which were probed by the root cDNAs. Freeze elution technique was used to extract three different partial pea root-specific genes which have been cloned in plasmid vectors pUC18 previously. These purified DNAs were radiolabelled and then used to probe with the library. One of the probes namely pPR179 was found to be highly specific and hybridized strongly to some of the root cDNA clones. This allowed full-length cDNAs that encoded root-specific protein(s) to be identified for subsequent analysis. Restriction patterns of the pea root cDNA-plasmid pUC19 recombinants revealed some artefactual cDNAs were synthesized and possible explanations were attempted.

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Dedicated to my parents and my sister for heartily support and encouragement.

ABBREVIATIONS

The abbreviation used throughout the dissertation are based on those that recommended by the Biochemical Society (1983) in the Biochemical Journal "Policy of the Journal and Instruction to Authors", volume 209, pp. 1-27. Notations that have been used but not listed in the Biochemical Journal are given below.

bp: base pairs

kb: kilobase pairs

cDNA: complementary DNA

ss/ds-cDNA: single-stranded/double stranded cDNA

mRNA: message RNA

tRNA: transfer RNA

dNTPs: deoxynucleoside triphosphates

poly(A)$^+$ RNA: polyadenylated RNA

BSA: bovine serum albumin

SDS: sodium dodecyl sulphate

SSC: saline sodium citrate

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CHAPTER I

INTRODUCTION

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INTRODUCTION

1.1 General Introduction

Peas (*Pisum sativum* L.) constituted one of the four most important seed legumes. It was grown most extensively in cool countries, flourishing in northern Europe, parts of Russia and China and the northwestern USA, though also having an important role at high altitudes in the tropics and as a winter crop in some hotter regions. As such, the crop constituted an important source of protein for human consumption. Traditionally, the crop has been grown for harvesting as fresh peas or as a dry mature product but, in many area of the world, the use of the crop has changed materially. A substantial proportion of the crop grown in northern Europe and North America was harvested as immature peas for freezing; this product has become one of the most important 'convenience protein foods' demanded by the twentieth-century people (Davies 1976).

The heterogeneous proteins contained in pea seeds were used as storage reserves for the germination of seeds. They, as well as many other legumes and cereals seeds storage proteins, represented a very large protein-synthesizing capacity in nature that provided a significant nitrogen stores as a main protein source for human and animal nutrition. There were some good reviews concerning the storage proteins in seeds that no detail elaboration was necessary here (Gatehouse et al., 1984; Higgins 1984; Payne and Rhodes 1982; Derbyshire et al., 1976).

The economic importance of legume seed proteins to human were unquestionable. The large proportion of food for mankind consumption in well-developed countries were derived from animals reared on diets enriched with legume (also cereal) seed proteins, whereas in developing countries, they were nearly the only protein component in people's daily diet (Payne 1983). Except the momentous nutritional role played by seed proteins nowadays, they also function as food additives during food processing by providing properties like texture improvement and emulsion stability. Although most of the legume seed proteins were highly consumable (ranging from 5% to 50% of total dry seed weight), their quality were generally poor because they tended to be deficient in methionine and cysteine (Burr 1975; Eggum and Beames 1983). Besides, their poor digestibility and the presence of toxic or anti-nutritional/metabolic components were factors that prevent them from becoming nutritionally excellent proteins (Gatehouse 1984a; Pusztai et al., 1983).

1.2 Application of Genetic Engineering to Plant Biotechnology

Because of the problems existed in legume proteins, attempts were made to improve their nutritional and functional qualities. Plant breeders have sought to introduce high-yielding and high-quality varieties that also carried the appropriate resistance to certain diseases and adverse climatic conditions through conventional plant breeding by transferring genes between different plant species. Until the past decades, rapid progress was being made in developing the tools for manipulating genetic information in plants by genetic engineering/recombinant DNA technology (Barton and Brill, 1983; Cocking et al., 1981). Plant genes were being cloned (Old and Primrose, 1986), genetic regulatory signals deciphered, and genes transferred

from similar or even entirely unrelated organisms (e.g. bacteria) to confer new agricultural useful traits on crop plants like legumes and cereal (Cocking and Davey 1987). These significantly increases the gene pool accessible for crop improvement (Goodman et al., 1987) and assisted the understanding of their physiological mechanisms in molecular level.

The development of plant genetic engineering despite the apparent success of traditional plant breeding programmes was indispensable because the latter suffered from several disadvantages. For instance, long testing and cultivation time (12 generations of backcrossing) were needed together with a following of a successful hybridization of two varieties was essential. Also, only limited gene pool was available by the range of plants with which they were sexually compatible if only sexual hybridization between them were undergone for qualities improvement. Moreover, the increase in crop yield would sooner or later slowed down to plateau, after the maximum productivity had been achieved, provided that the improvement of the crop plants was merely by shuffling the existing available characters. Finally, intensive care and accurate timing such as well-planned application of fertilizers, pesticides, herbicides and plant growth regulators were important in cultivating modern high yielding varieties. These were always costly to farmers and also a drain on energy sources and a potential source of pollutant (Shaw 1984; Mantell et al.,1985). The elucidation of the molecular basis of genetics and the progress in recombinant DNA technology promised the ability to make controlled changes to the genetic complement of plants.

1.3 The Study of Peas: From Cotyledons to Roots

Despite the advent in recombinant DNA technology and molecular biology, many traits that were of interest to plant scientists/breeders were poorly understood in terms of molecular basis. However, one exception was the seed storage proteins (e.g. peas) which have been intensively investigated with the aid of genetic engineering techniques (Sorenson 1984; Gatehouse et al., 1984; Larkins 1983; Brown et al., 1982). It was because each seed storage protein was the product of a single or a small family of genes. They were tissue specific and stage specific (under strict developmental control) so that those genes were only transcribed in a specific time during seed development. Based on these characteristics, the studies on pea seed storage proteins could be undertaken without affecting the plants' normal metabolism provided that the genes encoding the storage proteins were cautiously handled. Such advantage together with the economic importance of the seed proteins to human contributed to their intensive study throughout the past decade.

Since much of the early work on seed storage proteins were done, their structures, biosyntheses, role-played in seeds as well as the evolution of their encoding genes were, though not of 100%, being revealed tremendously. Thus it was a good idea to relocate the strategic point of research to some other part(s) of the plants. The roots of pea then came to one of the choices.

1.3.1 Nitrogen-fixing Ability

It was because the roots of pea (leguminous plant) possessed nitrogen-fixing nodules induced by different strains of the bacterium *Rhizobium* through their

symbiotic partnership. Legumes could grow well without the addition of expensive nitrogen fertilizers as long as they were infected with the correct strain. By studying the mechanisms and relationship between legumes' roots and *Rhizobium* in molecular level, it was possible to manipulate the nitrogen-fixing bacteria or their genes to alter other arable crops so that they became capable to fix their own nitrogen without too great a drain on photosynthates to provide the necessary energy (Gutschick 1980). The lack of knowledges on nodule-specific proteins in host plants (e.g. leghaemoglobin, glutamine synthetase, uricase and xanthine dehydrogenase, etc.) encoding by specific genes could be further investigated using pea roots as a model. Downie et al. (1984) cloned nodulation genes of *R. leguminosarum* that normally nodulated pea have been transferred to *R. phaseoli* (normally nodulated bean) that the latter then nodulated pea. Despite the apparent complexity of the nodulation sequence and symbiotic relationship, relatively few bacterial genes (10 kb) were required. Therefore, with the further studies on pea roots' nodules and *Rhizobium*, expansion of the host range of symbiosis to crops other than legumes is not impossible (Jones 1986).

1.3.2 Uptake of Nitrates and Other Solutes

For most of the crops, a linear increase in yield with increased nitrogen application was found. Hence the improvement of nitrates (or even phosphate and potassium) utilization by plants by manipulating the roots' uptake and transport parameters could be done, via the gene cloning of nitrate and nitrite reductase. Certain improvement in added nitrogen fertilizer utilization by recently bred cereals was reported by Bingham (1981), it is the time to extend such progress to leguminous pea plant which were of vital importance to human.

1.3.3 Pests and Diseases Resistance

Twenty-five million pounds per annum was spent in the UK on pesticides and herbicides in order to prevent yield losses caused by pests and diseases of agricultural crops. Thus, the breeding for diseases and pest resistance was important part in plant breeding programmes. However, the continual requirement for new sources of diseases/pests resistance species and the possibility that the breeding might be present in nonagronomic line or sexually incompatible wild type species could both complicated the matters. Transformation of crop plants with pest and disease resistance genes were therefore an attractive goal (Shaw 1984; Jones 1986).

Genes or group of genes encoding proteins such as lectins (Gatehouse et al., 1984a) or enzyme inhibitors (e.g. trypsin inhibitor) (Gatehouse et al., 1979), viral factors or phytoalexins (Day et al., 1983), "pathogens-related" proteins (White and Antoniw 1983; Van Loon 1980) and bacterial insecticidal toxins (Martin and Dean 1981) such as that produced by *Bacillus thuringiensis* could be manipulated and transferred to the desired plants. In case of roots, resistance to insects, nematode pathogens and fungal invasion might be induced by introducing genes whose products interfered with digestion (e.g. protease inhibitor) or with the nervous system (e.g. bacteria-derived inhibitors of acetylcholinesterase). Moreover, if the plants could be induced to have fungicide resistant ability by the cloning of specific resistant gene(s), fungicides application to eliminate the fungi on root without reducing the crop yield and quality would be more viable. Because of these reasons, the studies on pest and disease resistance on aerial part as well as the subaerial part (i.e. the root) of the plants were essential.

1.3.4 Stress Tolerance

The study of root systems by genetic engineering techniques in molecular basis might allow the transferrence of the ability to tolerate environmental stress from xerophytes or halophytes to crop plants. Proteins involved in biosynthesis of related furano coumarins and flavanoids that protected plants from excess UV irradiation (Kruezalar et al., 1983) as well as heat-shock genes involved in several multigenic families (Schoffl and Key 1983) to against the environmental heat stress have been investigated. Hence, the other major stress such as water content in soil and high salinity that encountered the roots of plants in first hand could be studied by using suitable root system as a model.

1.3.5 Pea Root-Specific Genes Isolation

Three types of pea root cDNAs inserted into EcoRI sites of plasmid vector pUC18 namely pPR179 *, pPR287(A) ** and pPR340† were constructed. They were all pea root specific as they were selected from those cDNAs showing significantly stronger hybridization to a mixture of total pea cotyledons, total pea leaves and total dark grown pea leaves cDNAs. Such cDNA provided messages expressed mainly in pea root. The corresponding full length gene was then isolated from a gene library. After characterizing this gene, the 5' non-coding region which was thought to control the activity of the gene could be used to control the activity of foreign gene(s) inserted back into the original host plant (i.e. pea). This allowed tissue and probably time-specific expression of any desire gene cloned into the

* 450 bp, hybridized to a mRNA of about 650 bases of moderate abundance.

** subclone of 250 bp insert of pPR287 which had multiple insert hybridized to a mRNA of about 1200 bases of low abundance.

† 480 bp, hybridized to a mRNA of about 3300 bases at very low abundance.

sequence.

1.4 Methods in Construction of a cDNA Library

The enzymatic conversion of poly(A)$^+$ RNA to double-stranded cDNA and the subsequent insertion of this DNA into vector (e.g. plasmid pUC19, phage λgt11) has become a basic technique in eukaryotic molecular biology. It was the primary step in molecular cloning to construct a cDNA library of a particular poly(A)$^+$ RNA before further investigation like DNA sequencing or in vitro mutagenesis could take place. It enable rapid progress in the study of gene organisation, structure, and expression (Catterall et al., 1979; Tilgham et al., 1978). A cDNA library was useful because it contained fewer clones than a complete genomic library so that screening could be done easier. Every cDNA clone contained a mRNA sequence so that false positive clone could not be selected easily. Besides, the expression of cloned genes in bacteria to produce eukaryotic proteins was possible after successful transformation. A cloned cDNA was by far the most suitable probe for hybridization to eukaryotic DNA because it contained no non-coding sequence (intron) other than the cloned mRNA sequence. This allowed comparison between the nucleotide sequence of genomic DNA and the cDNA copy so that precise determination of introns position and RNA splicing (Tilgham et al., 1978) was possible (for reviews see Maniatis et al., 1982; Forde 1983; Williams 1981; Efstratiadis and VillaKomaroff 1979).

Different poly(A)$^+$ RNA were copied into DNA with different efficiencies. Hence, the conditions that were optimal for copying one species of poly(A)$^+$ RNA did not guarantee another might work as well. Generally, the conditions that

lead to the greatest overall yield of cDNA from a heterogeneous population of poly(A)$^+$ RNA would be employed and the following parameters were important: (1) The quality and purity of reverse transcriptase (Myers et al., 1980) as well as DNA polymerase I (Wickens et al., 1978). (2) Optimal maintenance of pH at 8.3 and a suitable concentration of monovalent cation (e.g. Na^+/K^+). (3) An optimal concentration (6-10 mM) of Mg^{2+} divalent cation. (4) High concentration of deoxynucleotide triphosphates (dNTPs) (Retzel et al., 1980).

A variety of methods has been used to construct dscDNA and linked them to plasmid vectors and they were well-developed.

1.4.1 Homopolymer Tailing Method of cDNA Cloning

Until recently, this was the most widely used dscDNA preparative method (Wickens et al., 1978; Buell et al., 1978). Synthesis of the first cDNA was primed from oligo-d(T) annealed to the poly-A tail on the 3' terminus of the poly(A)$^+$ RNA by using the enzyme reverse transciptase. The RNA template was then alkaline hydrolysed and the second strand synthesis primed from hairpin structures which were formed at the 3' terminus of the first strand (Higuchi et al., 1976; Efstratiadis et al., 1976). Second strand synthesis could be carried out using either DNA polymerase or reverse transcriptase. The hairpin and any ssDNA at the other end of the cDNA molecules were then cleaved by S1 nuclease and the dscDNA could be cloned in the desired vector by homopolymer tailing technique. With the assistance of terminal transferase (Michelson and Orkin 1982) oligo d(A) sequence and oligo d(T) sequence (or dC.dG sequence) could be annealed to the cDNA and the vector so that the recombinant plasmid was constructed (Jackson et. al., 1972;

Lobban and Kaiser 1973).

1.4.2 Synthetic DNA Linkers or Adaptors Addition Method of cDNA Cloning

Synthetic double-linkers (Kurtz and Nicodemus 1981) containing one or more restriction endonuclease recognition site(s) or adaptors (Wu et al., 1978) with preformed cohesive end might be added to the cDNA using T_4 DNA ligase. The linkers or adaptors were than cleaved with the appropriate restriction enzyme and ligated to a plasmid vector that has been cleaved with a compatible enzyme (Heynecker et al., 1976, Bahl et al., 1978).

1.4.3 Okayama and Berg Method of cDNA Cloning

dC tailing of sscDNA followed by oligo dG priming of second strand synthesis eliminated hairpin formation and the use of S1 nuclease was first reported by Land et al. (1981). Later, Okayama and Berg (1982) devised a protocol in which full length cDNA could be efficiently cloned without using S1 nuclease since the latter inevitably degraded some terminal nucleotides from the dscDNA. Synthesis of the first strand was primed from an oligo-d(T) covalently attached to one end of the linearised plasmid. The cDNA was therefore immediately attached to the vector in the first step of its synthesis. The synthesis of the second strand was not the next step, instead oligo-d(C) tails are added to the DNA-RNA duplex. The oligo-d(C) tailed end of the plasmid vector, opposite to that joined to the cDNA, was removed and replaced by a similar restriction fragment tailed with oligo-d(G). The molecule can then be cyclized. Finally the mRNA was digested from the DNA-RNA duplex using RNase H. The large gap that was left was repaired using DNA

polymerase I and DNA ligase.

1.4.4 Heidecker and Messing Method of cDNA Cloning

Heidecker and Messing (1983) also proposed an efficient method for generating full length cDNA clones. The poly(A)$^+$ RNA was annealed to linearised and oligo-d(T) tailed plasmid DNA, which then primed synthesis of the first cDNA strand using reverse transcriptase. Oligo-d(G) tails were added to the cDNA-plasmid molecules, which were then centrifuged through an alkaline sucrose gradient. This step removed small molecules, hydrolysed the mRNA and separated the two cDNAs which were formly attached to the same duplex plasmid. Denatured, oligo-d(C) tailed plasmid DNA was added in excess and conditions adjusted to favour circularization by the complementary homopolymer tails. The excess oligo-d(C) tailed plasmid might simply renatured, but could not recircularized. The circular molecules have a free 3'-hydroxyl on the oligo-d(C) tail which primed second strand synthesis of the cDNA to create duplex recombinant plasmids which transformed *E. coli*. Clones could be obtained with the cDNA inserted in both orientations.

1.5 An Overview of the Method for the Synthesis of cDNA Library Used in this Project

The method for the synthesis of dscDNA for cloning in plasmid vectors was adopted from Gubler and Hoffman (1983). A similar proposal was also available from Watson and Jackson (1985). Such method was convenient and efficient that offered several advantages over the conventional one (see section 1.4.1). Such method did not depend on hairpin loop priming, nuclease S1 treatment was therefore not necessary and also a high percentage full length cDNA library could

be constructed (Schneider et al., 1984; Howells et al., 1984). The first strand cDNA using poly(A)$^+$ RNA as a template was catalysed by reverse transcriptase (see footnote in section 2.2.8). Second strand synthesis was performed using the mRNA-cDNA hybrids as substrate. By the utilization of *E. coli* ribonuclease H (RNase H) which was an endoribonuclease that digested the mRNA in the hybrid only (Leis et al., 1973), nicks in the mRNA strand were produced. DNA polymerase I used these nicks to replace RNA with DNA by nick translation type reaction. Klenow fragment was then added to remove any 3' small overhangs on the first strand cDNA to ensure they were blunt-ended. Afterward, preformed EcoRI adaptors were ligated with the dscDNA so as to prepare the cDNA for insertion into the site of a suitable plasmid vector. The cDNA produced was then available for transformation in competent *E. coli* cells and subsequent cloning (Efstratiadis and VillaKomaroff 1979; Young and Davis 1983). Detail procedures of the above cDNA synthesis were available in Pharmacia (1985) and Amersham (1985) cDNA synthesis kit instruction.

1.6 Methods of Screening a cDNA Library

Once a library was established, the identification and characterization of individual plasmid containing specific cDNA sequence was carried out in two distinct stages. Firstly a broad screen of the complete library to identify colonies likely to contain recombinant plasmids of interest was carried out, followed by the detailed characterization of the cDNA inserted into the selected plasmids.

Several primary screening methods were available. Through genetic methods, selection for the presence of plasmid vectors could be achieved using their drug

resistance ability or nutritional markers. In case of phage vectors plaque formation was itself the selected property. For certain replacement type λ vectors or cosmid vectors, size selection by the phage particle could select recombinants (Old and Primrose 1986). However, if an inserted foreign gene in the desired recombinant carried biosynthetic gene was expressed, they could be identified by complementation of nonrevertible auxotrophic mutations in the host strain (e.g. *E. coli*) (Ratzkin and Carbon 1977; Chang et al., 1978).

In situation where expression of cDNA sequences within a cDNA library has been sought, immunochemical primary screening could be done using radiolabelled antibodies (Broom and Gilbert 1978; Dahl et al., 1981; Williams 1981). On a replica filter containing the transformed cells, the colonies were lysed so that the antigen from the positive colonies could be released. A sheet of polyvinyl coated with the appropriate unlabelled antibody was applied to the filter. Antigen-antibody complex were then formed. The sheet was removed and exposed to radiolabelled antibody (e.g. by ^{125}I) which binded to another antigenic determinant site. Subsequent washing and autoradiography allowed the identification of positive clones (VillaKomaroff et al., 1978; Young and Davis 1983).

In the absence of expression, the method of Grunstein and Hogness (1975) was most widely adopted. Possible transformants were picked, placed on gridded nitrocellulose filter disc and then onto fresh agar containing the appropriate antibiotic. This formed the master plate from which each colony might be replica plated onto other nitrocellulose filter over-laid on agar plates, grown, lysed with alkali, and the denatured DNA baked onto the filter (Craig et al., 1981). Once the colonies have been fixed, the filter might be screened using a variety of radi-

olabelled hybridization probes, dependent on the circumstance. For example, a previously cloned DNA fragment, whole genomic DNA, synthetic oligonucleotides specifying a particular amino acid sequence, RNA or cDNA. In this project, such colony hybridization protocol using radiolabelled cDNA as probes was employed.

Secondary screening of candidate plasmids from cDNA library required the identification of the inserted cDNA sequence such that nucleotide sequence determination might be performed with confidence. Selected colonies were cultured in small scale, plasmid DNA were then isolated using alkaline lysis method (Birnboim and Doly 1979; Holmes and Quigley 1981) and the size of the cDNA inserts was determined by horizontal agarose gel electrophoresis (McDonell et al., 1977). Ideally, if the design of the recombinants resulted in reconstruction of restriction sites at both ends of the inserted cDNA, then the size of the insert might be determined after restriction by comparative electrophoresis with restriction fragments of known size (Sutcliffe 1978).

1.7 Aims of the Project

The rationale for this project was to construct a complete cDNA library of pea root by a recent innovative method (Gubler and Hoffman 1983; see also section 1.6). Through ligation with appropriate plasmid vector and competent cells transformation, sizing of cDNA inserts by horizontal agarose gel electrophoresis could be done. The relatively abundant cDNA clones were selected by hybridization with radiolabelled pea root cDNA probe. The choice of construction of a cDNA library rather than a genomic library laid on the fact that the former was generally easier to screen. It was because the cDNA were direct copies of cytoplasmic

mRNA transcripts with the absence of introns.

The second part of the project concerned with probing the pea root cDNA library with some previously constructed root specific DNA. Three types of pea root cDNA inserts (pPR179, pPR287(A) and pPR340) in cDNA-plasmid chimaeras corresponded to part of a complete full length pea root DNA were isolated and fractionated. Specific radiolabelled probes were prepared from them and were hybridized with the preformed root cDNA library. The aim was to isolate full length gene clones that encoded root-specific protein(s), so that further analyses like their possible functions, characterization of such root-specific gene(s), subsequent gene(s) engineering and insertion of foreign gene for expression could be achieved.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Biological and Chemical Reagents

Most of the chemical reagents were supplied by BDH Chemicals Ltd., Poole, Dorset, UK and were of AnalaR (analytical) grade or the finest available. Some other biological and chemical reagents were supplied by other major suppliers as listed below.

Ethyleneglycobis(β-aminoethyl)ether tetraacetic acid (EGTA), acridine orange, proteinase K, lysozyme, spermidine, bovine serum albumin (BSA), dithiothreitol (DTT), ampicillin (sodium salt), herring sperm DNA, ethidium bromide (EtBr) were all supplied by the Sigma Chemical Co., Poole, Dorset, UK.

Guanidinium hydrochloride and guanidinium thiocyanate were supplied by Fluke Chemie AG, CH 9470 Bucks.

Glyoxal gel and gelbond film were supplied by FMC Bioproducts, ICN Biomedical Ltd., Free Press House, Castle St., High Wycombe, Bucks, HP13 6RN.

Sephadex G-50, Ficoll 400 and cDNA Synthesis Kit were supplied by Phamacia Fine Chemicals, Uppsala, Sweden.

Yeast extract was supplied from Bio-Life, Milan, Italy.

Nitrocellulose filters (BA85, 0.45μm) were supplied by Schleicher and Schull, Anderman and Co., Ltd., Kingston-upon-Thames, Surrey, UK.

Oligo d(T)-cellulose (Cat. No. 20002) was supplied by Collaborative Research Inc., Waltham, M.D., U.S.A.

Bactoagar and tryptone were supplied by Difco Laboratories, Detroit, Michigan, U.S.A.

3MM papers were supplied by Whatman Ltd., Maidstone, Kent, U.K.

All the restriction endonucleases were supplied by Northumbria Biologicals Ltd., Northumbria, UK. and Bethesda Research Laboratories UK. Ltd., Cambridge. UK. The latter one also supplied agarose (gel electrophoresis grade) and Klenow polymerase.

Tris(hydroxymethyl) aminomethane (Tris), 5-dibromo-4-chloro-3-indoylgalactoside (X-gal), T_4 DNA ligase, glycogen and calf intestinal alkaline phosphatase (CIF) were supplied by Boehringer Mannheim Corporation (London) Ltd., Lewes, East Sussex, UK.

Plasmids pUC19, pBR322 and recombinant DNA pPR179, pPR287(A), pPR340 and NM258 lambda bacteriophage were supplied by personnel in D2 and C2 laboratories in Botany Department, University of Durham.

2.1.2 Bacterial Strains, Plasmids and Bacteriophage

The bacterial strain employed throughout the project was a derivative of E. coli K-12 namely DH5α.

The plasmid vector used for cloning was pUC19 (Vieira and Messing, 1982; Norrander et al., 1983) with a size of 2.7kb. It contained many cloning sites, for example, HindIII, SphI, PstI, SalI, AccI, HincII, XbaI, BamHI, XmaI, SmaI, EcoRI, HaeIII, etc. Recombinants could be selected by inactivation of β -galactosidase gene (white colonies on X-gal medium) and ampicillin resistant. Plasmids could accept inserts larger than 10kb but transformation efficiency and DNA yield decrease (Arrand, 1986). Plasmid pBR322 (Bolivar et al., 1977) was used as a size marker after being digested with suitable restriction enzyme(s).

Bacteriophage lambda NM258 was used as a standard size marker as well after restricted with appropriate restriction endonuclease(s).

2.2 Methods

2.2.1 Biochemical Techniques

The followings were collection of a set of biochemical techniques that were frequently used in molecular biology and gene cloning.

1. Glasswares and Plasticwares

All glasswares and plasticwares should be sterilized by autoclaving (20 min at 120°C) as well as Eppendorf tubes and disposable tips for pipettemans. For those items that autoclaving was impossible should be sterilized by rinsing in 80% ethanol and flaming if possible. Or otherwise, sterilized by 1% hot (60-65°C) SDS and rinsed thoroughly with sterile water afterward.

2. Reagents

All water and solutions used for molecular cloning in which they came in contact with nucleic acids, enzymes, bacteria, etc. should be autoclaved if appropriate. Stock solutions were made up using sterile water under clean conditions. The growth medium Yeast-Tryptone (YT) broth and Yeast-Tryptone-Ampicillin-X-gal (YT-amp-X-gal) agar as well as RNase solution (free of DNase) were prepared by methods mentioned by Maniatis et al.(1982).

3. Purification of Nucleic Acids by Phenol/Chloroform Extraction

The standard protocol to remove proteins from nucleic acids solution was based on that of Brawerman et al.(1972). "Phenol" meant phenol equilibrated with buffer containing 0.1% hydroxyquinoline and 0.2% β-mercaptoethanol where "Chloroform" meant a 24:1 (v/v) mixture of chloroform and isoamyl alcohol. Procedures could be obtained from Maniatis et al. (1982).

4. Concentration of Nucleic acids by Precipitation with Ethanol

The reagents required for precipitation were -20°C absolute ethanol and 3M ice cold sodium acetate (pH 4.8). The procedures were listed in Maniatis et al. (1982).

5. Storage of DNA, RNA and Bacterial Strains

DNA and RNA samples could be stored in sterilized TE buffer (10mM Tris.Cl, pH 7.5; 1mM EDTA) or sterile water at -20°C and -80°C respectively. However, for a longer period of storage, DNA samples should be placed in -80°C freezer whilst RNA samples should be stored in liquid nitrogen. For the storage of bacteria

(including bacterial colonies on nitrocellulose filters), they were kept on agar plates in an inverted manner sealed with Nescofilm at 4°C temporarily. For long term storage, bacteria strain should be kept in 25% YT-glycerol agar plates. In 500 ml of YT-glycerol agar solution, it contained 343.75 ml distilled water, 156.25 ml 80% glycerol, 2 g of tryptone, 1.25 g of yeast extract, 1.25 g of NaCl and 3.75 g of bactoagar. It should be autoclaved and after cooling down to 55°C, 12.5 mg (1.25 ml of 10mg/ml stock solution) of ampicillin solution was added. Then 20 plates could be made in laminar flow. It should be notified that we have to incubate the master nitrocellulose filters (section 2.2.9) at 37°C for 2.5 hours on YT-amp-X-gal agar plate and then transferred to YT-glycerol agar plate for another hour 37°C incubation before we could preserve them at -20°C.

6. Quantitation of DNA and RNA

Two widely used methods could be employed to quantitate the amount of DNA or RNA in a preparation (Maniatis et al., 1982).

A. Spectrophotometric Quantitation of DNA and RNA

By using a PHILIPS PU 8700 Series UV/Visible Spectrophotometer and 1-cm path length quartz cells, the concentration and the purity of both DNA and RNA could be estimated after a series of calculation.

B. Ethidium Bromide Fluorescent Quantitation of Double-stranded DNA

Less than 250ng/ml of ds-DNA after appropriate agarose gel electrophoresis could be visualized and quantitated under a UV transilluminator.

2.2.2 Preparation of Pea Roots from Pea Plants

The fibrous roots were cut with scissors from the pea plants after growing in culture fluid for 2-3 weeks. After 100 g of roots in fresh weight were collected, they were washed with distilled water several times and blotted dry by tissue papers. After that, they were put into liquid nitrogen to deep frozen quickly and then wrapped in small batches with known weight in aluminium foil separately. They were stored at -80°C for RNA extraction later.

2.2.3 Preparation of Total RNA from Pea Roots/Cotyledons

2.2.3.1 Hot SDS/Proteinase K Method

The method was adopted from Hall et al. (1978). In general, 50 g of frozen pea cotyledons/roots from -80°C freezer were homogenized in 130 ml homogenization buffer (0.2 M boric acid, 1% SDS, 30mM EGTA, 5mM DTT and adjusted to pH 9.0 by NaOH). 0.5 mg of proteinase K per ml of buffer was added to digest the linearized proteins (including RNase). After the removal of excess SDS by KCl precipitation, RNA was insolubilized by LiCl. RNA was then washed and precipitated by ethanol precipitation overnight. It was followed by phenol extraction and ethanol precipitation again!

2.2.3.2 Guanidinium/Cesium Chloride Method

Guanidinium hydrochloride and thiocyanate (Cox, 1968) dissolved protein readily and released nucleic acids from nucleoproteins as their cellular structures disintegrated and the ordered secondary structured lost. Hence, even RNase could be denatured in 4 M guanidinium thiocyanate (Chirgwin et al., 1979; Ullrich et

al., 1977; Cathala et al., 1983) and reducing agent β-mercaptoethanol (Sela et al., 1957) and then the separation of total RNA from genomic DNA was achieved by centrifugation through a 5.7 M cesium chloride cushion (Glisin et al., 1974).

7.5 g of thoroughly grinded pea roots was placed in a sterilized (by 1% hot SDS at 65°C) 100 ml MSE centrifuge tube with 25 ml extraction buffer (4 M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0; 0.5N-lauroylsarcosine; 0.1 M β-mercaptoethanol). It was then polytron mixed for 20 seconds at maximum speed with probe and the centrifuge tube being wrapped in Nescofilm. Afterwards, the probe was washed with 10 ml of extraction buffer to make the final volume to become 35 ml. The homogenate was spinned at 4°C , 15000 rpm for 30 min in a MSE 18 centrifuge. The supernatant was then layered equally onto 12 ml per tube cesium chloride cushion (5.7 M CsCl; 0.1 M EDTA, pH 7.0) in three 23 ml prepspin tubes. They were ultracentrifuged at 30000 rpm at 4°C for 24 hours. The supernatant and the floating debris were removed by water aspiration. The RNA pellet at the bottom was resuspended in 1 ml per prepspin tube 7.5 M guanidinium hydrochloride solution (25mM sodium citrate, pH 7.0; 5mM DTT; 7.5 M guanidinium hydrochloride). The solution was transferred to a 30 ml sterilized Corex tube and was centrifuged for 15 min at 6000rpm. The supernatant was poured into another sterilized Corex tube carefully. 0.025 volume (about 75μl) of 1 M acetic acid and 0.5 volume (about 1.5 ml) of cold absolute ethanol were added and left to precipitate at -20°C overnight. RNA could then be recovered by centrifugation at 9000 rpm for 15-30 min at 0°C. After washed with 70% ethanol, recentrifuged and vacuum dried, it was resuspended in 500 μl of 0.1% DEPC-treated (Ehrenberg et al., 1976) double-distilled water and stored at -80°C.

2.2.4 Preparation of Polyadenylated RNA from total RNA

The method in preparation of polyadenylated RNA – poly(A)$^+$ was adopted from Maniatis et al. (1982). Several techniques have been developed to separate poly(A)$^+$ RNA from total RNA and some important points should be taken into account when considering the method to be used (Maniatis et al., 1982; Taylor, 1979). The method of choice here was affinity chromatography using oligo d(T)-cellulose (Edmonds et al., 1971; Aviv and Leder, 1972) which could be obtained commercially. The poly(A)$^+$ RNA was selected by passing the total RNA through an oligo d(T)-cellulose column twice (Arrand, 1986; Evans et al.,1980) and then the poly(A)$^+$ could be eluted by using a different salt concentration elution buffer (Craig et al., 1976).

2.2.5 Standard Enzymatic Methods Used in DNA Manipulation

2.2.5.1 Restriction of DNA and Determination of Fragments Size

One of the three buffers namely low salt, medium salt or high salt restriction buffer were used in DNA restriction. The choice of the right buffer was depending on the requirements of the restriction enzymes used. To obtain good restriction results usually 3-5 units of the desired restriction enzyme was required to completely digested 1 μg of DNA sample. 5x or 10x restriction buffer and suitable amount of sterile water were added to make the buffer concentration reduced to 1x (assuming a total restriction volume to be 20μl). Besides, 2μl of RNase (10mg/ml) could be added to digest the RNA (e.g. tRNA) and so as 2μl of 0.1 M spermidine which could enhance the digestive efficiency of the restriction enzymes. They were all contained in Eppendorf tube, well mixed and briefly spinned down by a bench

centrifuge. Afterwards, the restriction was done at 37°C for at least 2 hours. It was then stopped by heating at 70°C for 5 min. For every 4μl of DNA sample 1μl of agarose loading dye was added for tracking (for preparation of materials mentioned above see Maniatis et al., 1982).

When estimation of the DNA fragments was necessary, various standard size markers for instance, phage lambda NM258 restricted with HindIII, plasmid pBR322 cut with Alu I, etc. could be used. The logarithm (base 10) of fragments' size (in bp) of the restricted size marker were plotted against the distance of migration of each of them. A straight line could be obtained for accurate determination of dsDNA fragments sized from 100-10000 bp.

2.2.5.2 Ligation Reaction

After suitable restriction, phenol extraction and ethanol precipitation, the DNA which was dissolved in appropriate amount (20-100μl) of TE buffer (10mM Tris.Cl, pH7.6;1mM EDTA, pH 8.0)/sterile water was ready for ligation reaction accomplished in vitro through the action of DNA ligase (Engler and Richardson, 1982). At least 1μl of 1u/μl T_4 DNA ligase was added to ligate 1μg DNA for 12-16 hours at 15°C (Ferretti and Sgaramella, 1981) in 1x ligation buffer made from a 10x stock solution (Maniatis, et al., 1982). During the ligation reaction ATP was hydrolysed to AMP and PPi (inorganic pyrophosphate) (Weiss and Richardson, 1968) while the phosphodiester bond was generated between 5'-phosphate and 3'-hydroxyl groups. After the ligation, DNA was stored at -20°C.

2.2.5.3 Dephosphorylation of Plasmid DNA at the 5' Ends Using Alkaline Phosphatase

This technique was employed to remove the protruding terminal 5'-phosphates to, for example, prevent the recircularization of the plasmid DNA (Ullrich et al., 1977). Calf intestinal alkaline phosphatase (CIP) was used (Chaconas and Van de Sande, 1980) because it could be inactivated by heating to 68°C in SDS. The procedures used followed that in Maniatis et al. (1982). However the spun column purification step was omitted as it was unnecessary in our case.

2.2.6 Agarose Gel Electrophoresis

The simple and rapid to perform technique used to separate, identify and purify DNA was agarose gel electrophoresis (Sharp et al., 1973; Helling et al., 1974) in which different size and conformation of DNA migrated at different rate (Thorne, 1966,1967). When used in concentrations from 0.1-2.5%, agarose gels could resolve DNA from 880000 down to 150 bp (Yang et al., 1979; Bostian et al., 1979). Horizontal slab gels submerged in buffer in gel electrophoresis tanks were used throughout.

2.2.6.1 Full Size Agarose Gel Electrophoresis

In order to make a full size agarose gel (approx. 20x15x0.6 cm), 1.4 g of agarose was added to 180ml of distilled water and heated to dissolved. After the sol had cooled down to below 60°C, 20 ml 10x electrophoresis buffer (Alec's buffer) and 20μl ethidium bromide (from a stock solution of 10mg/ml, stored at 4°C in a light-proofed bottle) were added and then mixed by stirring gently. Before that, a plastic frame with size for making the full size agarose gel was stuck to a

clean glass plate with the application of vacuum grease. Then the sol was poured into the levelled frame with a perspex comb suspended across the mould which was 1 mm above the glass plate to produce slots. After the gel was completely set, carefully removed the comb and the frame and mounted the gel in the gel electrophoresis tank filled with well mixed 1x Alec's running buffer (200 ml 10x Alec's buffer plus 1.9 L distilled water and 200μl of 10mg/ml ethidium bromide). The trapping of air bubbles should be avoided and the gel should be completely covered by electrophoresis buffer. All DNA samples were then loaded into the slots with the addition of 1/4th volume of agarose loading dye. The the power pack was connected to the tank in correct polarity according to the DNA migration and was then switched on. The gel was electrophoresed for 3-16 hours at 30-120 V. The DNA bands were visualized by the fluorescence of the ethidium bromide-DNA intercalated complex under a short wave UV transilluminator (Sharp et al., 1973). The results could be photographed by using an orange-red Kodak 22A Wratten Filter mounted on a camera equipped with sensitive film of Polaroid Type 667 (ASA 3000).

2.2.6.2 Agarose Minigel Electrophoresis

For quick detection of DNA bands, especially those with size between 250-1000 bp, 0.7% agarose minigel was used. 0.35 g of agarose was heated to dissolve in 45 ml of distilled water. After cooled down to below 60°C 5 ml 10x Tris-Borate-EDTA (TBE) buffer (108 g Tris base; 55 g boric acid; 20 ml 1 M EDTA, pH 8.0 in 1 L of distilled water) and 5 μl ethidium bromide (10mg/ml stock) was added. The sol was mixed by swirling and was poured into minigel apparatus for setting. 50 ml of 1x TBE buffer was made from the 10x stock and 5 μl of ethidium bromide was

again added. The gel was electrophoresed at 50 mA for 30-60 min, checked on a UV transilluminator and photograph could then be taken.

2.2.6.3 Glyoxal (RNA) Gel Electrophoresis

RNA should be denatured by glyoxalation in order to linearize the fragment for accurate determination of its size (McMaster and Carmichael, 1977). The method used here was given by R. Swinhoe (pers. comm.) simplified from other available method (Miller, 1987). Samples was glyoxalated by placing in an Eppendorf tube in order: 20 μl redistilled dimethylsulphoxide (DMSO); 2 μl 0.2 M sodium phosphate buffer, pH 6.8; 5.7 μl 6 M deionized glyoxal and 12.3 μl RNA (about 20 μg). They were altogether incubated at 50^{0}C for 1 hour. For the preparation of half sized gel, 1.125 g of high gelling temperature agarose was heated to dissolve in 75 ml of 10 mM sodium phosphate buffer (pH 6.8) and after cooling down the sol was poured into the gel-forming frame adhered to a sheet of gelbond by vacuum grease. The glyoxalated RNA samples were then loaded with the addition of 1/5th volume of agarose beads (50% glycerol; 10 mM sodium dihydrogen phosphate, pH 7.0; 0.4% w/v bromophenol blue). The gel was electrophoresed in 10 mM sodium phosphate buffer at 100 V for 4 hours with slow stirring and buffer circulation. Afterwards, the gel was stained in freshly prepared acridine orange dye (30 mg of acridine orange in 1 L of running buffer) for 5-10 min in dark and thence destained in running buffer overnight. The results could be visualized under short wave UV transilluminator.

2.2.7 Recovery of DNA from Agarose Gels by Freeze Elution

The wanted DNA fragment was cut from the agarose gel using a sterilized razor blade after suitable restriction. Then the fragment was put into a 0.5 ml Eppendorf tube with a hole at the bottom pierced with fine forcep. In addition, the hole should be plugged with small amount of sterilized glass wool. Such Eppendorf tube was then placed into a 1.5 ml Eppendorf tube with the cap removed. They were frozen at -80°C for 1/2 hour. Afterwards, the whole complex was spinned in an Eppendorf centrifuge for 20 min *. The liquid containing the DNA was then extracted with equal volume of phenol/chloroform (1:1) and then three times with equal volume of chloroform. Finally 1 ml of cold absolute ethanol was added to the purified aqueous DNA solution for precipitation. The pellet could be recovered by centrifugation and then resuspended in 50 μl sterile water/TE buffer.

2.2.8 Construction of a Pea Cotyledon/Root cDNA library

Double-stranded complementary DNA (ds cDNA) can be made and cloned into plasmids or bacteriophages to produce a complete cDNA library. The synthesis of cDNA from pea cotyledon/root polyadenylated RNA – poly(A)$^+$ RNA was based on the cDNA Synthesis Kit supplied by Pharmacia. The protocol used here was the same as the manual enclosed in the synthesis kit.

5 μg of cotyledon/root poly(A)$^+$ RNA and suitable amount of diethylpyrocarbonate (DEPC) treated RNase-free water were heated at 65°C for 10 min in an Eppendorf tube. 1 μl DTT solution and first-stranded reaction mix † primer,

* the gel should be checked at the first 10 min to ensure it have not thawed yet or otherwise stopped at there.

† Contained FPLC Cloned Murine, Moloney Murine Leukemia Virus (MMLV), Reverse Transcriptase, RNA guard, RNase/DNase-free BSA, oligo d(T)$_{12-18}$

dATP, dCTP, dGTP and dTTP in aqueous buffer. (both supplied in the kit) were mixed with the denatured mRNA and incubated. The first single strand cDNA was then synthesized.

Second strand reaction mix** and 1 μl of Klenow fragment (both supplied in the kit) were added to the Eppendorf tube containing the first strand cDNA. Hence the complete blunt-ended double strand cDNA was synthesized. It was then purified by phenol/chloroform extraction and the ds cDNA was separated from the unreacted nucleotides by passing down a spun column of Sephacryl S-200 using ligation buffer [66 mM Tris.Cl, pH 7.6; 1 mM spermidine; 10 mM $MgCl_2$; 15 mM DTT; 0.2mg/ml BSA (RNase/DNase-free)] as elution buffer.

To the purified ds cDNA, 5 μl EcoRI adaptor solution, 1 μl ATP solution and 3 μl of T_4 DNA ligase (all supplied in the kit) were added and incubated at 12^{0}C overnight. Both the blunt ends of the ds cDNA would ligate with synthetic EcoRI adaptors [oligonucleotides with specific preformed restriction sites that did not require cleavage in order to create a cohesive end (Bahl and Wu, 1978)]. Then 10 μl of ATP solution and 1 μl of T_4 polynucleotide kinase (both supplied in the kit) were added to phosphorylate the terminals of the ds cDNA. After purification through the spun column again, the cDNA was ready for insertion into suitable plasmid vector.

Plasmid pUC19 was restricted with EcoRI followed by dephosphorylation reaction using calf intestinal alkaline phosphatase. A minigel was done to ensure the dephosphorylated vectors were presence after a series of treatment.

** Contained *E. coli* RNase H, *E. coli* polymerase I in aqueous buffer with dNTPs.

Finally insertion of cDNA into a plasmid vector could be performed by adding 1 μl of T_4 DNA ligase, 3 μl of 10x diluted ATP, 2 μl of 0.05 μg/μl plasmid vectors and a series of different concentration of cDNA (concentration adjusted by ligation buffer). A control was also set up with all the conditions the same but only with no cDNA included. This provided the evidence that the dephosphorylated vector would not recircularized. The remainder of cDNA solution was stored at 4^{0}C. After incubation at 12^{0}C overnight, recombinants formed and were able to transform competent cells (Hanahan, 1985). Positive transformants could be screened by their resistance to YT-amp-X-gal selective agar.

On the basis of the number of recombinants obtained, the concentration of cDNA to plasmid that gave best results of ligation/transformation was determined. By using the remainder of the cDNA solution, a scaled up version of the ligation/transformation was performed to generate a complete cDNA library.

2.2.9 Transformation of Competent Cells

E. coli competent cells for high efficiency transformation were kindly provided by Mr. Dave Bown (Botany Dept., Uni. of Durham). They were suspended in 0.1 M $CaCl_2$ and stored in 200 μl aliquots in 0.5 ml Eppendorf tube at -80^{0}C. After the cells were taken out from the freezer, they were thawed in hand until just thawed and then left on ice for 10 min. Then they were mixed with DNA sample (e.g. DNA-plasmid chimaeras). Up to 2/5 volume of cells could be added but no more than 100 ng per 200 μl of cells. They were left on ice for another 45 min following by heat shocked at 42^{0}C for 90 seconds. 800 μl of YT-medium was added and then incubated at 37^{0}C for 1 hour to allow antibiotic resistance to express. Eventually

the cells were plated out by glass spreader on selective agar (e.g. YT-amp-X-gal agar plate) at certain different concentration to achieve the best transformation results.

2.2.10 Alkaline Minipreparations of Plasmid DNA – Minipreps

The protocol used here was derived from D. Ish-Horowicz (1981) which was a modification of the method of Birnhoim and Doly (1979). Overnight culture of plasmid-containing strain was grown up in sterilized McCartney bottle containing 10 ml of YT-medium plus the appropriate antibiotic (e.g. ampicillin). The culture was centrifuged down using a bench centrifuge. The broth was decanted off and the cell pellet was dried by inverting the samples over paper towelling. The pellet was resuspended in 200 μl of lysozyme solution freashly prepared (4 mg/ml lysozyme; 50 mM glucose; 10 mM EDTA; 25 mM Tris.Cl pH 8.0) and then placed on ice for 30 min. 400 μl of NaOH/SDS mixture (0.2 N NaOH, 1% SDS) was added, gently mixed and placed on ice for 5 min. The sample was transferred to a 1.5 ml Eppendorf tube. At this stage the sample should be fairly viscous with a tendency to form "strands" at the tip of the pipetteman. 300 μl of 5 M ice cold potassium acetate was added, mixed with the solution gently and was placed on ice for 30 min. It was then centrifuged for 30 min in an Eppendorf centrifuge. 0.7 ml of phenol/chloroform (1:1) mixture was added and mixed by inverting. It was centrifuged for 2 min to separate the layer and the lower organic layer was discarded. Such extraction was repeated using 0.7 ml of chloroform and again the lower layer was discarded. 1 ml of -20°C absolute ethanol was added, mixed by inverting and stored at -20°C for 15-60 min. The DNA pellet was then collected by centrifugation for 15 min. After the supernatant was poured off, 1 ml of 70%

ethanol was replaced to wash the precipitate. It was then centrifuged for 5 min. The supernatant was discarded and the sample was dried over tissue paper by inverting the Eppendorf tube on it. Finally the last trace of ethanol was removed by vacuum dry and the pellet was redissolved in 50 μl of TE buffer (10 mM Tris.Cl, pH 7.6; 1mM EDTA, pH 8.0).

2.2.11 Transfer of Bacterial Colonies onto Nitrocellulose Filter

After the selection of possible transformants was completed using selective agar, the transformed colonies were picked out from the agar plate with sterile cocktail sticks carefully and transferred onto nitrocellulose filters on selective agar by streaking diagonally on the gridded filters (Hanahan and Meselson, 1980;1983). They were then be incubated at 37^{0}C overnight.

2.2.12 Replication of Nitrocellulose Filter from Master Filter

This method enable the production of replica filter sets for different hybridization was described by Maniatis et al. (1982) and Davis et al. (1980). Three pieces of sterilized 3MM paper were laid down on a clean, alcohol wiped glass plate with the top piece being wet with sterile water. A master filter from the selective agar was removed and placed with colonies up on the wet 3MM paper. A nitrocellulose filter was then put accurately onto the master filter and was marked carefully the orientation according to the master filter. A wet piece of 3MM paper was then placed on the filters followed by two other dry 3MM papers. A glass plate was then placed on the whole stack and was pressed gently and evenly. The filter copy was removed and grown on selective agar overnight at 37^{0}C whilst the master filter should be kept at -20^{0}C in YT-glycerol agar plate as described in section 2.2.1.

2.2.13 Lysis of Bacterial Colonies on Nitrocellulose Replicas

Replica filter was removed from the selective agar plate and was placed on 3MM papers soaked with 10% SDS for 3 min, then denaturing solution (0.5 M NaOH, 1.5 M NaCl, 1 mM EDTA) for 5 min, neutralizing solution (3 M NaCl; 0.5 M Tris.Cl, pH 7.0; 1mM EDTA) for 5 min, and followed by 2x SSC (0.15 NaCl; 15 mM sodium citrate, pH 8.0) for 5 min. The replica filter should be dried on 3MM paper in between each step. The filter with released plasmid DNA was then air dried, baked at 80°C for 2 hours in a vacuum oven and was ready for hybridization.

2.2.14 Radiolabelling DNA Using Random Oligonucleotides as Primers

The method of "random primed" DNA labelling could produce DNA labelled to high activities and was developed by Feinberg and Vogelstein (1983, 1984). The principle was based on the hybridization of a mixture of all oligonucleotides to the DNA to be labelled. This method enables the labelling of DNAs available only in very small amount. The "Random Primed DNA Labelling Kit" was supplied by Boehringer Mannheim. 5 μl (about 25 ng) DNA in an Eppendorf tube to be labelled was denatured by heating for 10 min at 95°C and subsequent cooling on ice. Following that 3 μl double-distilled sterile water, 1 μl of each of the dATP, dGTP, dTTP, 2 μl of reaction mixture, 2 μl of Klenow fragment and 5 μl (about 50 μCi) of [α^{32}P]dCTP were added. Then the mixture was incubated for 30 min at 37°C. The reaction could be terminated by an addition of 2 μl 0.2 M, pH 8.0 EDTA.

2.2.15 Chromatography Through Sephadex G-50 Column

This technique employed gel filtration to segregate radiolabelled DNA from

unincorporated , labelled deoxynucleotide triphosphates (Maniatis et al., 1982). 0.5 g of Sephadex G-50 (DNA grade) was slowly added to 15 ml of elution buffer (150 mM NaCl; 10 mM EDTA; 0.1% SDS; 50 mM Tris.Cl, pH 7.5) and was soaked overnight at room temperature. A Sephadex G-50 column in a disposable 5 ml STERILIN plastic pipette plugged with sterile glass wool was prepared. Bubbles trapped should be avoided. It was then washed with several column of buffer. Radiolabelled DNA sample was then applied to the column and was eluted with the buffer. The process should be done behind perspex/lucite protective screen to shield personnel from radioactivity exposure. 12-15 fractions (300 μl) were collected into Eppendorf tubes and were checked with Geiger-Muller counter at a fixed distance. The leading peak of the radioactivity consisted of nucleotides incorporated into DNA, while the trailing peak consisted of unincorporated [α^{32}P]dNTPs. The results could be confirmed further by a liquid scintillation counting (see section 2.2.15). The radioactive fractions in the leading peak were pooled together and stored at -20^{0}C in lead vial in the radioactive materials storage box. All the remaining solutions, column, Sephadex G-50, glass wool, etc. should be disposed properly in the radioactive waste sink or bin after thoroughly washed with 1% DECON and checked with a Geiger-Muller counter.

2.2.16 Liquid Scintillation Counting of Radiolabelled DNA

The column effluent from the Sephadex G-50 column was checked with a Packard PL Tri-Carb Liquid Scintillation Counter. 2 μl out of 300 μl of the radiolabelled DNA sample was taken out from each Eppendorf tube. It was then mixed with 4 ml of scintillant (Ecoscint A) in a plastic counting vial. For [α^{32}P] counting, protocol no. 7 was employed and the whole process would be undergone

automatically.

2.2.17 Colony Hybridization with Radiolabelled Nucleic Acid Probes

The immobilization of denatured nucleic acids onto a nitrocellulose filter and allowed direct hybridization of radioactive complementary nucleic acid probes (Grunstein and Hogness, 1975) without interference from renaturation of the DNA enable the studying of hybridization kinetics in mixed-phase reactions (Gillespie, 1968; Gillespie and Spiegelman, 1965; Nyegaard and Hall, 1964). Denhardt (Denhardt, 1966) later extended this technology to single stranded (denatured) radiolabelled DNA probes and eliminated non-specific filter-binding of the probes. The DNA blot hybridization method used here was based on the Amersham Nucleic Acids Hybridization Bulletin (1985), except the step of high stringency wash (50 ml 0.1x SSC) before air drying the filters was omitted.

2.2.18 Southern Blotting

This technique was used for transferring DNA from agarose gels to nitrocellulose filter. It was first developed by E. Southern (1975). The following protocol was kindly suggested by Mr. R. Swinhoe (pers. comm.) or similar reference could be obtained from Amersham Nucleic Acids Hybridization Bulletin (1985). After electrophoresis was completed, the gel was shaken gently in denaturing solution (1.5 M NaCl, 0.5 M NaOH) twice for 15 min. The gel should be free from any vacuum grease which might hinder the blotting. Then the gel was shaken twice for 15 min in neutralizing solution (1.5 M NaCl; 0.5 M Tris.Cl, pH 7.2; 1 mM EDTA). A nitrocellulose filter was wet thoroughly in distilled water and then 20x SSC (3 M NaCl; 0.3 M sodium citrate, pH 7.0). The filter was then laid carefully onto

the gel without trapping any bubbles. The whole capillary blotting apparatus was shown in figure 2.1 (pp. 47). The blotting took place at 4°C overnight (about 16 hours) and after its completion, the position of the slots and the gel's outline were marked on the filter with a pencil before removed. The nitrocellulose filter was sandwiched between two pieces of clean 3MM paper for drying and then oven dried in vacuum for 2 hours at 80°C. The filter was stored at room temperature and was ready for hybridization.

2.2.19 Autoradiography

The radioactive nitrocellulose filter to be autoradiographed was taped to a backing of Whatman 3MM paper. Radioactive ink was dotted at several locations around the edge of the filters in a random pattern for easier distinguishing/orientation after the film was exposed. The sample together with the backing sheet were wrapped in clingfilm when the ink dried. They were then placed in a Kodak X-omatic cassette. During the processing in the dark room all the light should be turned off in the dark room except the safety red diffuse lamp. A Fuji X-ray film of appropriate size was activated by flashing and then the Flashed side of the film was placed down facing the radioactive filter. The cassette should be completely closed before the light was switched on. It cassette was stored at -80°C for several hours to several days depending on the strength of the radioactivity on filters. Before the film was developed, the cassette should warmed up to room temperature for at least half an hour. Afterward, the film was removed from the cassette and developed in phenisol developer under safety diffuse red light for 8 min with occasional turning. It was then rinsed with tap water, drained and immersed into fixer for 3 min. Eventually the film was rinsed completely for 1/2 hour

and hung up to dry.

2.2.20 Identification of Positive Clones

After the development of the autoradiograph, it was aligned with the master filter in accurate and correct orientation. The dark dots on the film represented the positive recombinants and the corresponding colonies on the master filter were picked out.They were streaked on selective agar plate to obtain single colony and subsequently plasmid DNA minipreparation was done to extract the plasmid DNA for restriction and size determination. The autoradiograph obtained via Southern Blotting contained different bands. They could be used to compare and analyse with the photograph taken after the gel electrophoresis.

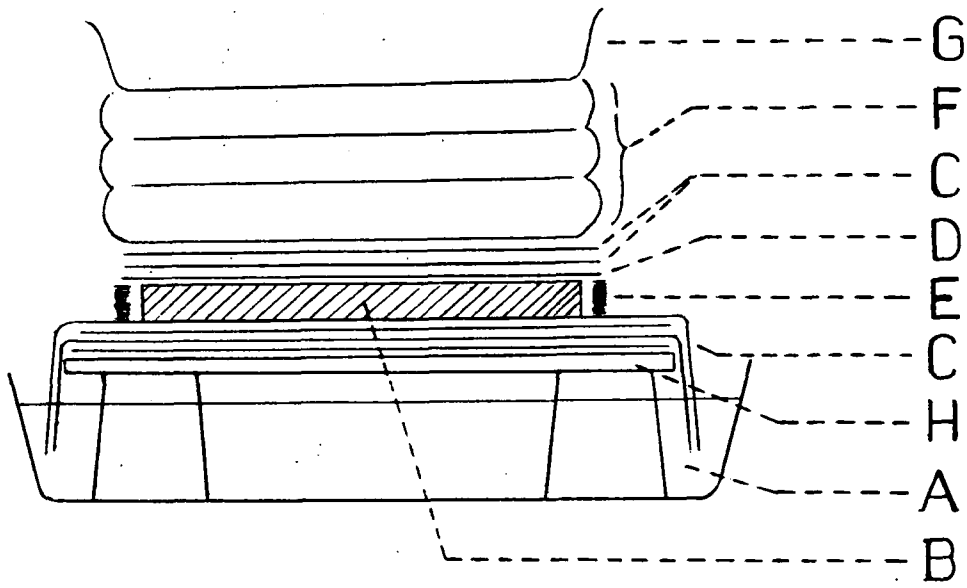


Fig. 2.1 The set-up used in Southern Blotting.

A: 20x SSC Buffer B: Agarose Gel C: Sterilized 3MM Papers D: Nitrocellulose Filter E: Clingfilm F: Nappies (as Absorbent) G: Weight H: Glass Plate

CHAPTER III

RESULTS

CHAPTER III

RESULTS

3.1 Preliminary Investigations

3.1.1 DNA Manipulation Practises

Before the project was started, some preliminary experiments were performed to improve the skills and techniques used in DNA manipulation and handling which played a vital role in the project. A series of experiments were done.

Plasmid pUC18 was restricted with BamHI while λ DNA was restricted with Sau3A. The restricted vector and the restricted fragments were ligated together using T_4 DNA ligase. After the ligation reaction, the recombinants were transformed into *E. coli* competent cells and by antibiotics resistance screening, the possible transformants were identified and selected. Plasmid DNA minipreparation was then performed. The inserts size were determined after suitable restriction and agarose gel electrophoresis.

3 μg of λ DNA (0.5μg/μl) was digested with 10u of Sau3A (4u/μl) using high salt restriction buffer while 2.5 μg of pUC18 (0.5μg/μl) was digested with 5u of BamHI (5u/μl) using the same buffer as well. The results are shown in Fig. 3.1.

Since the digestion of pUC18 with BamHI was incomplete, more restriction

enzyme, 15u (5u/μl) was used to give a completely linearised pUC18 fragment (track no. 2, Fig. 3.2). Meanwhile, ligation between the restricted λ DNA and the restricted pUC18 was done using 1 μl (1u/μl) T_4 DNA ligase with the addition of 2 μl 6mM ATP. After the ligation reaction 10 μl of λ/pUC18 recombinants and a control, 1 μg of pUC18 restricted with 2 μl (5u/μl) of EcoRI were checked on agarose gel (Fig. 3.3).

The λ/pUC18 recombinants were then transformed with *E. coli* cells (Maniatis et al., 1982) and after the antibiotic resistance selection using YT-amp-X-gal agar plate, possible transformants (white colonies) and two non-transformants (blue colonies) were picked out for plasmid DNA minipreparation by alkaline lysis method (Maniatis et al., 1982). After the minipreps, the extracted plasmid DNA were restricted with suitable restriction enzyme for the assessment of inserts size.

Sample no.	DNA(μl)	Enzyme (5u/μl)	H_2O (μl)	10x buffer (μl)
1 (λ DNA)	4	5u EcoRI + 5u HindIII	12	2 (EcoRI buffer)
2 (λ DNA)	4	5u EcoRI + 5u HindIII	12	2 (HindIII buffer)
3 (DNA from blue colony A)	5	5u EcoRI	12	2 (EcoRI buffer)
4 (DNA from white colony)	5	5u EcoRI	12	2 (EcoRI buffer)
5 (DNA from blue colony B)	5	5u HindIII	12	2 (HindIII buffer)

Table 3.1 Restriction set-up for λ DNA and pUC18 after miniprep using restriction endonuclease(s) EcoRI and/or HindIII.

3.1.2 Pea Cotyledons Poly(A)$^+$ RNA Extraction Practises

Frozen pea cotyledons were kindly provided by Dr. J. A. Gatehouse. 10 g of cotyledons were used as starting material. The method of direct extraction of mRNA in Hot SDS/Proteinase K (section 2.2.3.1) was employed with all the amounts of materials used being scaled down to a factor of 5 since the weight of the cotyledons used was 1/5th of the recommended in the protocol.

After the pea total RNA was extracted, it was quantitated by spectrophotometric method (section 2.2.1). Pure sterile water provided the background baseline and 10 μl of pea RNA extract was dissolved in 2.5 ml of sterile water for checking (Fig. 3.6).

The absorbance was 0.889 and peaked at λ = 260.8. Because an OD of 25 corresponded to approximately 1 μg/μl for ssRNA (Maniatis et al., 1982), the concentration of RNA extract was,

$$0.9/25 \times 2.5 \times 1000 \times 1/10 = 9\mu g/\mu l$$

Since the total volume of the RNA extract was more than 500 μl, the total amount of RNA obtained exceed (9 x 500) μg = 4.5 mg. While the dual λ ratio (OD260/OD280) was 1.175 (Fig. 3.6).

For further confirmation a glyoxal gel electrophoresis (section 2.2.6.3) was run. 12.3 μl (9μg/μl) of RNA extract was used as suggested and the two ribosomal RNA bands together with the smears of mRNA and tRNA were clearly shown on the gel (Fig 3.7). After the extraction of total RNA, selection of poly(A)$^+$ RNA by oligo d(T)-cellulose affinity chromatography (section 2.2.4) was done. All the RNA

(approx. 4.5 mg in 500 μl) was applied to the column. mRNA pellet recovered was then dissolved in 400 μl of sterile water. 100 μl out of 400 μl of mRNA solution was dissolved in 2.5 ml sterile water for spectrophotometric scanning (Fig. 3.8). The absorbance was 0.483 peaked at λ = 259.5. Therefore the concentration of pea cotyledon poly(A)$^+$ RNA was,

$$0.483/25 \times 2.5 \times 1000 \times 1/100 = 0.483\ \mu g/\mu l$$

total mRNA remained was 0.483 μg/μl x 300 μl = 145 μg. Approximately 5.8 μg (12.3 μl of 0.483 μg/μl) of mRNA was used to run glyoxal/DMSO RNA gel electrophoresis. The poly(A)$^+$ RNA bands were shown (Fig. 3.9, track no. 1).

3.1.3 Pea Cotyledons cDNA Library Construction Practise

Pharmacia cDNA Synthesis Kit was used for production of pea cotyledon cDNA library (section 2.2.8) The method used followed the established protocol enclosed in the cDNA synthesis kit. 4.5 μl corresponded to 5 μg of intact poly(A)$^+$ RNA (1.12 μg/μl) was kindly supplied by Mr. D. Bown as starting material.

After the dscDNA was synthesized and before purification in the spun column, the volume of dscDNA obtained was approximately 100 μl with theoratically 10 μg of dscDNA formed. 5 μl of EcoRI adaptor solution was added to provide adaptors to ligate with the blunt-ended dscDNA. Meanwhile, plasmid vector pUC19 was digested with 3 μl (5u/μl) of EcoRI. After the restriction, 4 μl (0.5 μg) of restricted pUC19 was mixed with 1 μl of agarose loading dye to perform agarose gel electrophoresis (Fig. 3.10, track no. 1). The remaining restricted vectors (2 μg) were purified by extraction and then dephosphorylated (section 2.2.5.3) with calf intestinal alkaline phosphatase (CIP). 0.5 μl of enzyme (1u/μl) was used for

dephosphorylation during each incubation. Noticed that the spun column purification step in the recommended method was omitted because the CIP used was highly purified and contained no ammonium sulphate. The dephosphorylated vector was then dissolved in 20 μl of sterile water. Later an agarose minigel (section 2.2.6.2) check shown that the plasmid was viable.

The final volume of cDNA produced after all the spun column purification steps was 115 μl and 35 μl of ligation buffer was added to make up the volume to 150 μl. Four cDNA/vector ligation reactions were set up before parallel transformation of competent *E. coli* cells (section 2.2.8) in order to find out the appropriate amount of cDNA solution to optimize the transformation (Table 3.2).

Reaction	1	2	3	4 (Control)
cDNA solution (μl)	6	3	2	0
Ligation Buffer (μl)	24	27	28	30
pUC19 (0.05 μg/μl) (μl)	2	2	2	2
Diluted ATP (μl)	3	3	3	3
T_4 DNA ligase (μl)	1	1	1	1
Total (μl)	36	36	36	36

Table 3.2 Ligation reactions set-up suggested in the Pharmacia cDNA Synthesis instruction manual to find out the optimal transformation conditions.

The resulting ligation reaction mixtures were used to transform *E.coli* DH5α competent cells. For each 36 μl of mixture, it was added to 200 μl of competent cells suspended in 0.1 M $CaCl_2$ and 800 μl of YT-medium. After the transformation, two aliquots (10 and 100 μl) of each transformed cells were plated out on YT-amp-

X-gal agar for selection and screening. The results of colonies counting were listed in table 3.3.

Sample no. – vol. of aliquot/μl)	white colonies no. (w)	blue colonies no. (b)	w/b ratio
1-100	420	400	1.05
1-10	360	280	1.29
2-100	1440	576	2.5
2-10	440	320	1.38
3-100	800	760	1.05
3-10	248	108	2.3
4-100 (control)	1	460	0
4-10 (control)	0	720	0

Table 3.3 Results of colonies counting after *E. coli.* transformation.

From the above results, the optimal transformation condition was sample 2-100. Hence this could be choosen as model for subsequent scale up transformation reaction. Later, plasmid DNA minipreparations using alkaline lysis method (section 2.2.9) was done on some selected possible colonies and the cDNA inserts size were determined on agarose gel electrophoresis after EcoRI restriction (Fig. 3.11). 10 μl of each of the DNA samples was digested with 1 μl (5u/μl) of EcoRI while 2.4 μg of pBR322 was digested with 1 μl (3u/μl) of Alu I as a size marker.

From Fig. 3.11, the restriction was unsuccessful. It might due to inadequate restriction enzyme used. Then another restriction was re-performed. This time 2 μl (5u/μl) of EcoRI was used and the results were photographed and shown on Fig. 3.12. The restriction was complete and the plasmids with inserts could be seen clearly. Track no. 2, 3, 4, 7, 10 corresponded to non-transformants without inserts

while track no. 5, 6, 9, 11, 12, 13 were real transformants with cDNA inserts of different size. A calibration curve was plotted (Fig. 3.13) and the inserts size were determined (Table 3.4).

Sample no.	insert migration distance (cm)	size (bp)
5	9.7	1000
6	11.7	398.1
9	9.3	1202.3
11	11.4	457.1
12	8.6	1659.6
13	12.1	335.0

Table 3.4 Pea cotyledons cDNA inserts size calculated after the restriction using EcoRI restriction endonuclease.

3.2 Extraction of Pea Roots Poly(A)$^+$ RNA

Fresh pea roots were prepared as described in section 2.2.2. Total RNA was attempted to extract from 50 g of root materials by the method mentioned in section 2.2.3.1. After the RNA extract was dissolved in 700 μl of sterile water and stored in aliquots at -80°C, 10 μl of them was added to a clean cuvette contained 2.5 ml of sterile water for UV spectrophotometric scan (Fig. 3.14).

The curve peaked at λ = 270.4 nm with absorbance of 0.656 which was deviated from an ideal case of pure RNA which should peak at λ = 260 nm. However, based on the value of λ = 260 nm on the graph, an absorbance of 0.367 was read. Possible concentration of pea root RNA extracted was,

0.367/25 x 2.5 x 1000 x 1/10 = 3.67 μg/μl

total possible amount of RNA was, 3.67 μg/μl x 700 μl = 2.57 mg. The yielding sounded reasonable but the purity of the sample was not good (according to the peak position and the dual λ ratio of RNA in which the value of the latter one was 1.043, Fig. 3.14).

Subsequent glyoxal (RNA) gel electrophoresis check confirmed that the quantity of RNA in the extract was not enough for poly(A)$^+$ RNA extraction (Fig. 3.9, track no. 3 and 4).

The extraction of pea roots RNA was therefore repeated using guani- dinium/cesium chloride method (section 2.2.3.2). 7.5 g of pea roots was used. Transparent gelly-like RNA was extracted and solubilized in 600 μl of sterile water. 10 μl of RNA solution was scanned by UV spectrophotometer. The values of peak λ and absorbance were 258.0 and 0.097 respectively (Fig. 3.15). Concentration of RNA obtained was,

0.097/25 x 2.5 x 1000 x 1/10 = 0.97 μg/μl

total amount of RNA extracted was, 0.97 μg/μl x 600 μl = 582 μg. The dual λ ratio was 2.135 (Fig. 3.15). Afterward, 5 μg of RNA was used to perform glyoxal gel and the result was shown on Fig. 3.16.

Track no. 1 corresponded to poly(A)$^+$ RNA of pea pods (25 μg of sample was loaded). Track no. 2 corresponded to RNA of pea root. The weakness of that two bands was due to small amount of RNA loaded and its denaturation upon time elapsed.

The pea roots RNA (600 μg) was then passed through the oligo d(T)-cellulose

column (section 2.2.4) to isolate the poly(A)$^+$ RNA. Through the UV monitor (λ = 254 nm), two peaks were seen with the first and second peaks referred to poly(A)$^-$ RNA and poly(A)$^+$ RNA respectively (Fig. 3.17). Noticed that the scale of the poly(A)$^+$ peak was 10x more than that of poly(A)$^-$ for easier observation.

11.7 ml of poly(A)$^+$ RNA column effluent was collected. However, after ethanol precipitation, the mRNA could not be recovered due to too small quantity of mRNA as well as too much diluted in large volume of elution buffer. Therefore, another trial to isolate root poly(A)$^+$ RNA was done with 250 μg of total root RNA in 270 μl of TE buffer (concentration = 0.93 μg/μl). It was kindly provided by Mr. R. Swinhoe. Oligo d(T)-cellulose affinity chromatography was run and the UV absorption profile was shown in Fig. 3.18.

170 μl of possible poly(A)$^+$ RNA column effluent was ethanol precipitated and resuspended in 10 μl of sterile water. 0.5 μl of the RNA solution dissolved in 0.5 ml of water in a quartz cell was checked with a PHILIPS PYE UNICAM SP8-150 UV/Visible Spectrophotometer. Reading at λ = 260 nm showed an absorbance of 0.002 (Fig. 3.19). The concentration of mRNA was,

0.002/25 x 500 1/0.5 = 0.08 μg/μl

total mRNA obtained was, 0.08 μg/μl x 10 μl = 0.8 μg. The mRNA recovered was not much enough to ensure a successful cDNA synthesis, hence another attempt to isolate pea root mRNA was undergone. The guanidinium/cesium chloride extraction method was used again but with a larger amount (20 g) of root as starting material.

The RNA extracted was solubilized in 550 μl of sterile water with 5 μl of

it being taken for spectrophotometric scan. The absorbance was 0.083 and was peaked at λ = 258.6 nm. Hence, the concentration of RNA was,

0.083/25 x 2.5 x 1000 x 1/5 = 1.66 μg/μl

total amount amount of yielding was, 1.66 μg/μl x 550 μl = 913 μg. The dual λ ratio was 2.251 (Fig. 3.20).

All the total RNA extracted was then loaded into an oligo d(T)-cellulose column and the UV absorption profile was in Fig. 3.21. 8.6 ml of poly(A)$^+$ RNA extract was collected, 5 μl of glycogen together with 19 ml of ethanol was added to it to enhance precipitation. But according to the UV absorption profile (Fig. 3.22) on mRNA recovery check, there was no peak at λ = 260 nm.

3.3 Construction of Pea Root cDNA Library

Fortunately, a generous gift of pea root poly(A)$^+$ RNA which was extracted previously was given by Dr. M. Evans so that the cDNA library construction could be proceeded. 5 μg of pea root poly(A)$^+$ RNA in 5.2 μl sterile water (concentration: 0.96 μg/μl) was used for synthesizing the cDNA (see section 2.2.8).

In addition, 2 μg of pUC19 was restricted with 3 μl (5u/μl) of EcoRI. After the restriction, phenol extraction, ethanol precipitation and terminal 5' phosphate removal by dephosphorylation (see section 2.2.5.3) were done. The purified and concentrated pUC19 plasmid was dissolved in 20 μl of water. 1 μl of it was checked with agarose minigel (see section 2.2.6.2) and was shown in Fig. 3.23. From the comparison between the brightness of the bands, more than 75% of dephosphorylated vector was recovered. Assuming 75% recovery, the concentration of plasmid

solution was,

$2\mu g$ x 75% = $1.5\mu g$ in $20\mu l$ = $0.075\mu g/\mu l$

Hence 10 μl more sterile water was added to dilute the plasmid concentration to 0.05 $\mu g/\mu l$ for cDNA insertion as recommended in the manual of cDNA synthesis (see section 2.2.8).

The final volume of cDNA solution synthesized after all the spun column purification steps was 100 μl, therefore 50 μl of ligation buffer was added to make up the final volume to 150 μl. Four cDNA/pUC19 ligation reaction were set up as suggested in Table 3.2. *E.coli* competent cells were than transformed (see section 2.2.9) and was plated out on YT-amp-X-gal selective agar for subsequent screening. The results were listed in Table 3.4.

Sample no. – vol. of aliquot/μl	white colonies no. (w)	blue colonies no. (b)	w/b ratio
1-10	1	100	0.01
1-100	2	790	0.003
2-10	0	55	–
2-100	8	1844	0.004
3-10	0	14	–
3-100	18	1142	0.015
4-10 (Control)	0	5	0
4-100 (Control)	1	448	0

Table 3.4 Results of colonies counting after *E. coli.* transformation.

From the results above, the optimal transformation condition was sample 3-100. The number of transformants was not many. Nevertheless, 12 white colonies

were selected from the plates for plasmid DNA minipreparation (see section 2.2.10) and subsequently 10 μl of DNA extract from each sample was restricted with 2 μl (5u/μl) EcoRI. pUC19 restricted with EcoRI and λ DNA restricted with HindIII were used as size markers (Fig. 3.24). From the figure, it showed that the EcoRI restriction did not work properly and RNA was present. However, promising results were seen because almost half of the minipreps DNAs showed more than one band on the photograph. Hence, another restriction reaction was done using 2 μl of BamHI to digest with 10 μl of plasmid DNA extract. This time 1 μl of RNase (1 μg/μl) was added to every selected sample. Again the same markers were used as before. The restriction was completed and the photograph was shown in Fig. 3.25. A graph of $\log_{10}$ size of markers' fragments (bp) against distance migrated was plotted (Fig. 3.26) and the inserts size were determined (Table 3.5).

Sample no.	Fragments' migration distance (cm)	Total fragments' size (bp)	Insert size (bp)
1	4	4365.2	1665.2
3	3.8, 8.2	5269.3	2569.3
4	2.9, 3.6, 4.6	16833	14133
5	4.8	2754.2	54.2
7	4.1	4168.7	1468.7
10	4.7, 7.1	3591.9	892
12	4.9, 4.5	5747.8	3047.8

Table 3.5 Pea roots cDNA inserts size calculated after the restriction using BamHI restriction endonuclease.

As far as to obtain higher number of transformants (white colonies on YT-amp-X-gal agar plate), the ligation/transformation reaction was re-performed. 30

μl of cDNA column effluent was added to 3 μl of diluted ATP, then 1 μl of T_4 DNA ligase and 2 μl (0.05 μg/μl) of dephosphorylated pUC19 vector. Competent cells transformation was done and the cells were plated out on selective agar in different aliquots. (i.e. two plates in 10 μl aliquots, two plates and four plates in 100 μl and 200 μl aliquots respectively). The colonies counting results were listed in Table 3.6.

Samples (μl)	White colonies no.	Blue colonies no.
A. 10	4	140
B. 10	6	159
C. 100	12	520
D. 100	9	477
E. 200	31	exceed 800
F. 200	28	exceed 800
G. 200	26	exceed 800
H. 200	35	exceed 800

Table 3.6 Results on colonies counting after *E. coli.* transformation.

White colonies including the dubious one were transferred onto gridded nitrocellulose filters (see section 2.2.11) and were incubated overnight on selective agar. White, blue and a combination of both colonies were found. The filters were then replicated (see section 2.2.12) for hybridization later on.

Another two plasmid (dephosphorylated pUC19) – cDNA ligation/transformation reactions were undergone to raise more white colonies (transformants) using the same experimental conditions/procedures as described before. Totally 184 white colonies were found and they were all transferred onto nitrocellulose

filters and replicated. After the replication, the master filters were stored at -20°C in YT-glycerol agar plate (see section 2.2.1).

3.4 Colony Hybridization and Inserts Size Determination

5 μl of cDNA (exceed 25 ng) column effluent was radiolabelled by random primed labelling method (see section 2.2.14) followed by Sephadex G-50 gel filtration (see section 2.2.15) to purify the radiolabelled cDNA from the unincorporated nucleotides. 12-15 (300 μl each) fractions were collected in Eppendorf tube and they were checked with Geiger-Muller counter and liquid scintillation counter (see section 2.2.16) subsequently. The results on scintillation counting were shown in Fig. 3.27.

According to the results, fractions 7-9 contained the radiolabelled cDNA were stored in lead vials at -20°C for hybridization. One set of four nitrocellulose replica filters (with all the white colonies collected through various ligation/transformation reactions) were lysed and treated (see section 2.2.13) to prepare for colony hybridization (see section 2.2.17). Three fractions (about 900 μl) of radiolabelled cDNA column effluent collected after the Sephadex G-50 gel filtration were used to probe with the nitrocellulose filter containing the plasmid DNA after the bacterial colonies were lysed. Autoradiograph was taken then (see section 2.2.19). After eight days exposure, the film was developed (Fig. 3.28). Based on the autoradiograph, positive colonies were identified by aligned with the master filter (see section 2.2.20). Nine possible colonies (some are white and some are blue/white mixing colonies) were picked out, streaked on selective agar to separate single white colony and then minipreps were done. For each of the sample

(sample 2-10), 10 μl of miniprep DNA extract was restricted with 2 μl of BamHI (5u/μl). λ/HindIII and pUC19/EcoRI were used as size markers so that the inserts size could be determined. 1 μl of RNase (1 μg/μl) was added to remove the RNA. The results were shown in Fig. 3.29. A standard calibration line was plotted and the inserts size of the cDNA were calculated (Fig. 3.30 and Table 3.7).

Sample no.	Fragments' migration distance (cm)	Total fragments' size (bp)	Insert size (bp)
2	4.7	6309.6	3609.6
3	5.8, 7.8	5315.5	2615.5
4	4.4, 6.1	10555.7	7855.7
5	6.4	2730.3	-
6	6.2. 8.0	4542.7	1842.7
7	5.8, 6.4, 7.7	8270.8	5570.8
8	5.4, 5.6	8739.6	6039.6
9	5.7	3981.1	1281.1
10	5.3, 7.1	6875.6	4175.6

Table 3.7 Pea roots cDNA inserts size calculated after restriction using BamHI restriction endonuclease.

After that, the set of plasmid DNA was then restricted with 2 μl (5u/μl) EcoRI using the same size markers as that in the BamHI miniprep DNA restriction. However, the results was not satisfactory (Fig. 3.31). Partial digestion was suspected, hence the restriction was repeated. The conditions were changed slightly : only 5 μl instead of 10 μl of miniprep DNA solution was used in each sample, restriction buffer and enzyme were obtained from another source as well as 2 μl of 0.1 M spermidine was added to enhance the digestion efficiency of the

restriction endonuclease. However, the results was similar to the former one (Fig. 3.32). Because of this, the quality of the miniprep DNA was suspected. It might contained various contaminants and impurities, therefore, another miniprep was then done with great cautious especially the phenol/chloroform extraction steps. The resulting DNA extract was restricted with EcoRI using the same experimental conditions stated above and the photograph was shown in Fig. 3.33.

According to the position of fragments shown, they were similar to the two restrictions done before. This meant that the minipreps and restriction reactions performed so far have no problem, but the plasmid-cDNA chimaeras were unexpectedly different from the prediction in which the two EcoRI cutting sites on the plasmid recombinants were altered that did not allow a proper EcoRI restriction to take place.

3.5 Southern Blotting

Owing to the results obtained in EcoRI restriction, a Southern blotting was performed in order to confirmed cDNA inserts were definitely cloned into the plasmid despite the alteration of the EcoRI restriction sites. Thus 5 μl of cDNA solution was random primed radiolabelled with [α^{32}P]dC TP and purified along the Sephadex G-50 column. Liquid scintillation counting was used to select the Eppendorf tubes containing the labelled cDNA (tube no. 6-8) based on the results of Fig. 3.34.

After the Southern Blotting (section 2.2.18), the nitrocellulose filter blotted with the restricted DNA fragments were hybridized with the radiolabelled cDNA probe. The autoradiograph developed after the hybridization was shown in Fig.

3.35 after 16 hours exposure. Various dark bands were seen clearly. By referring to the photograph taken after the EcoRI restriction, the corresponding cDNA inserts were identified (the bands with arrows pointed to in Fig. 3.35).

3.6 Probing with Inserts of Plasmids pPR179, pPR287(A) and pPR340

The inserts of plasmids pPR179, pPR287(A) and pPR340 were DNA of size 450, 250 and 480 bp respectively. All of them used pUC19 as the plasmid vector and ligated with the vectors in EcoRI sites. Large amount of each of them were produced by maxiprep and purified by CsCl centrifugation to get rid of all the proteins, chromosomal DNA, etc.

In order to ensure enough DNA inserts were available to be radiolabelled as probes, at least 2 μg of each of the DNA inserts from the plasmid recombinants were required. Let A be the amount of plasmid recombinants needed to give 2 μg of inserts after suitable restriction,

For pPR179: A = 2 x (450+2700)/450 = 14 μg

For pPR287(A): A = 2 x (250+2700)/250 = 23.6 μg

For pPR340: A = 2 x (480+2700)/480 = 13.25 μg

where 450, 250, 480 and 2700 were size in bp of DNA inserts of pPR179, pPR287(A), pPR340 and vector pUC19 respectively. The plasmid-DNA chimaeras were kindly provided by Dr. M. Evans with concentrations :

Plasmid recombinant	Concentration (μg/μl)	Total amount given (μl)
pPR179	2.85	10
pPR287(A)	5.5	30
pPR340	1.4	20

Hence the plasmid-DNA chimaeras were restricted with EcoRI with reference to the amount required to give adequate amount of DNA inserts for probing as calculated before.

Sample	Amount needed (μl)	10x EcoRI buffer (μl)	EcoRI (5u/μl)(μl)	water (μl)
pPR179	5	2	4	9
pPR287(A)	4.5	2	6	7.5
pPR340	10	2	4	4

Besides, 3 μl (1.2 μg/μl) of pBR322 restricted with 2 μl of Alu I (3u/μl) and 2 μl (1 μg/μl) of pUC18 were used as size markers for reference. The results were shown in Fig. 3.36. The wanted DNA inserts from the three samples were cut out from the agarose gel and extracted by freeze elution method (see section 2.2.7). Noticed that sample pPR287(A) gave two fragments beside the vector after the restriction, but only the 250 bp fragment was needed. Each of the extracted DNA samples was then dissolved in 50 μl of 1x TE buffer and 15 μl of each of them was used to check on agarose minigel electrophoresis. Again 1 μl (1.2 μg/μl) of pBR322 restricted with 1 μl (3u/μl) of Alu I was used as a size marker. The minigel photograph was shown on Fig. 3.37.

Meanwhile, three more sets of replica filters were produced from the master filters. The bacterial colonies were then lysed and fixed on nitrocellulose filters

by baking at 80°C for two hours so that the insert probes could later hybridized with them. The three different types of insert extract were then radiolabelled by random primed labelling method separately. Each of them was purified along Sephadex G-50 column and by liquid scintillation counting (Fig. 3.38-3.40), the suitable radiolabelled DNA samples were kept in different lead vials. The labelled DNA probes of pPR179, pPR287(A) and pPR340 were hybridized with three separate sets of replica filters and autoradiographed afterward. According to Geiger-Muller counter checked, the radioactive strength of the filters hybridized with probe pPR179 was strong (about 50 cps) while the other two sets that hybridized with probes pPR287(A) and pPR340 were much weaker (about 3 cps). Therefore, autoradiograph of pPR179 was exposed for only three hours while the other two were exposed for 5 days. Hybridization between the probes and the filters were revealed on the autoradiographs after being developed.

All the autoradiographs were superimposed onto the master filters. The bacterial colonies corresponded to the dark spots on the autoradiographs were picked out (8, 4 and 6 colonies from pPR179, pPR287(A) and pPR340 respectively), streaked on selective agar plates to obtain single colony. The selected white colonies from different streaked plates were inoculated in YT-broth containing ampicillin and plasmid DNA minipreparations were performed afterward. The photographs of the minipreps DNA (5 μl) restricted with 3 μl of EcoRI (5u/μl) with the addition of 1 μl of RNase (1 μg/μl) were shown in Fig. 3.41 and Fig. 3.42.

Fig. 3.1 Track no. 1 – Restriction of λ DNA with Sau3A. Track no. 2 – Partial restriction of pUC18 with BamHI.

Fig. 3.2 Track no. 1 – Unrestricted pUC18 as a control. Track no. 2 – Complete restriction of pUC18 with Bam HI.

Fig. 3.3 Track no. 1 – pUC18 restricted with EcoRI as a control. Track no. 2 – λ/pUC18 recombinants restricted with EcoRI.

The EcoRI site was in the multipurpose cloning region of pUC18, after the insertion of λ DNA into the plasmid and restricted with EcoRI, a linearised fragment was seen together with a smear of small restricted λ fragments. Noticed that the migration rate of the recombinant was slower than that of the linearised pUC18, this confirmed the ligation reaction was successful.

Fig. 3.4 Plasmid DNAs restricted with appropriate restriction enzyme(s) for insert size assessment. Track no. 1 – λ DNA restricted with EcoRI + HindIII in EcoRI restriction buffer. Track no. 2 – λ DNA restricted with EcoRI + HindIII in HindIII restriction buffer. Track no. 3 – Plasmid DNA from blue colony (pUC18) partially restricted with EcoRI. Track no. 4 – Recombinant DNA from white colony (λ DNA + pUC18) restricted with EcoRI completely. Track no. 5 – Plasmid DNA from blue colony completely restricted with HindIII.

Fig. 3·1

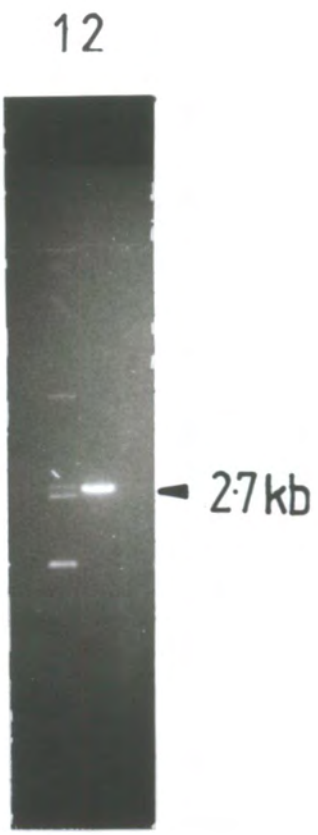


Fig. 3·2

Fig. 3·3

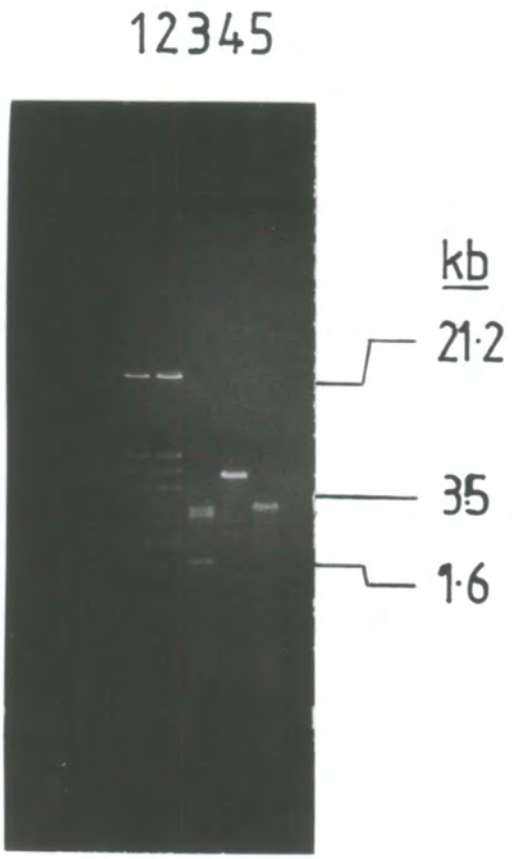


Fig. 3·4

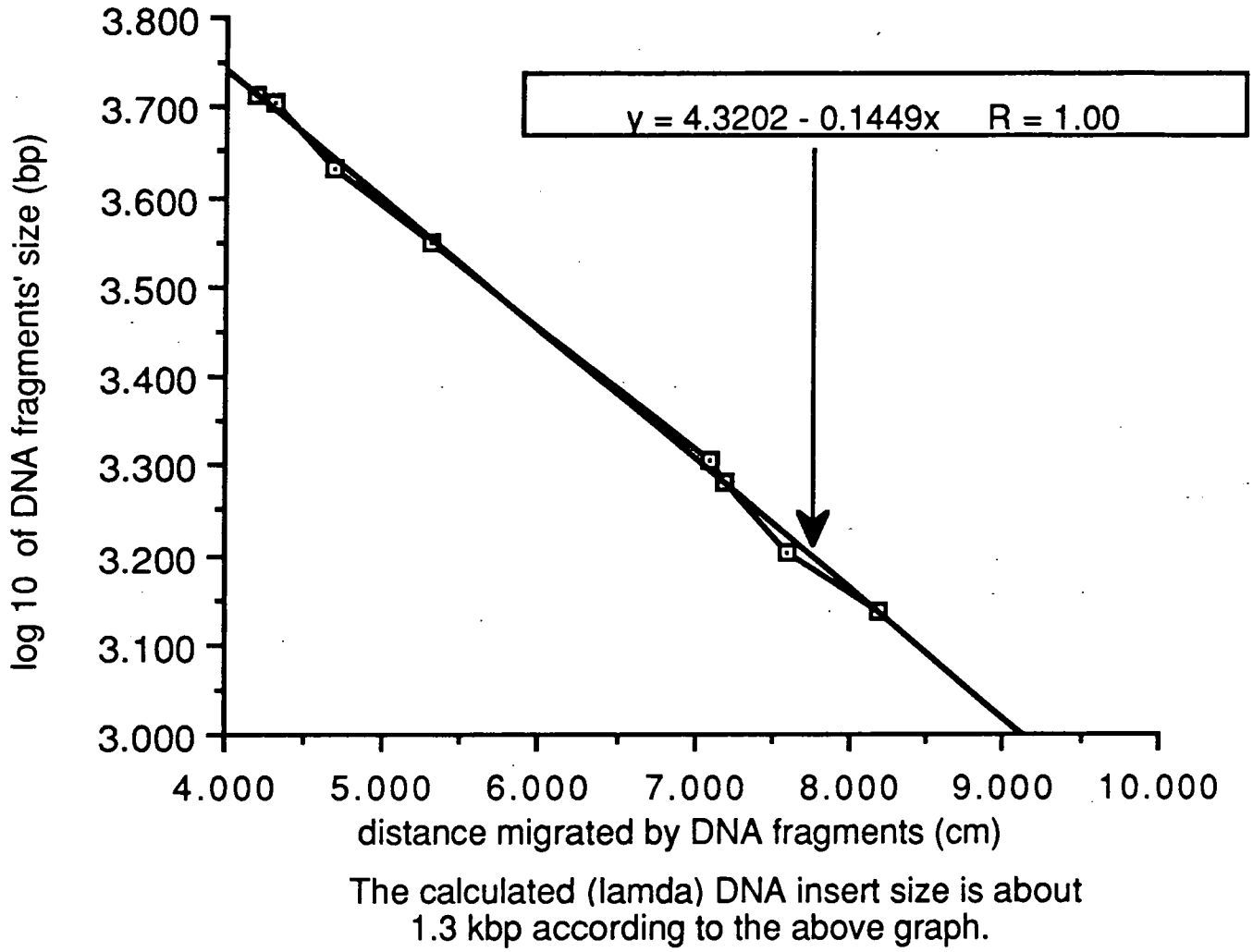
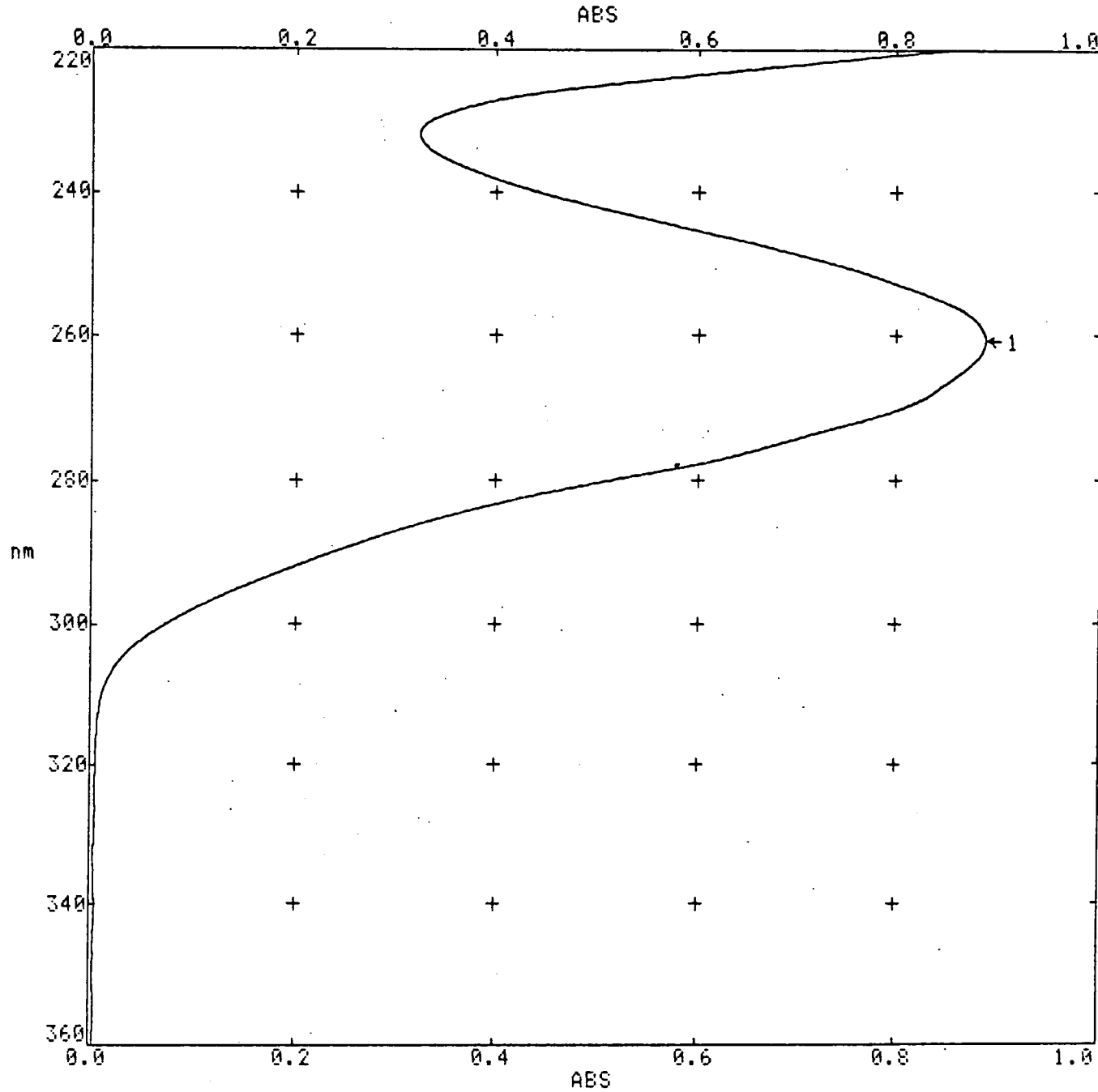


Fig. 3.5 Log_{10} of DNA fragments' size in bp (λ/EcoRI + HindIII) against their distance migrated in cm.



7 1 DNA/RNA CHECK SCAN

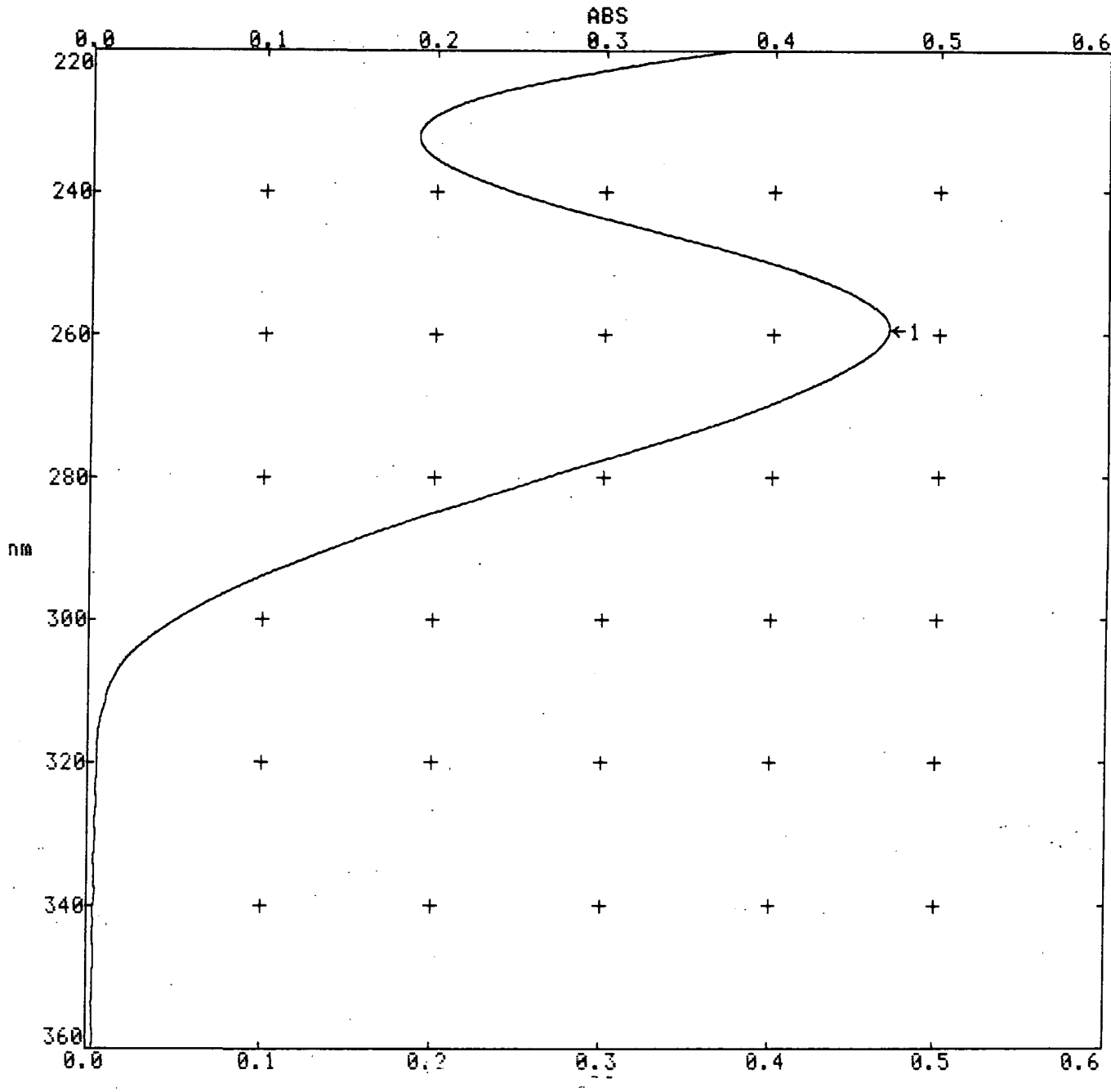
	1	2	3	4
λ	260.8			
ABS	0.889			

* DUAL λ DATA *

I.D. 2 1 260/280 RATIO FOR DNA/RNA

NO	λ1	λ2	λ1/λ2
	260.0	280.0	
1	0.889	0.518	1.715

Fig. 3.6 UV absorption spectrum of pea cotyledons total RNA (220nm - 360nm).



6 1 DNA/RNA CHECK SCAN

	1	2
λ	259.5	
ABS	0.483	

Fig. 3.8 UV absorption spectrum of pea cotyledons Poly(A)$^+$ RNA (220nm - 360nm).

Fig. 3.7 Glyoxal/DMSO gel electrophoresis of pea cotyledon RNA. The gel was stained with acridine orange. After staining, the gel appeared to be yellowish while the RNA bands became orange in colour under UV transilluminator. Two subunits of the RNA were separated on the gel.

Fig. 3.9 Glyoxal/DMSO gel electrophoresis of pea parts. Track no. 1 – Pea cotyledons mRNA. Track no. 2 – Total RNA of pea pods extracted by hot SDS/proteinase K method. Track nos. 3, 4 – Total RNA of pea roots extracted by hot SDS/proteinase K method.

Fig. 3.10 Restriction of pUC19 with EcoRI for subsequent cDNA insertion. Track no. 1 – restricted pUC19. Track no. 2 – unrestricted pUC19 as a control.

Fig. 3.11 Insert size assessment of pea cotyledons cDNA after plasmid DNA restricted with EcoRI. Track no. 1-11 – DNA from possible transformants (white colonies). Track no. 12 – DNA from a non-transformant (blue colony). Track no. 13 – pBR322 restricted with Alu I as a size marker.

Fig. 3·7

Fig. 3·9

Fig. 3·10

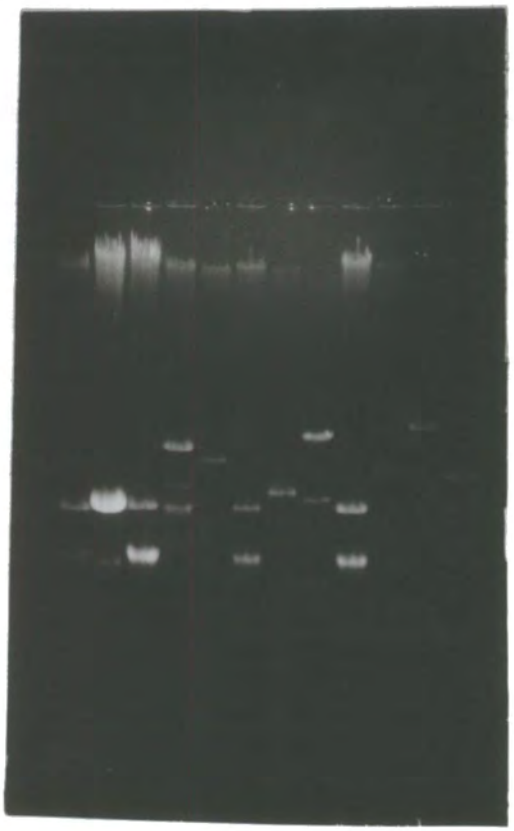


Fig. 3·11

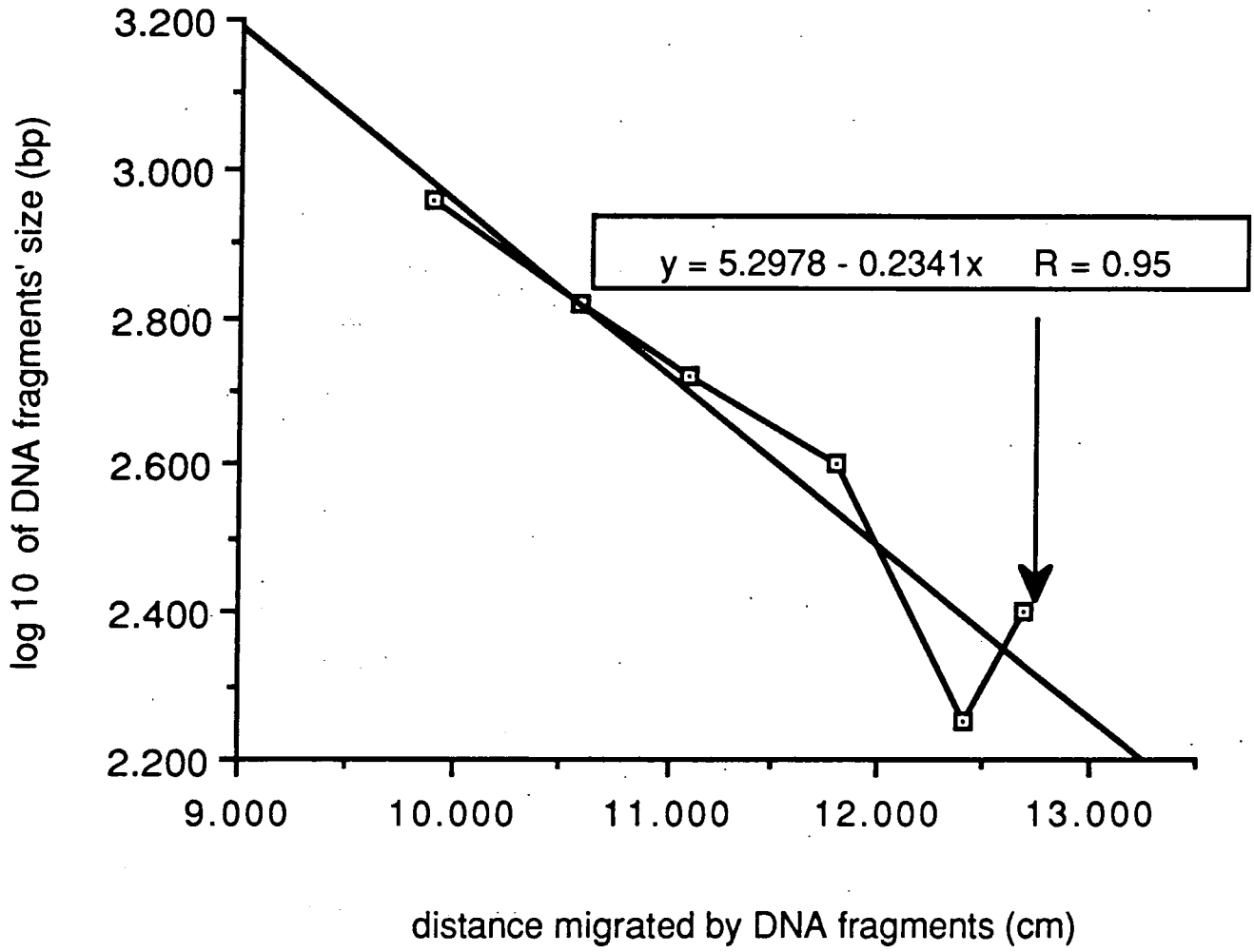
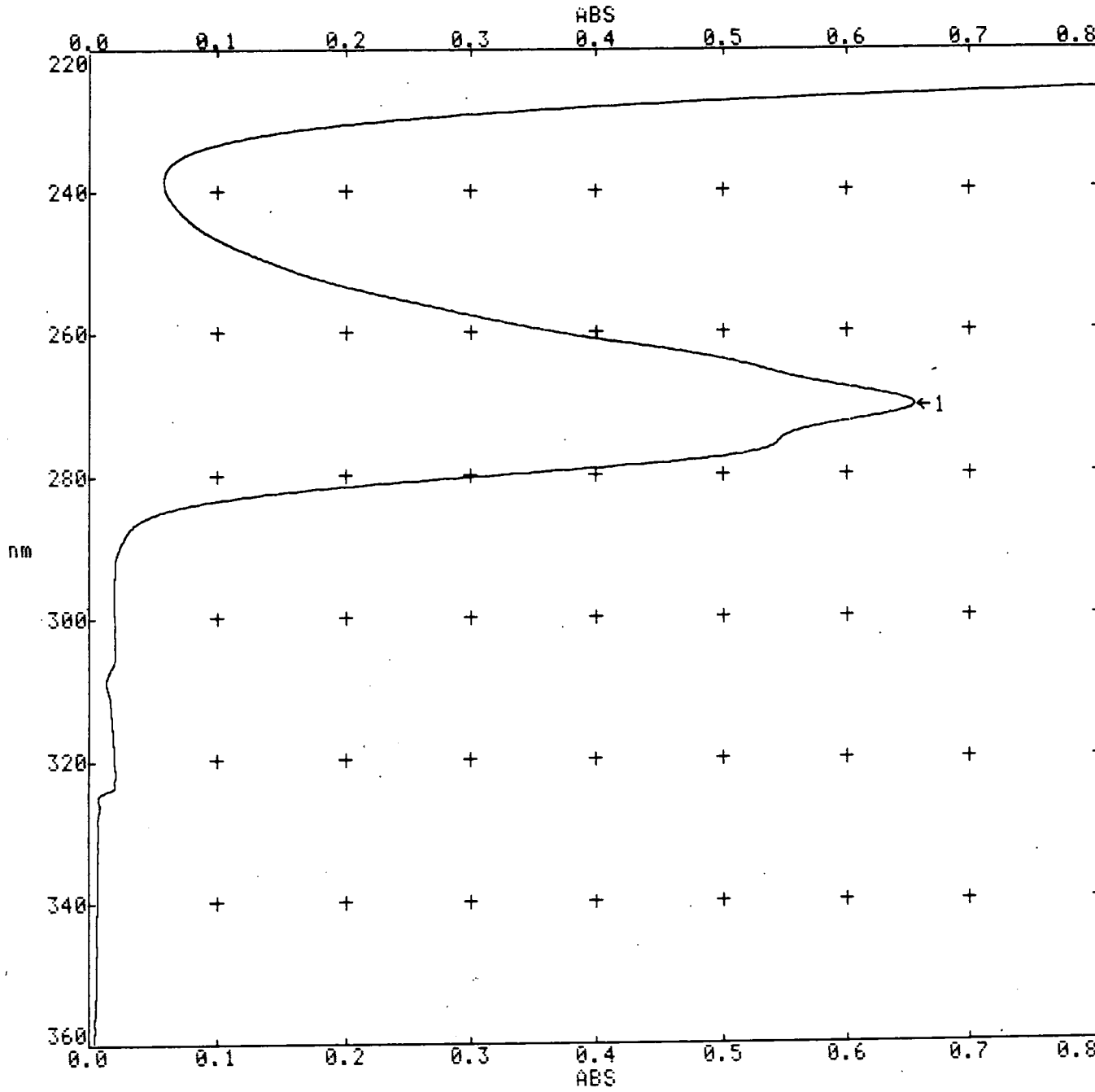


Fig. 3.13 Calibration line of log$_{10}$ of DNA fragments' size (bp) against distance migrated (cm) - pBR322 restricted with Alu I.



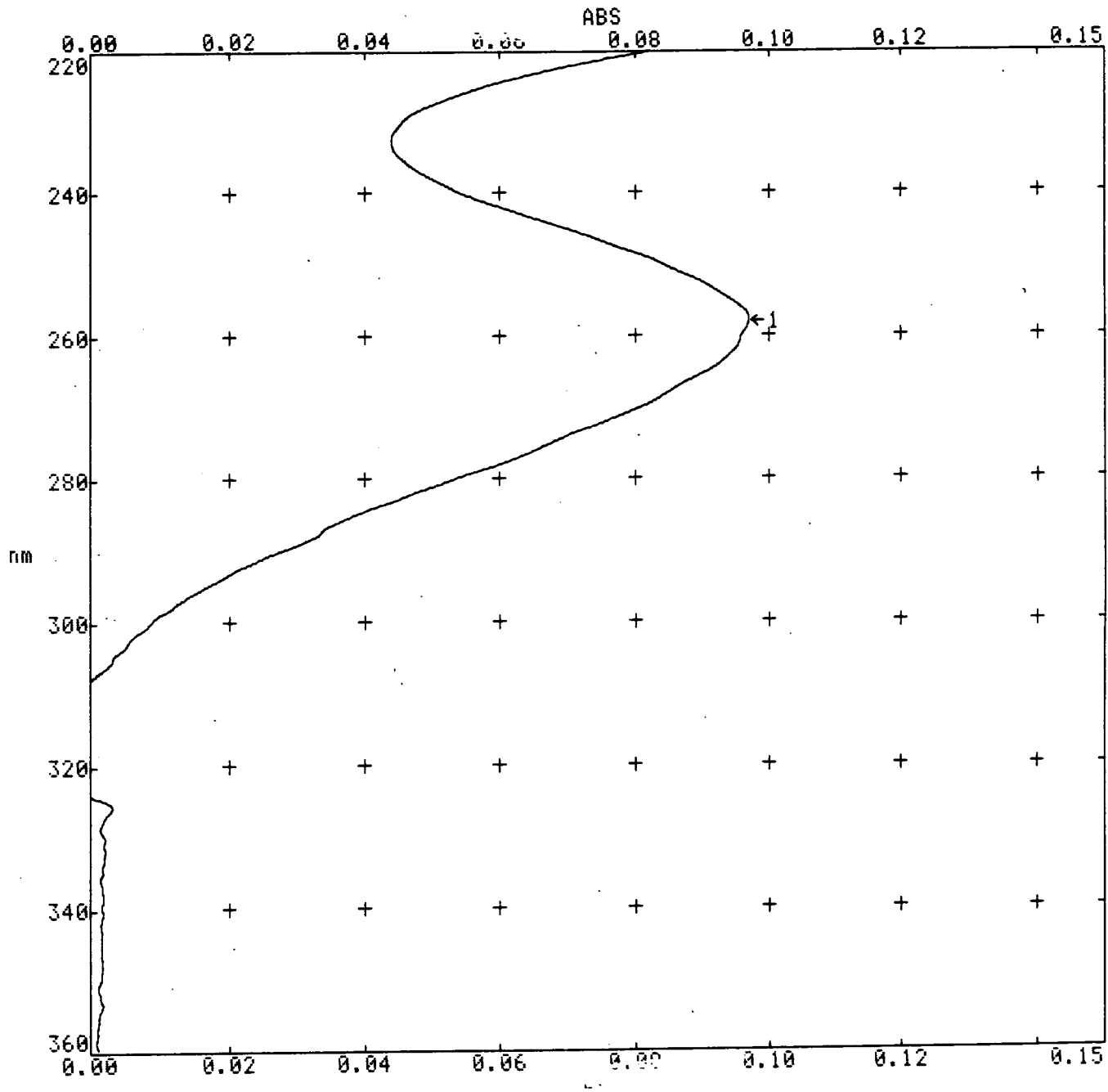
4 1 DNA/RNA CHECK SCAN

	1	2	3	4
λ	270.4			
ABS	0.656			

* DUAL λ DATA *

I.D. NO	1	RATIO	B2.0 ABS	
		λ1	λ2	λ1/λ2
		260.0	280.0	
1		0.36	0.752	1.043

Fig. 3.14 UV absorption spectrum of pea roots total RNA (220nm - 360nm).



1 DNA/RNA CHECK SCAN

	1	2	3	4
λ	258.0			
ABS	0.097			

* DUAL λ DATA *

I.D. 1 1 260/280 RATIO FOR DNA/RNA

NO	λ1	λ2	λ1/λ2
	260.0	280.0	
1	0.076	0.036	2.135

Fig. 3.15 UV absorption spectrum of pea roots total RNA (220nm - 360nm).

Fig. 3.12 Insert size assessment of pea cotyledons cDNA after plasmid DNA was restricted with EcoRI. Track no. 1 – pBR322 restricted with Alu I as a size marker. Track no. 2 – DNA from blue colony (pUC19) linearised by EcoRI as a control. Track nos. 3, 4, 7, 10 – non-transformants without cDNA insert. Track nos. 5, 6, 9, 11, 12, 13 – transformants with cDNA inserts. Track no. 8 – unrestricted transformant.

Fig. 3.16 Glyoxal/DMSO gel electrophoresis of pea root total RNA extracted by Guanidinium/Cesium Chloride method. Track no. 1 – poly(A)$^+$ RNA of pea pods as a control. Two bands were seen with the large subunit running slower and approximately 2x more abundant than the small subunit. Track no. 2 – pea roots total RNA. Two vague bands were shown. The weakness of the bands in track no. 2 when compared with that in track no. 1 might due to much lower concentration of sample loaded (5x less).

Fig. 3.23 Agarose minigel of dephosphorylated vectors pUC19 which have already restricted with EcoRI.

Fig. 3.24 Restriction of plasmid recombinant DNA with BamHI after miniprep. Track no. 1 – λ DNA restricted with HindIII and pUC19 restricted with EcoRI as size markers. Tracks nos. 2-13 – possible white colonies plasmid DNA restricted with EcoRI.

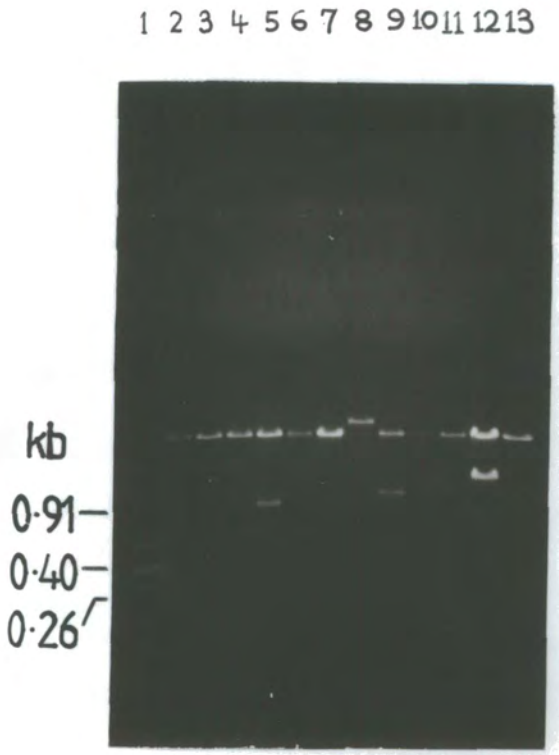


Fig.3·12

Fig.3·16

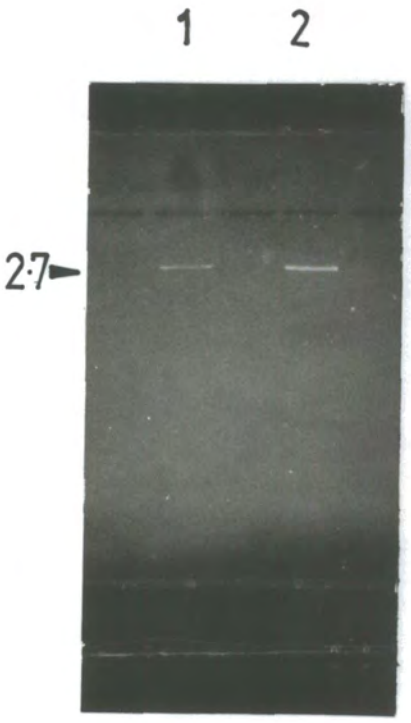


Fig.3·23

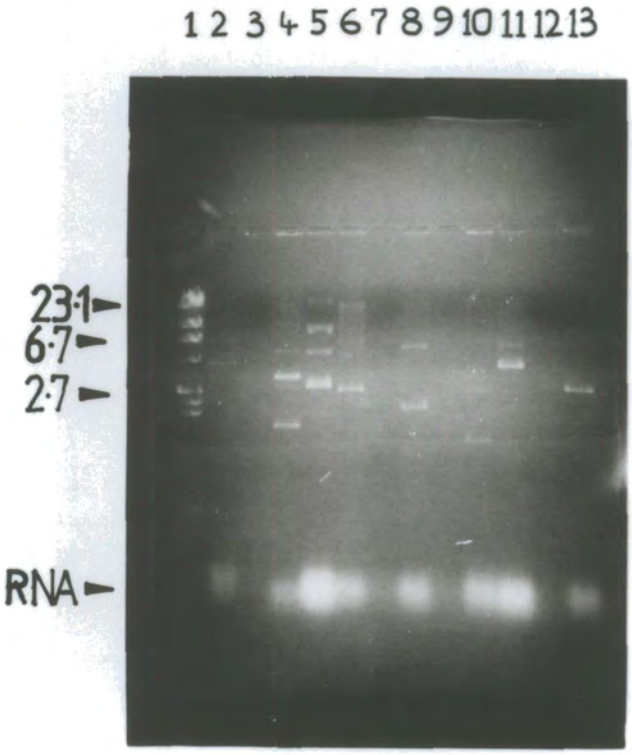


Fig.3·24

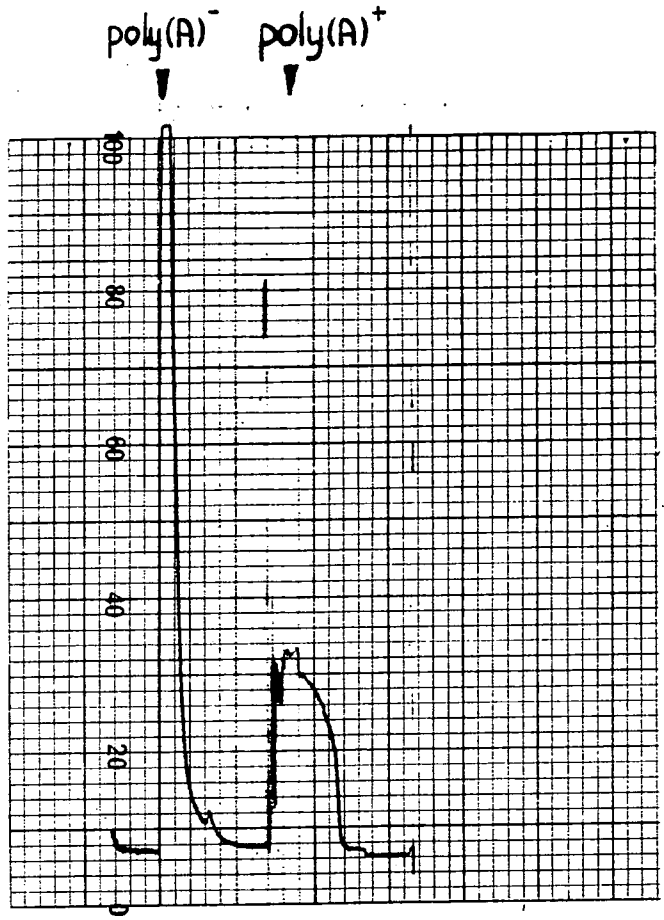


Fig.3·17

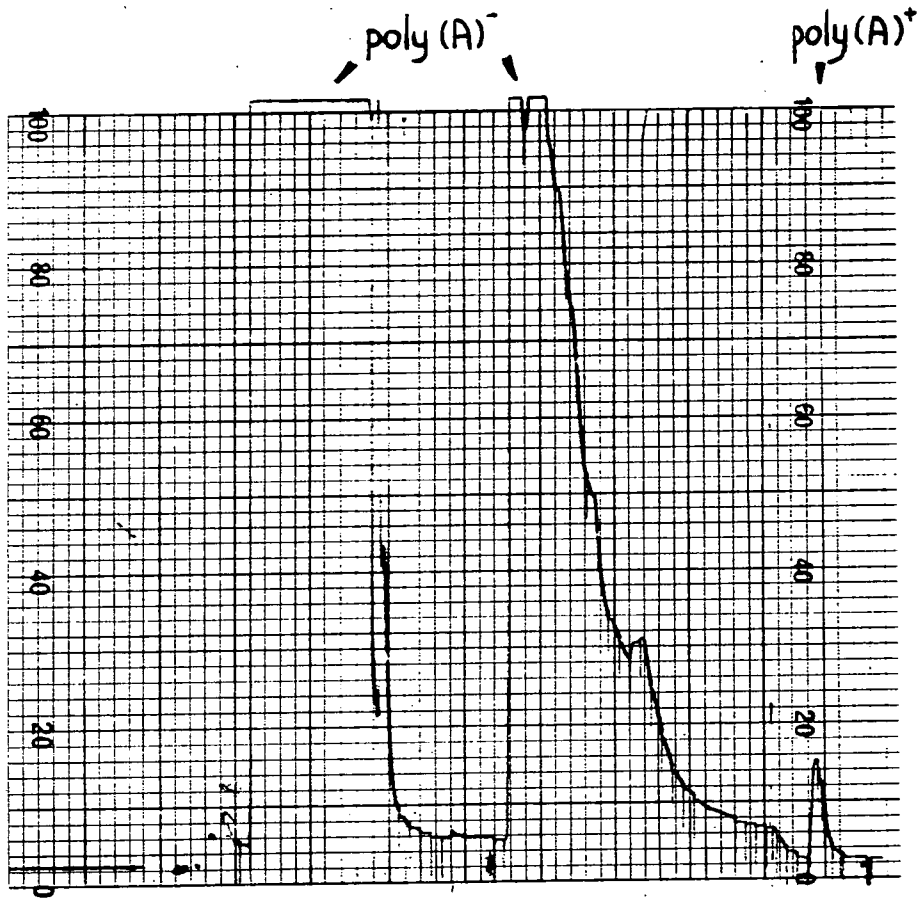


Fig.3·18

Fig. 3.17 and Fig. 3.18 UV absorption profile (λ = 254nm) of pea roots RNA after passing through the oligo-d(T) cellulose column. In Fig. 3.18, RNA has passed through the column twice to enhance the isolation of Poly(A)⁻ RNA.

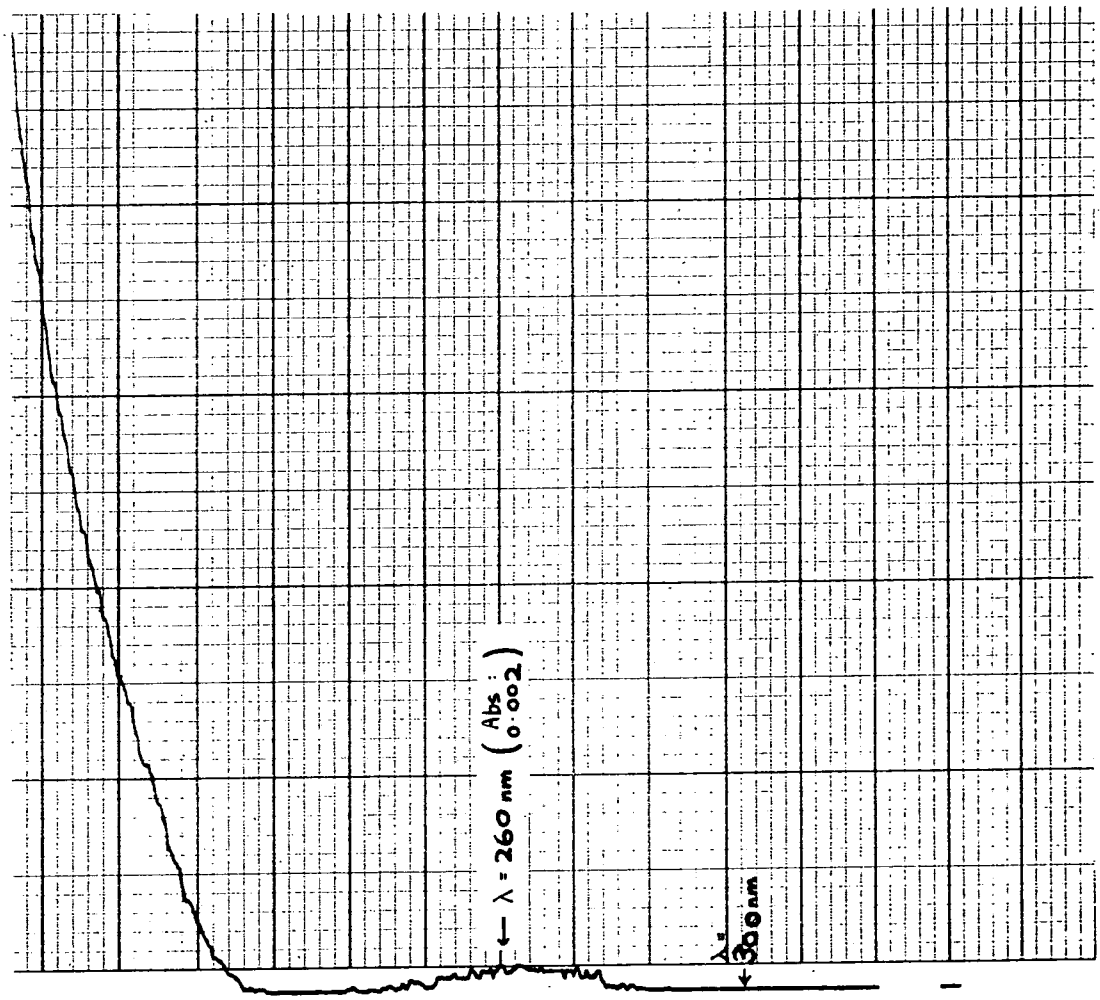
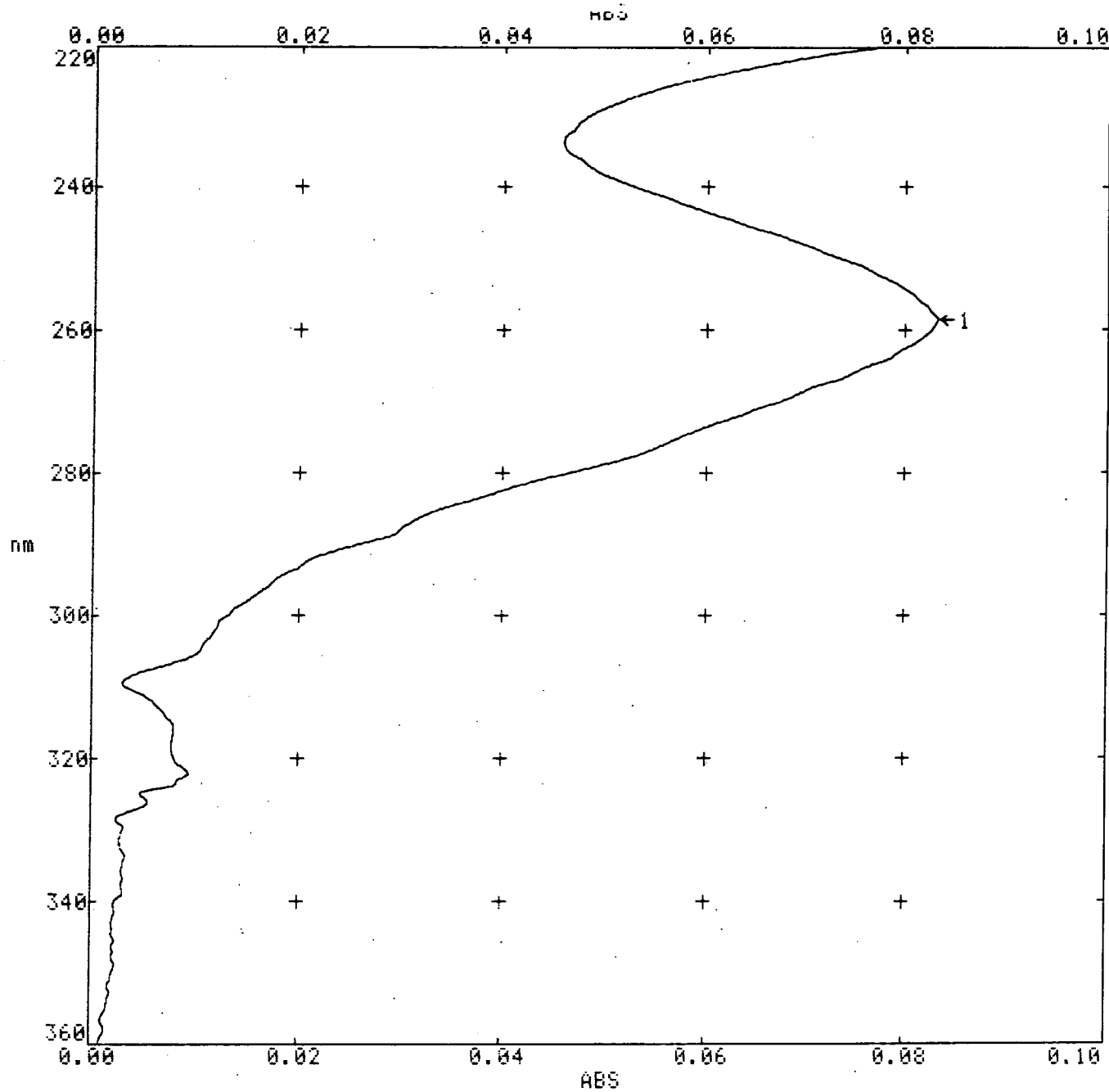


Fig. 3.19 UV absorption spectrum of pea roots Poly(A)$^+$ RNA (180nm - 320nm).



* DUAL λ DATA *

1 260/280 RATIO FOR DNA/RNA

λ1	λ2	λ1
260.0	280.0	
0.066	0.029	2.2

DNA/RNA CHECK SCAN

	1	2	3	4
λ	258.6			
ABS	0.083			

Fig. 3.20 UV absorption spectrum of pea roots total RNA (220nm - 360nm).

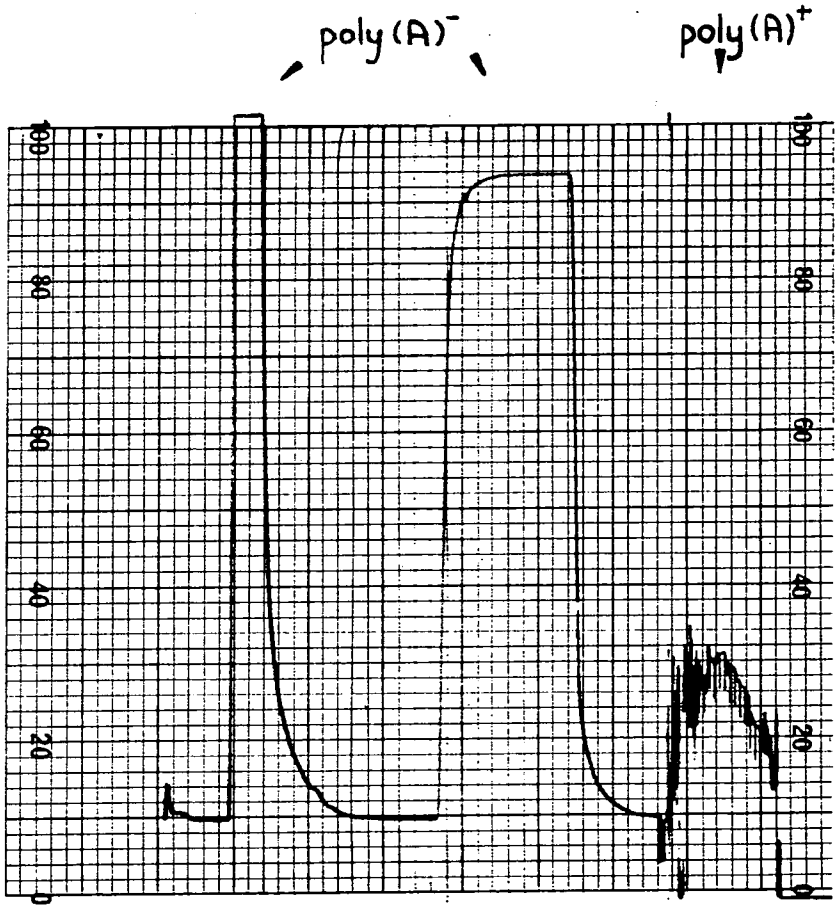


Fig. 3.21 UV absorption profile (λ = 254nm) of pea root RNA after passing through the oligo d(T)-cellulose column. RNA has passed through the column twice to enhance the isolation of Poly(A)$^-$ RNA.

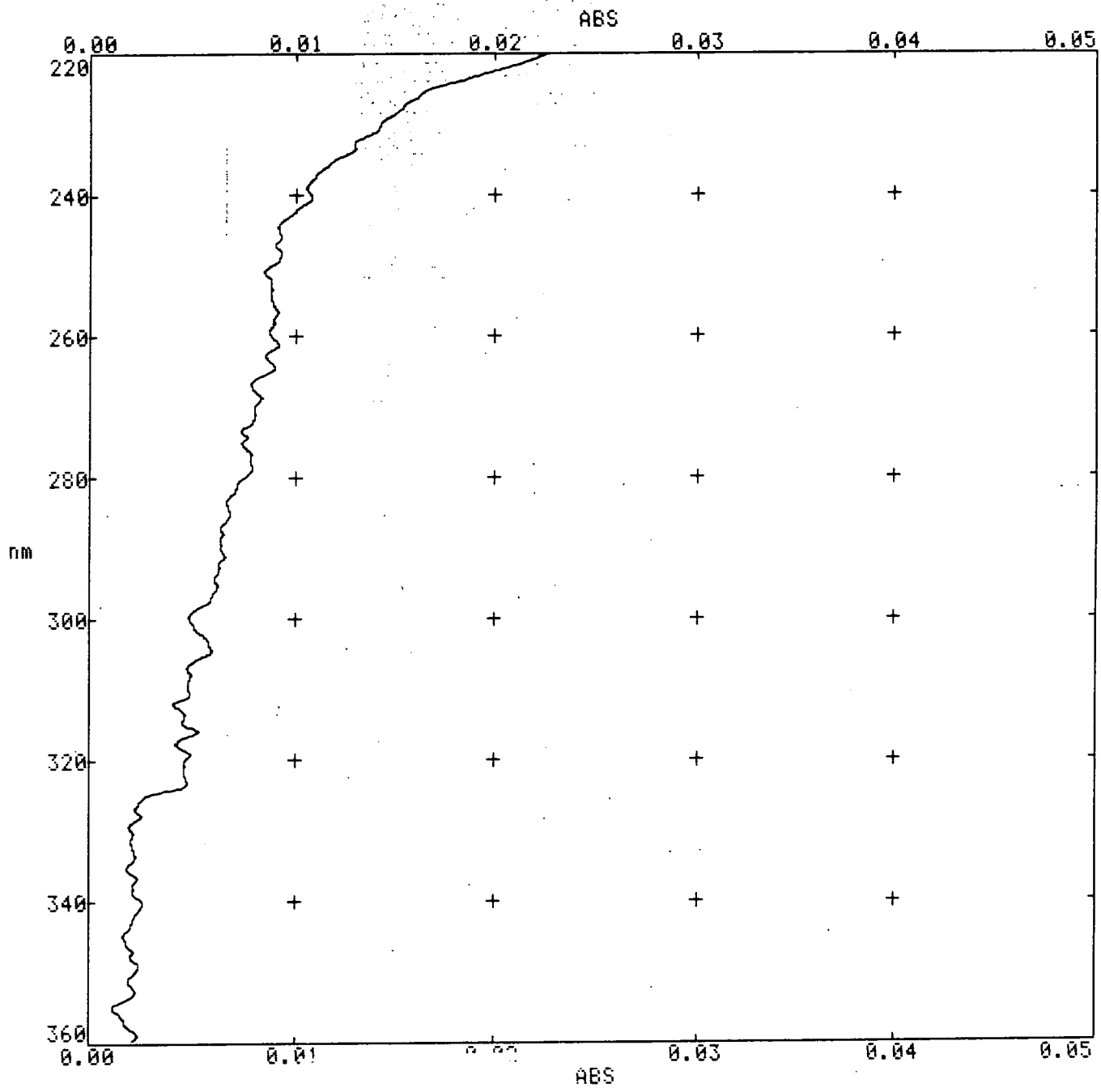


Fig. 3.22 UV absorption spectrum of pea roots Poly(A)$^+$ RNA (220nm - 360nm).

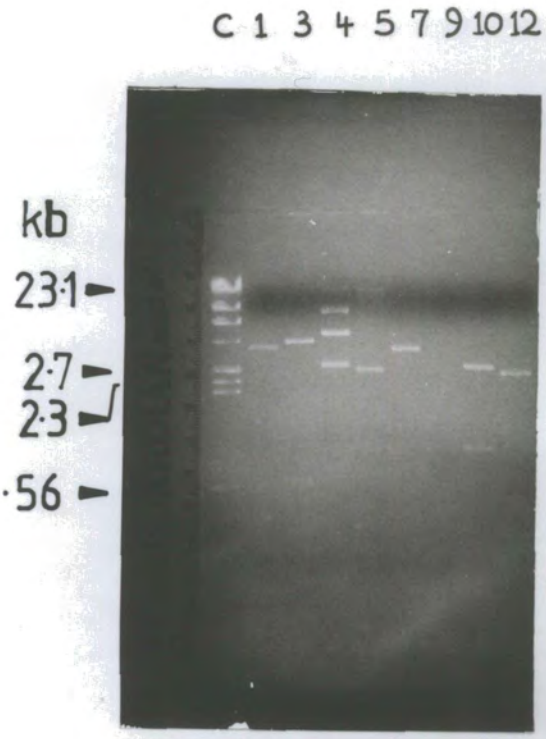


Fig.3·25

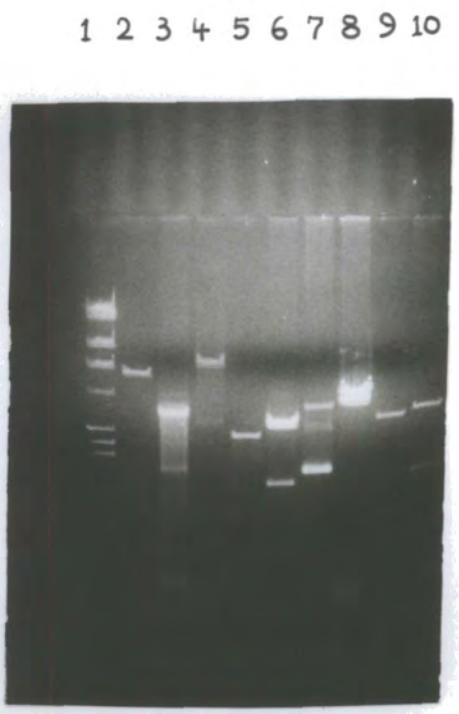


Fig.3·29

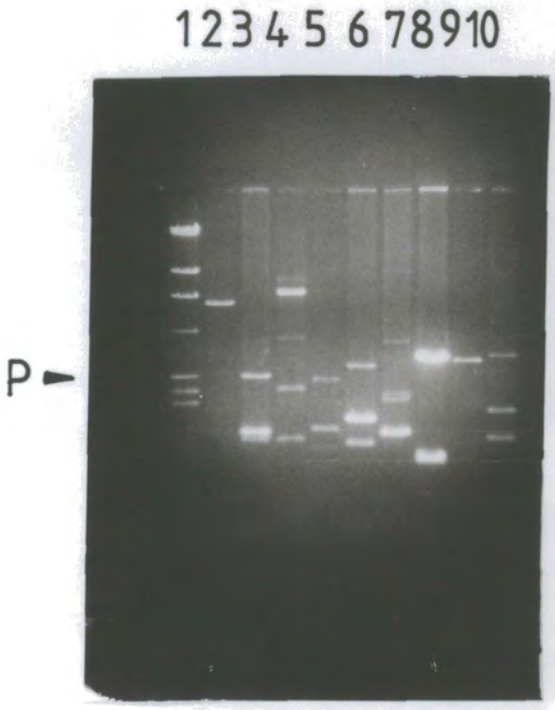


Fig.3·31

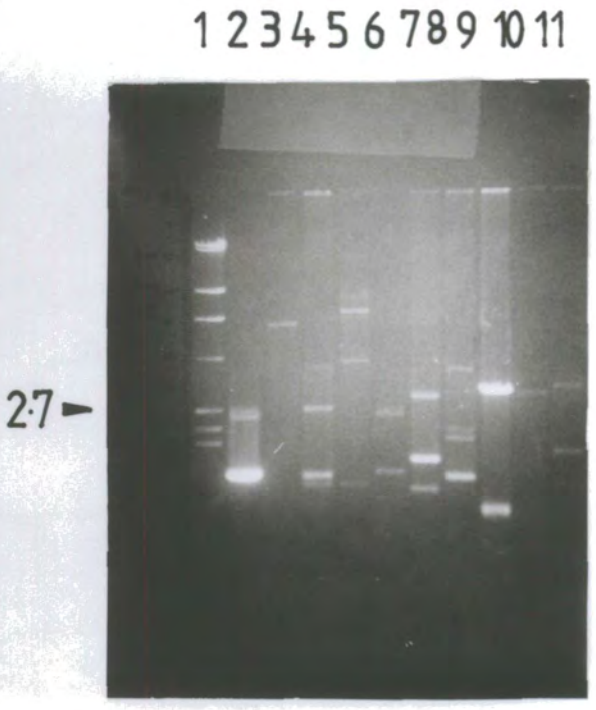


Fig.3·32

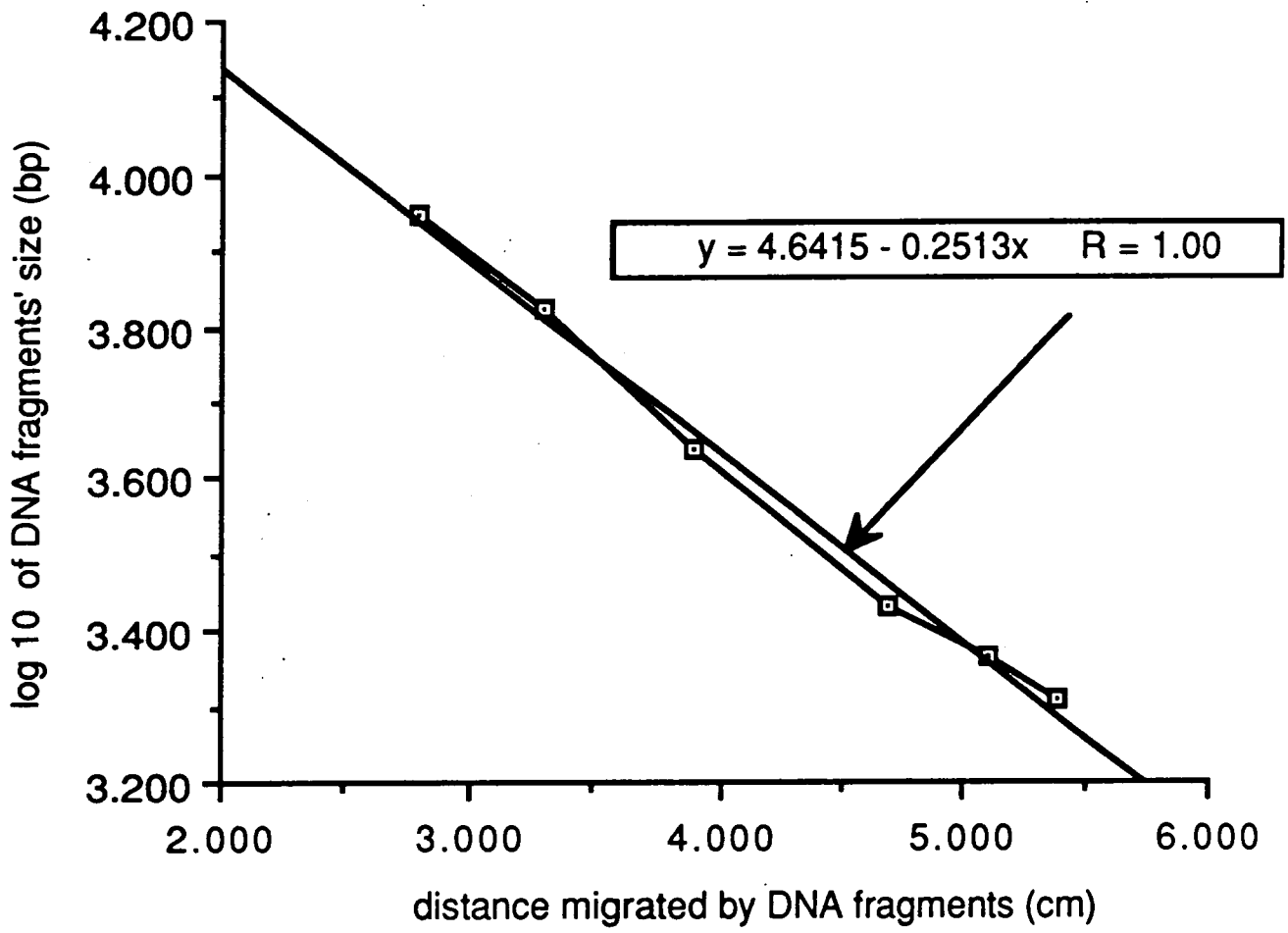


Fig. 3.26 Calibration line of $\log_{10}$ DNA fragments' size (bp) against distance migrated (cm) – λ DNA restricted with HindIII and pUC19 restricted with EcoRI.

Protocol #: 7 Name:32P 1min cpm 12-Jul-88 16:14
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	1106.00	6.01	668.00	642.93	534.
2	1.00	155.00	16.06	93.00	646.94	527.
3	1.00	112.00	18.89	57.00	459.12	532.
4	1.00	114.00	18.73	59.00	568.11	531.
5	1.00	126.00	17.81	39.00	282.81	530.
6	1.00	614.00	8.07	467.00	899.25	525.
7	1.00	29317.0	1.16	24400.0	1075.4	536.
8	1.00	42236.0	0.97	35029.0	1079.1	537.
9	1.00	30259.0	1.14	24273.0	1057.8	543.
10	1.00	14429.0	1.66	11460.0	1003.5	531.
11	1.00	39132.0	1.01	31938.0	1031.9	537.
12	1.00	144425.	0.52	121763.	1071.2	541.
13	1.00	151107.	0.51	133889.	1171.1	543.
14	1.00	119614.	0.57	101636.	1079.7	533.
15	1.00	92899.0	0.65	76556.0	1035.7	530.

Fig. 3.27 Liquid scintillation counts in count per minute (CPM) for checking and separation of radiolabelled pea roots cDNA probe.

Fig. 3.28 Autoradiographs displaying colony hybridization of radiolabelled pea roots cDNA probe to replica filters with pea roots cDNA/pUC19 chimaeras.

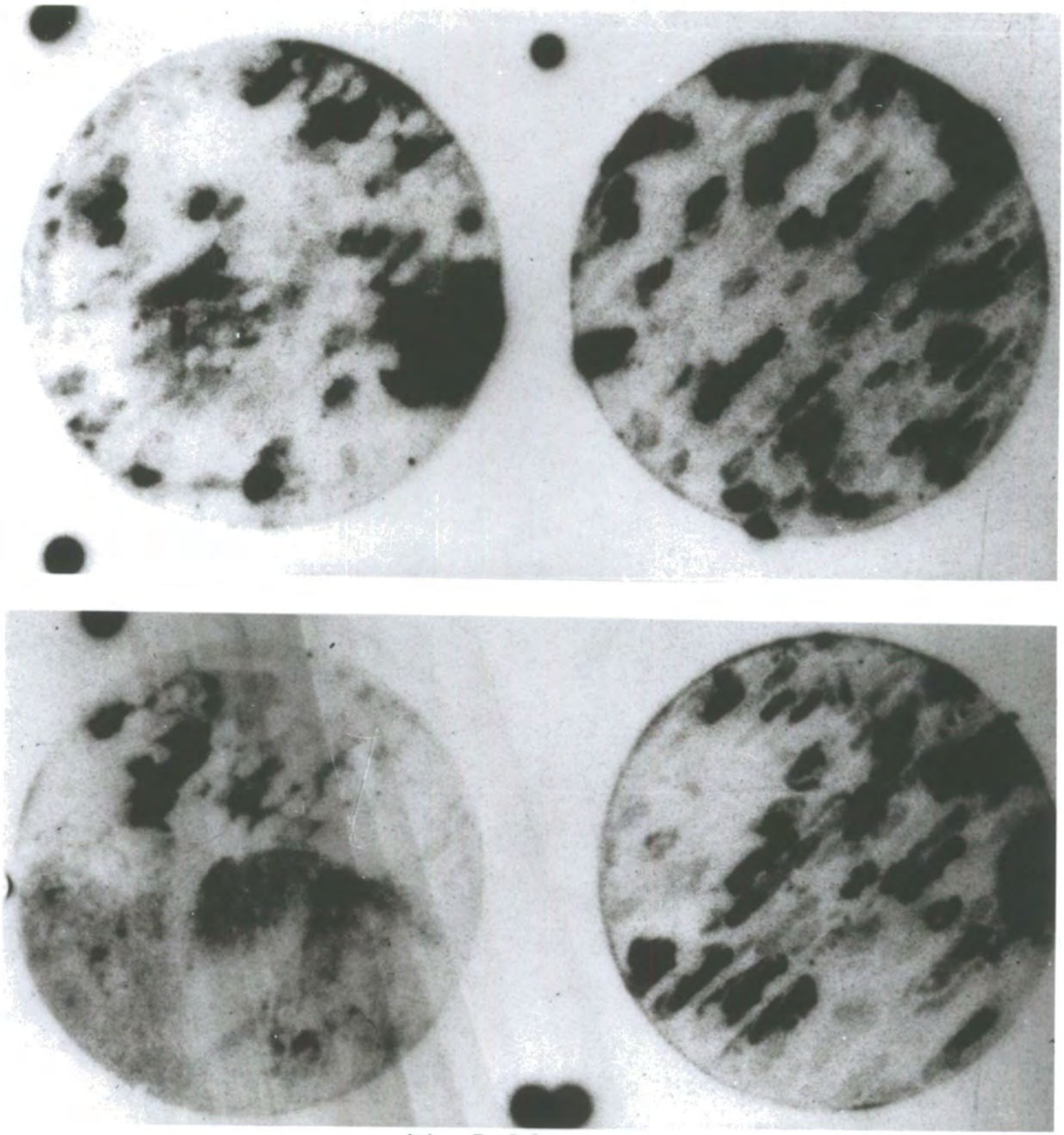


Fig. 3·28

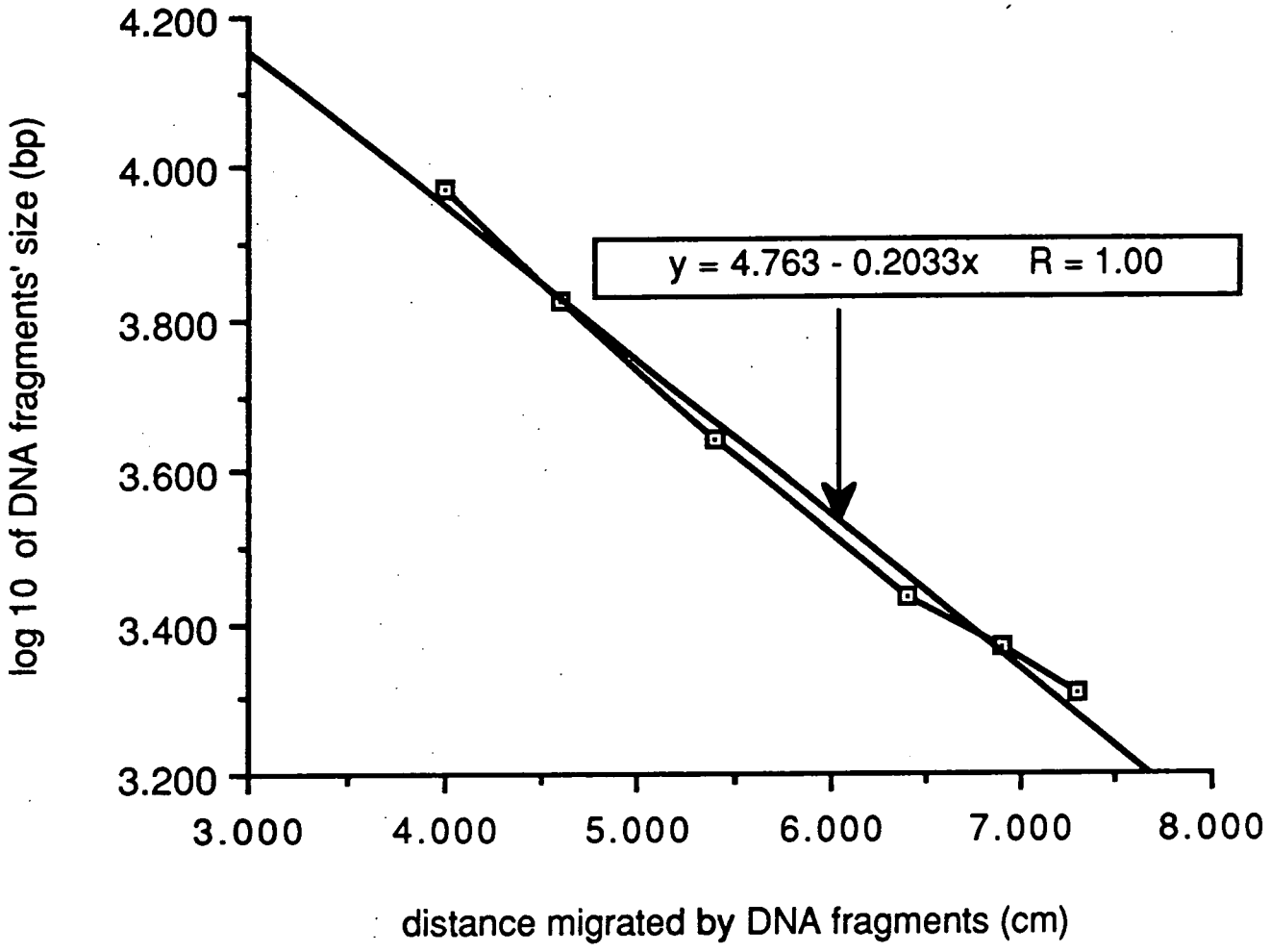


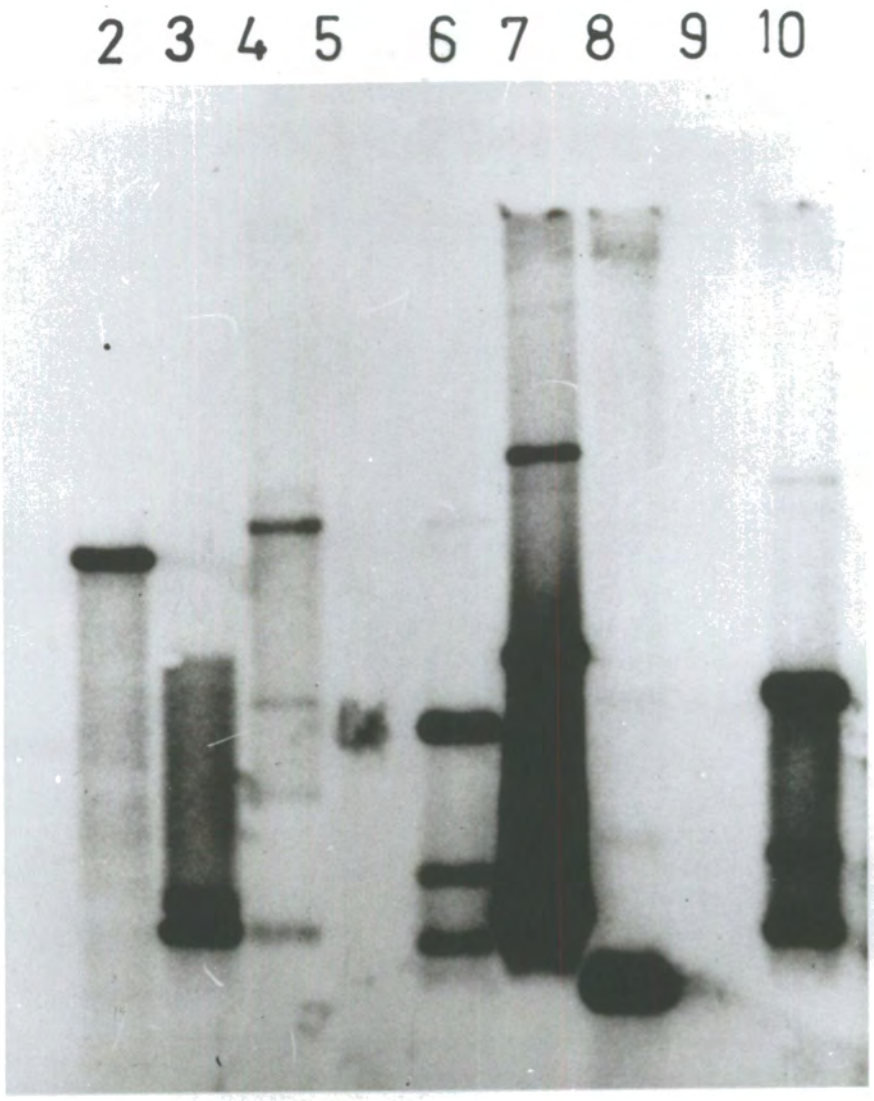
Fig. 3.30 Calibration line of $\log_{10}$ DNA fragments' size (bp) against distance migrated (cm) – λ DNA restricted with HindIII and pUC19 restricted with EcoRI.

Protocol #: 7 Name:32P 1min cpm 27-Jul-88 12:03
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	38.00	32.44	16.00	454.06	540.
2	1.00	61.00	25.60	23.00	333.25	537.
3	1.00	25.00	39.99	14.00	348.98	537.
4	1.00	77.00	22.79	40.00	403.42	535.
5	1.00	28.00	37.79	12.00	556.33	540.
6	1.00	17857.0	1.49	14982.0	1113.8	538.
7	1.00	58179.0	0.82	48236.0	1089.8	537.
8	1.00	11848.0	1.83	9246.00	1049.4	539.
9	1.00	11201.0	1.88	9219.00	1097.9	538.
10	1.00	58525.0	0.82	52536.0	1217.0	538.
11	1.00	136180.	0.54	121699.	1235.8	548.
12	1.00	147544.	0.52	125340.	1117.5	542.

Fig. 3.34 Liquid scintillation counts in count per minute (CPM) for checking and separation of radiolabelled pea roots cDNA probe.

Fig. 3.35 Autoradiograph of radiolabelled cDNA probe hybridized with its complementary DNA fragments after Southern Blotting. The arrows on the gel photograph corresponded to those fragments that hybridized strongly with the labelled cDNA probe.



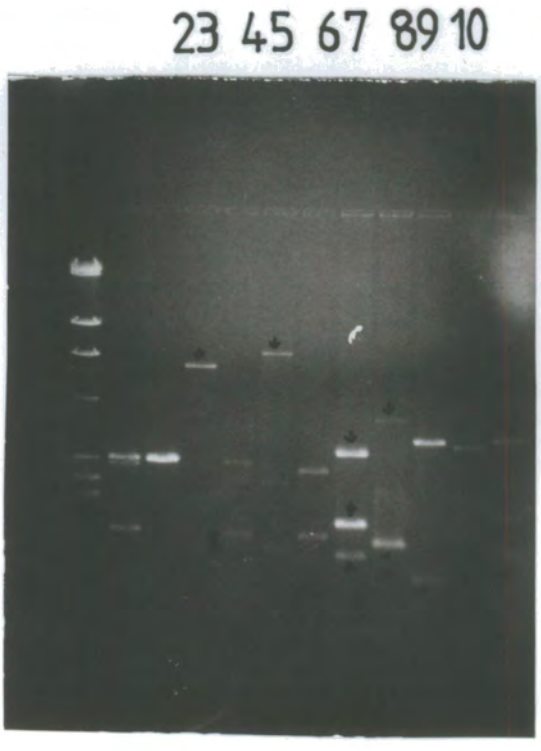


Fig. 3·35

Fig. 3.33 Restriction of selected recombinant plasmid DNA with EcoRI after miniprep. Track no. 1 – λ DNA/HindIII and pUC19/EcoRI size markers. Track A and track B – partial and complete restriction of pUC19 using different concentrations of EcoRI, 5u and 15u respectively.

Fig. 3.36 Restriction of plasmid-DNA chimaeras pPR179 (track no. 1), pPR287(A) (track no. 2) and pPR340 (track no. 3) by EcoRI to release the DNA inserts. Track C – pBR322 restricted with AluI as size marker.

Fig. 3.37 Agarose minigel electrophoresis to check the DNA inserts after freeze-elution extraction.

Fig. 3.41 Restriction of recombinant plasmid DNA that hybridized with the DNA insert from sample pPR179 with EcoRI after miniprep. Track A – pBR322/AluI size marker. Track C – λ DNA/HindIII size marker. Track P – pUC19/EcoRI size marker. Track nos. 1-8 – possible recombinant plasmid DNA containing the full length DNA sequence of pPR179 probe restricted with EcoRI.

Fig. 3.42 Restriction of recombinant plasmid DNA that hybridized with DNA insert from sample pPR287(A) (track nos. 1-4) and sample pPR340 (track nos. 5-10) with EcoRI after minipreps. For abbreviation for tracks P and C see explanation above.

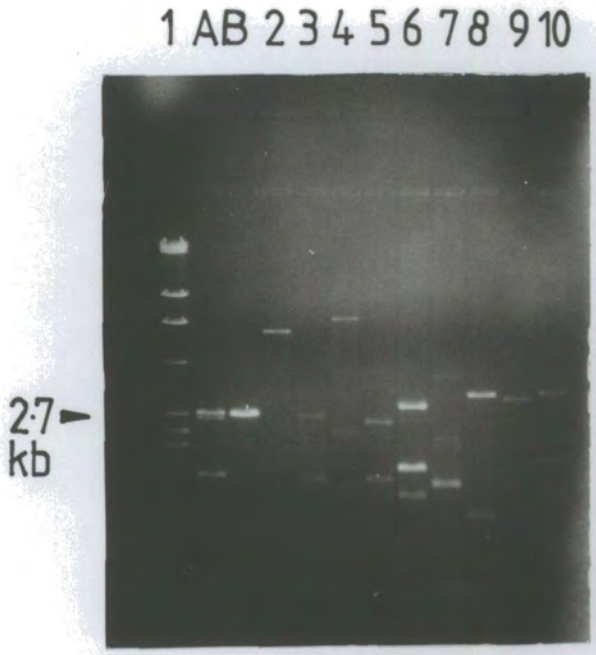


Fig. 3·33

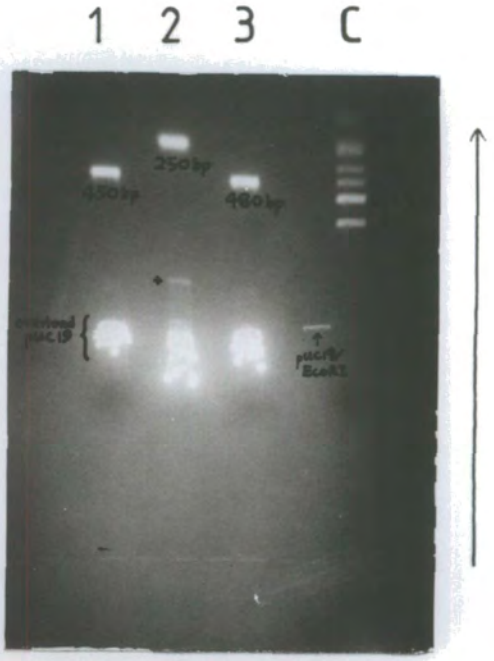


Fig. 3·36

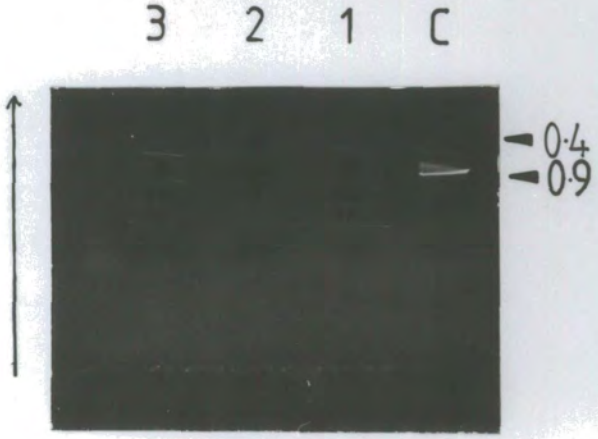


Fig. 3·37

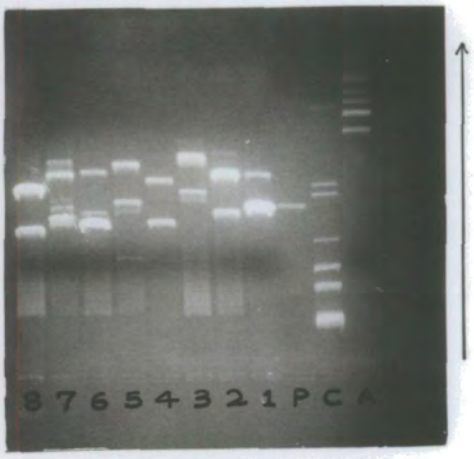


Fig. 3·41

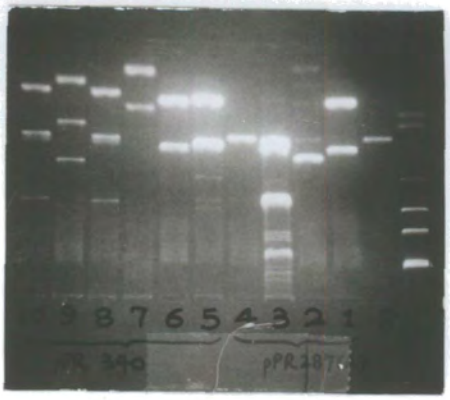


Fig. 3·42

Fig. 3.38 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPR170 DNA insert probe.

Fig. 3.39 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPR287(A) DNA insert probe.

Fig. 3.40 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPR340 DNA insert probe.

Protocol #: 7 Name:32P 1min cpm 08-Aug-88 13:58
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	36.00	33.33	16.00	389.08	521.
2	1.00	59.00	26.03	31.00	687.33	521.
3	1.00	55.00	26.96	19.00	339.94	526.
4	1.00	27.00	38.49	10.00	471.32	523.
5	1.00	24.00	40.82	9.00	469.11	533.
6	1.00	65.00	24.80	35.00	707.94	522.
7	1.00	3497.00	3.38	3112.00	1223.5	524.
8	1.00	5991.00	2.58	5193.00	1093.3	512.
9	1.00	6041.00	2.57	5370.00	1185.0	523.
10	1.00	3823.00	3.23	3444.00	1218.6	519.
11	1.00	3621.00	3.32	3249.00	1194.3	521.
12	1.00	5790.00	2.62	5217.00	1234.5	533.
13	1.00	23343.0	1.30	21023.0	1180.2	519.

Fig.3·38

Protocol #: 7 Name:32P 1min cpm 08-Aug-88 15:46
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	38.00	32.44	13.00	214.82	527.
2	1.00	35.00	33.80	20.00	638.25	523.
3	1.00	43.00	30.49	20.00	494.28	530.
4	1.00	27.00	38.49	10.00	401.99	519.
5	1.00	34.00	34.29	15.00	590.34	526.
6	1.00	241.00	12.88	201.00	955.81	510.
7	1.00	4082.00	3.13	3702.00	1220.4	521.
8	1.00	4354.00	3.03	4007.00	1247.3	524.
9	1.00	4573.00	2.95	4221.00	1266.1	525.
10	1.00	4355.00	3.03	3786.00	1069.4	510.
11	1.00	7054.00	2.38	6449.00	1241.6	526.
12	1.00	15608.0	1.60	14282.0	1217.3	520.

Fig.3·39

Protocol #: 7 Name:32P 1min cpm 08-Aug-88 16:51
Region A: LL-UL= 5.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region B: LL-UL=50.0-1700 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Region C: LL-UL= 0.0- 0.0 Lcr= 0 Bkg= 0.00 %2 Sigma=0.00
Time = 1.00 QIP = tSIE ES Terminator = Count

S#	TIME	CPMA	A:2S%	CPMB	SIS	tSIE
1	1.00	530.00	8.68	223.00	372.18	498.
2	1.00	308.00	11.39	95.00	284.96	506.
3	1.00	260.00	12.40	107.00	310.90	512.
4	1.00	435.00	9.58	254.00	551.19	513.
5	1.00	132.00	17.40	49.00	271.20	510.
6	1.00	935.00	6.54	709.00	929.73	506.
7	1.00	9310.00	2.07	7428.00	988.11	510.
8	1.00	12062.0	1.82	10896.0	1166.1	512.
9	1.00	8989.00	2.10	7916.00	1106.2	508.
10	1.00	9691.00	2.03	8151.00	986.00	506.
11	1.00	8935.00	2.11	6603.00	885.26	512.
12	1.00	19781.0	1.42	17394.0	1062.0	482.

Fig.3·40

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

4.1 Analysis on Preliminary Investigations

4.1.1 DNA Manipulation Practise

At the very beginning of the project, DNA manipulation practises were performed as described in section 3.1.1. Plasmid vectors pUC18 were restricted with BamHI and subsequently ligated with Sau3A restricted λ DNA fragments. The first restriction reaction of pUC18 with BamHI was incomplete as two bands were seen (Fig. 3.1, track no. 2) obviously because of insufficient restriction endonuclease was used. All restriction endonucleases cleaved their DNA substrates to form 5'-phosphate and 3'-hydroxyl termini on each strand, except NciI which has been reported to liberate 3'-phosphate and 5'-hydroxyl termini after digestion of DNA (A. W. Hu and A. H. Marshal unpub. observ.). Thus, a general rule of 5 units of restriction enzyme to digest 1 μg of DNA should be followed. Later the restriction was success by using more BamHI and the ligation with restricted λ DNA fragment posed no problem. Noticed that a higher concentration of λ DNA than pUC18 was used to enhance the ligation reaction. Plasmid recombinants were found (Fig. 3.3, track no. 1) after *E. coli* transformation followed by alkaline lysis plasmid DNA minipreparation. The size of the DNA inserts are checked with suitable restriction by using EcoRI and/or HindIII (Table 3.1).

Tricky points were already present at the beginning of the experiment in which

BamHI restricted plasmids were ligated with Sau3A restricted λ DNA. This was possible because BamHI and Sau3A were isoschizomers; restriction endonucleases which recognised identical sequences. Sau3A recognised a tetranucleotide sequence 5'-N_1GATCN_2-3' that was included within the hexanucleotide sequence recognised by a BamHI – 5'-GGATCC-3'. The cohesive termini produced by Sau3A would cohere with those produced by BamHI by T_4 DNA ligase. However, during DNA inserts size determination, EcoRI and/or HindIII was used instead of Sau3A or BamHI. It was due to the fact that after the restricted pUC18 and λ were covalently joined, the 'hybrid site' so produced would be once again sensitive to Sau3A, but might not constitute a target for BamHI (i.e. 5'-N_1GATCC-3') which would depend upon the nucleotides adjacent to the original Sau3A site. On the other hand, Sau3A should not be used to cut out the DNA insert in the plasmid recombinant since some other restriction sites (e.g. those restricted by PvuI: 5'-CGATCG-3') on pUC18 were vulnerable to Sau3A restriction too. λ fragments were cloned into the multiple cloning site of pUC18 within the HindIII and EcoRI restriction site, thus the inserts could be released by these two enzymes without problem.

4.1.2 Isolation of Total RNA from Pea Cotyledons

Extraction of RNA from pea cotyledons has also been practised. The hot SDS/proteinase K method was employed according to the protocol of Hall et al. (1978). The homogenisation buffer used containing supersaturated boric acid could maintain the stability of the nucleic acid and provided a buffering effect. SDS was used as a protein denaturant in which it binded to the seeds' polypeptide chains via hydrophobic interactions. The binding was almost uniform along polypeptides with an amount of approximately 1.4 g SDS bound/g of polypeptide. The

detergent linearised protein chains by breaking all the non-covalent interactions, hence allowed proteinase K digestion to facilitate easily and removed all proteins including endogenous nucleases.

The extraction of RNA was highly satisfactory that gave 4.5 mg of total RNA from 50 g of pea cotyledons (Fig. 3.6). Electrophoretic analysis of this RNA on agarose gel after glyoxal/DMSO denaturation revealed a diffuse band of mRNA and tRNA lying between 25S and 18S ribosomal RNA markers isolated from the seeds (Fig. 3.7). The extraction was success because the method used was tailored for cotyledon RNA preparation since pioneer work has been done on French bean (*Phaseolus vulgaris*). In addition, the large amount of storage proteins reserved for germination of pea seeds revealed that large quantities of RNA as well as mRNA were present since the latter functioned as a template for efficient translation of proteins. This meant that even slight degradation of RNA occurred, the significant quantity presence could compensate the lost and still provided a good recovery after the extraction processes. The extracted pea root RNA was then used for poly(A)$^+$ RNA preparation. However, when this SDS/proteinase K RNA extraction method was used on pea root, reasonable quality and quantity of RNA was not obtained (Fig. 3.9, track no. 3 and 4; Fig. 3.14). This will be discussed in section 4.2.

4.1.3 Selection of Poly(A)$^+$ RNA from Pea Cotyledon RNA

Preparation of poly(A)$^+$ RNA from pea cotyledon RNA employed oligo d(T)-cellulose affinity chromatography. Pioneer work on purification of biological active rabbit globin mRNA was done by Aviv and Leder (1972). The method depended

upon annealing polyadenylic acid rich mRNA to oligothymidylic acid residues immobilized on cellulose resin and subsequent its elution with buffer of low ionic strength. The abundant content of poly(A)$^+$ RNA in total pea cotyledon RNA allowed a recovery of 145 μg of the former after the oligo d(T)-cellulose chromatography. They were then used for construction of a pea cotyledon cDNA library. In contrast, such purification method did not work well on pea root mRNA selection and a more detail discussion was attempted in section 4.3.

4.1.4 Construction of Pea Cotyledon cDNA Library

A cDNA library was a mixture of clones constructed by inserting cDNA into a suitable vector. The term library implied the existence of large number of different recombinants. With the use of purified pea root poly(A)$^+$ as a template, cDNA cloning could be proceeded. As mentioned in section 1.4, several methods were available for generating a cDNA library. Thus the strategies used for selection of a proper cloning route were important. In the present work, since the library after construction would be screened with nucleic acid probes, basically any method outlined in section 1.4 would suffice. Nevertheless, preparation of a complete, full-length cDNA library (in pea seed as well as pea root later) was the chief aim. The ideal one should compose of greater than 5000 different cDNAs which have a probability of greater than 99% in finding a specific sequence that represented about 0.1% of the mRNA population. Besides, the magnitude of members in the library should range from 10^4-10^5 using plasmid as vectors. Therefore the choice of a suitable method should base on these criteria.

For cloning cDNA by homopolymer tailing (section 1.4.1), the used of S1 nu-

clease could eliminate unpaired sequences at the hairpin, nucleotides that were derived originally from the 5' ends of mRNA. Moreover, the enzyme might attack double-stranded termini and introduced nicks into double-stranded molecules. The homopolymer tails were also proved refractory to enzyme removal (Eden et al., 1982). Thus not only was it impossible to produce full-length cDNA but also the ability of terminal transferase to tail at nicks or gaps created branched structures that interfered with cloning. For the Heidecker and Messing method (1983) (section 1.4.4) and Okayama and Berg method (1982) (section 1.4.3) or its derivatives vector-primed cDNA cloning, they were effective methods for generating full-length cDNA clones. However, these approaches required more difficult and time-consuming initial preparation than traditional cDNA cloning protocols, so they were not the method of choice. Eventually, a method that obviated the use of S1 nuclease by employing the RNA replacement strategy which also allowed improvement of cDNAs length prepared was used. Also, adaptors instead of linkers to bridge the ligation between cDNAs and plasmid vectors were choosen. The difference stemed from the three steps of methylation, ligation of linkers, and cleavage of linkers as contrasted with the one step of ligation of adaptors that the former was obviously more labour intensive. The commercial cDNA synthesis kit provided all these advantages together with all the ready-to-use materials were therefore used for the library construction.

The vectors of choice for cloning were pUC19. Such plasmids have largely supplanted conventional pBR322 for the following reasons: (1) they had multiple cloning site (2) they could be propagated at a higher copy number and smaller in size (3) they contained a promoter for β-galactosidase which could direct attention

exclusively to clones with inserts; and (4) they did not contain sequences that inhibit transformation of eukaryotic cells (Kimmel and Berger 1987).

In this present project, a cDNA library was constructed by the method described in section 1.5 and 2.2.8, synthesized from pea cotyledon mRNAs, and cloned into the EcoRI site of pUC19 using EcoRI adaptors. The primary screening of the library made use of the inactivation of the β-galactosidase gene in the vector so that the recombinants with cDNA inserts could not utilize X-gal in the nutrient agar. The transformants which were white colonies could then be identified from the non-transformant blue colonies. Table 3.3 shown the results of colonies counting after the transformation and the cDNA inserts size were checked on agarose gel electrophoresis after a series of works like plasmid DNA extraction and EcoRI restriction. Based on the optimal transformation condition results, the transformation efficiency was 7.46 x 10^5 colony forming units (cfu)/μg of DNA insert. From the DNA inserts' size calculated (Table 3.4), their size ranged from 335 - 1660 bp. These data suggested the synthesis of pea cotyledon cDNA and ligation with pUC19, transformation of *E. coli* with recombinants and subsequent inserts size determination were all successful.

The necessity to perform several transformation reactions with different aliquots of cDNA (Table 3.2) was unquestionable. An effective way to optimize colony forming potential and colony density was to determine the saturation level of cotyledon cDNA plus pUC19 vector. Different preparation would give different saturation points even though they contained equal masses of total DNA. Hence it was advisable to determine the optimal conditions and saturation point prior to transformation.

4.2 Isolation of Total RNA from Pea Root

The isolation of undegraded ribonucleic acid from cells and tissue involved three steps: (1) inhibition of endogenous nucleases (2) deproteinization of the RNA and (3) physical separation of the RNA from the other components of the homogenate. In case of pea roots, total RNA was attempted to be extracted firstly by the SDS/proteinase K method which has been used pea cotyledon RNA extraction as mentioned before. However, the RNA yield was unsatisfactory. Hence a second trial using guanidinium salts/cesium chloride centrifugation method was proved to be successful. Although SDS/proteinase K was a popular RNA extraction method, it was incompatible with the preparation of subcellular fractions. The success rate with this approach varied with different tissues and did not equal that of guanidine-base methods. Obviously, SDS/proteinase K method was not suitable to pea root RNA extraction. Pea root tissues were basically a poor source of nucleic acids. It contained high level of nucleases and the nucleic acids might be complexed with secondary metabolites like phenols and carbohydrates. The ability of proteinase K to digest the high level of nucleases in plant cells was unquestionable. However, it would only be useful if peptides were covalently linked to the RNA. Moreover, it was anyway, a type of enzyme in which kinetics, pH, temperature, ionic interaction, etc. might affect its performance greatly. Inactivation of nucleases parallelled the kinetic efficiency of protein denaturation, and the very high efficacies of guanidinium thiocyanate and guanidinium chloride proved to be a more effective denaturant. Besides, selective precipitation based on solubility using lithium chloride (Auffray and Rougeon 1980) to physically separate the RNA from the other macromolecular components in the homogenate was nonquantita-

tive. Such problem was serious as the root RNA concentration in the homogenate was very low. Therefore, quantitative recovery of RNA in a very dilute RNA solution by cesium chloride dense solution ultracentrifugation (Chirgwin et al., 1979; Glisin et al., 1974; Ullrich et al., 1977) was the method of choice.

4.3 Isolation of Poly(A)$^+$ RNA from Pea Root Total RNA

Posttranscriptional polyadenylation was a common feature of the biogenesis of most eukaryotic mRNAs. To separate pea root poly(A)$^+$ RNA from all others, oligo d(T)-cellulose chromatography was the technique used throughout the cloning process, which had been used previously to extract pea cotyledon poly(A)$^+$ RNA. This method was generally effective in which it could reduce the content of rRNA and removed tRNA from mRNA preparations. Oligo d(T)-cellulose resin also has high binding capacity per gram and a noncollapsible matrix. This permitted the use of small columns and small-volume batch techniques for binding, washing and eluting. But unfortunately, the attempts to isolate pea root poly(A)$^+$ RNA from the bulk RNA were failed and the possible reasons were analysed as followed.

The yield of mRNA should approximate 1-3% of the input, unfractionated RNA in plants. Higher yields indicated contamination with non-poly(A) containing RNAs, in particular rRNA. The amount of starting material used might not sufficient to provide enough poly(A) RNA for mRNA extraction. The presence of rRNA intermingled with poly(A) RNA decreased the accessibility of the polyadenylated RNA tailed to the oligo d(T)-cellulose. Also, heated and quench-cooled the total RNA before running the column might not have done well so that

the mRNA has great chance of aggregating with rRNA and hence affected oligo d(T)-mRNA binding. Existence of rRNA in the sample could be readily confirmed by gel electrphoresis (Ogden and Adams 1987). However, owing to the time constraint and inadequate amount of precious RNA, confirmation by glyoxal/DMSO gel electrophoresis had not done in the last time preparation.

Another critical point was that Higgs et al. (1983) and Montell et al. (1983) reported a highly conserved sequence AAUAAA, found 11-30 nucleotides upstream from the poly(A) tail in most animal mRNAs (Proudfoot 1982) was necessary for a mature 3' end message formation before polyadenylation process started (Delauney 1984). But such polyadenylation signal sequence in plant mRNAs were more variable than in animal in terms of the actual sequences involved and their distance from the polyadenylation site. The initial size of poly(A) tract in pea root mRNA might considerably shorter than its animal counterparts (Palatnik et al., 1979, 1980) which lowered their binding efficiency to oligo d(T)-cellulose matrix. Hence mRNAs could be eluted together with the poly(A)$^-$ RNA by loading buffer. A careful examination of bona fide poly(A)$^-$ RNA has revealed the existence of a small amount of mRNA activity (Kaufmann et al., 1977; Sonenshein et al., 1976; Palatnik et al., 1979) . On the other hand, oligo d(T)-cellulose has been reported to have a requirement for a minimum poly(A) length of approximately 15 residues and has been shown to have significant contamination of its nonbinding fractions with polyadenylation mRNAs (Rosenthal et al., 1983). Moreover, a major disadvantage of oligo d(T)-cellulose was the short length of oligomers generally used to prepare the resin (usually a maximum of 18-30 dT residues). This lead to inefficient binding of mRNA molecules with relatively short poly(A) tails and hence, contamination

of the nonbound fractions [poly(A)$^-$ RNA] with some true poly(A)$^+$ RNA. This matrix was also unable to fractionate the poly(A) tracts of mRNA by their length difference because the oligo-d(T) tail was too short (Jacobson 1987).

In addition, most newly synthesized mRNAs contained long, posttranscriptionally added poly(A) tails which shortened as the mRNAs aged in the cytoplasm (Palatnik 1979). This occurred in aged pea roots which were collected for mRNA extraction as well as their declined in metabolism. Hence the isolation of poly(A)$^+$ RNA became more difficult. Meanwhile, mRNAs with poly(A) tails shortened below 40-65 residues have increased rates of degradation (Nudel et al., 1979). These factors altogether might contribute to the unsuccessfulness in recovery of pea root mRNAs. Suggestion was therefore made to try to use poly(U) filters or poly(U)-Sepharose chromatography (Lindberg and Persson 1987). The latter was especially effective as it has molecules of poly(U) which were approximately 100 nucleotides long, providing more efficient binding of short poly(A) tract of pea root poly(A)$^+$ RNA than oligo d(T)-cellulose did, giving a more legitimate poly(A)$^-$ fraction. Also, the use of fresh, young pea roots which contained more newly synthesized, long poly(A) tail mRNAs for extraction of poly(A)$^+$ RNA was highly recommended. Finally, purification of mRNA by affinity chromatography resulted in a highly enriched but nevertheless impure population of polyadenylated molecules since contaminants, principally rRNA, were present in variable but often significant amounts. Therefore quantifying the poly(A)$^+$ RNA by the method of Krug and Berger (1987) and assessment of their quality were advised before the construction of cDNA libraries.

4.4 Construction of a Pea Root cDNA Library

The principle, procedure and reasons of choice of the method for synthesis of pea root cDNA were described in section 1.5, 2.2.8 and 4.1.4 respectively. After the synthesis and ligation to plasmid vector pUC19, the DNA-plasmid recombinants were used to transform *E. coli*. The colonies counting results were listed in table 3.4 and the transformation efficiency was 1.4 x 10^4 cfu/μg of DNA insert based on the optimal transformation conditions result. When comparing the transformation efficiency in case of pea root with that of pea cotyledon (7.46 x 10^5 cfu/μg DNA insert, section 4.1.4) and pea shoot segments (6 x 10^4/μg DNA insert; Young, R., unpub. result), the shoot and the root were of the same magnitude which was 10x lower than that of cotyledon. This could roughly accounted for the much higher abundance of mRNA in cotyledon than in root and shoot.

From the results of colonies counting, the number of non-transformants was higher than transformants. Such a high percentage of non-transformed blue colonies indicated that the cells might have transformed with either recircularized or oligomeric plasmids. Although the plasmid vectors had been dephosphorylated using alkaline phosphatase to prevent self-ligation and subsequent minigel checked to confirm the absence of recircularized or oligomeric plasmids, they might present in a very low quantities to be discovered on minigel but high enough quantities to give a high background of non-transformant (Delauney 1984). This, together with the possibility of incomplete restriction of pUC19 plasmids before the cloning of cDNA synthesized, contributed to the results of low recombinants to non-recombinants ratio. Besides, the quality and quantity of intact mRNAs template might have declined due to degradation as they were extracted and stored for certain period

of time. The low efficiency of cDNA synthesized – 20-30% of the starting mass of poly(A)$^+$ RNA (Dr. I. M. Evans, pers. comm.) and degradation during the lengthy procedures should not be excluded too.

Seven of the transformants were picked for plasmid DNA extraction. After the BamHI restriction, the size of the cDNA inserts was found ranged from 900 - 3050 bp (table 3.5). The results of sample no. 4 and 5 were excluded since the former one gave an insert sized over 14 kb while the latter one was only 54 bp. The unreasonable large DNA insert size was due to miscalculation on partially restricted plasmid DNA fragments and the small DNA insert probably contained no cDNA fragment but only the EcoRI adaptors.

After another two ligation/transformation reactions, 184 more recombinants were raised (Table 3.6) and they were all transferred to nitrocellulose filters for colony hybridization.

4.5 Colony Hybridization of Pea Root cDNA and Autoradiography

Molecular hybridization was the formation of double-stranded nucleic acid molecules by sequence-specific base pairing of complementary single strands (Meinkoth and Wahl 1984) which have been denatured. Under appropriate conditions, only clones containing DNA sequences that shared homology with the probe would hybridize. Positive recombinants could be identified by autoradiography of the filter replicas. In the present work, the pea root cDNA containing filters were first pre-hybridized in a solution containing heterologous herring sperm DNA which were rendered single-stranded after denaturation, SDS and Denhardt's solution. The purpose of this step was to saturate binding sites on the nitrocellulose filters that

would otherwise lead to an acceptable background (Hanahan and Meselson 1983). The filters were then incubated in the same solution containing the radioactively labelled probe and the heterologous DNA overnight to allow hybridization to take place. The filters were then washed under conditions of fixed temperature but varying salt concentration such that only specific hybrids were stable. Such a series of post-hybridization washes of increasing stringency at lower ionic strength aimed to remove those non-hybridized DNAs. SDS was also used to assist the removal of non-specifically bound probes. The location of the hybrid molecules was then determined by autoradiography.

SDS added in the prehybridization/hybridization solutions generated a lot of bubbles, removal of them were necessary to prevent inefficient hybridization. Since the cDNA probe from pea root was used to identify its related cDNA sequence, the high stringency wash using low SSC concentration was omitted to prevent dehybridization. However, the exact relationship between stringency and sequence homology depended on base composition, the length of the probe and the their homologous regions. Hence it was difficult to determine other than empirically (Wahl et al., 1987).

From the autoradiography taken (Fig. 3.28), the numbers of colonies of different intensity of hybridization from strong, medium, weak to very weak were 10, 24, 41 and 65 respectively. The background was not serious and those strongly hybridized recombinants could be identified easily. The most frequent cause of background was the cDNA probe itself but not the time of prehybridization. Therefore careful preparation and labelling of probe were essential. Moreover, it was sometime difficult to distinguish true positive signals from "spontaneous" spots,

speckles, and smears that appeared on the film. The causes of false spots might be simply a cosmic ray, a contaminated cassette or screen, or the static electricity released by crushing plastic wrap. It was thus recommended a pair of duplicate filters from each master plate could be made. Spurious dark spots which present on only one filter of a pair could safely be ignored.

The use of pea root cDNA probe to hybridized with its own cDNA library aimed to identify the clones which were abundant in pea root. The sequence could be responsible for encoding important root specific proteins. The abundant clones could be picked out by superimposing the film and the master filter. However, not all of the abundant sequences were abundant enough to give a clear signal, cDNA which were short relative to the length of the probe could give a relatively weak signals for their abundance class. Also, some non-cDNA inserts contaminant and some colonies without insert might present. Hence the cDNA probe should have identify all the very abundant cDNA clones but only about half of the abundant cDNAs (Gatehouse 1985).

Nick-translated ^{32}P-labelled nucleic acids were the most widely used probe in nucleic acid hybridization. Recently developed in vitro transcription systems to generate ssRNA probes (Green et al., 1983) were claimed to have advantages over nick-translated DNA. In addition, non-radioactive probes like nick-translated DNA containing biotin-labelled nucleotides (Brigati et al., 1983; Langer et al., 1981) and enzyme-linked nonradioactive assay (Leary et al., 1983) were developing which eliminate some of the disadvantages of radioactive probes (e.g. short half-life, high cost, hazardous nature, inconvenience, etc.).

4.6 Analysis on Pea Root cDNA Inserts

After aligning the film with the master filters, nine most intensely hybridized colonies were picked out for minipreps and agarose gel electrophoresis for inserts size assessment (Fig. 3.29). The restriction endonuclease first used was BamHI. The restriction was done and the inserts size were ranged from 1.28 - 7.85 kb (Table 3.7). One BamHI site was found in the multiple cloning region of pUC19. After the insertion of pea root cDNA into such region, the number of fragments generated followed the BamHI restriction would be one or more depended on whether the cDNA inserts happened to have BamHI site(s) on or not. From table 3.7, sample no. 5 contained no cDNA insert but just linearised plasmid pUC19 vectors. This could be seen clearly on the gel which were comigrated at same position with the EcoRI restricted pUC19 (Fig. 3.29, track no. 1, the 5th band from top).

Afterward, the extracted DNA were restricted with EcoRI (Fig. 3.31). However, one or more DNA bands were seen on each sample with no linearised pUC19 vector bands except on track no. 3 (Fig. 3.31) which shown a possible vector band and two inserts. Hence incomplete digestion was suspected. Since minipreparation of plasmid DNA by alkaline method might cause some enzymes including EcoRI worked less well on it than on highly purified DNA (Maniatis et al., 1982). This problem could be avoided, as suggested by Maniatis, by increasing the amount of enzyme twofold to threefold and/or by carrying out the digestion in larger reaction volumes (20-50μl) so that any inhibition in the DNA were diluted. Then another two similar restrictions using more EcoRI and a freshly prepared miniprep DNA were performed respectively (Fig. 3.22 and 3.33). Out of expectation, the results shown were similar to the one done at the first time. This proved that the

minipreps and the restriction done so far have no problem. Because of the odd results, a Southern Blot was done using radiolabelled pea root cDNA as a probe to confirm cDNAs were definitely cloned into the plasmids. Various dark hybridized bands were shown on film after development, supporting the above argument.

According to the Southern Blot autoradiograph in Fig. 3.35, the restriction patterns were primarily investigated. Sample no. 2 might contain the vector-cDNA recombinants that were unrestricted by EcoRI. Sample no. 3 contained pUC19 vectors with two cDNA inserts in which the smaller size inserts were more abundant. Sample no. 4, 6 and 10 contained cDNA inserts which could be existed alone and/or linked with some other DNA fragments because DNA bands with different size were hybridized. In sample no. 5 and 9, no cDNA insert was found. This proved that sample no. 9 actually contained no insert but only the pUC19 vectors and the insert size calculated in table 3.7 could be a mistake on migration distance measurement because the DNA bands on the gel were distorted after a high voltage (110V) electrophoresis. Sample no. 7 and 8 contained cDNA inserts which were highly abundant in pea root since the hybridization was exceptionally intense. For sample 7, similar situation was happened as that in sample no. 4, 6 and 10 while in sample no. 8, cleavage site might have altered that generated a slightly larger vector with a very small but abundant cDNA insert.

4.7 Possible Explanations on the Unexpectable Restriction Patterns

The possibility of partial restriction by EcoRI to give the results shown on Fig. 3.31, 3.32 and 3.33 was low. It was because no vector band was seen on the gel except only one sample. Hence an alteration in EcoRI restriction sites might have

taken place. The reason behind was not certain but possible explanations were attempted to make as followed.

During the cloning between plasmid and cDNA, adaptors were added to bridge them together. Ready-made adaptors which formed a duplex containing a phosphorylated blunt end and a non-phosphorylated EcoRI overhang were used (Bahl et al., 1978; Rothstein et.ai., 1979). Use of synthetic oligomers for cloning the blunt end pea root cDNA fragments involved two ligation reactions. The first was the ligation of the adaptors to the fragments to be cloned. The second was the ligation of these tailored fragments to plasmids pUC19 vectors. The last step before ligation to the vector molecules was the removal of excess adaptors by gel chromatography. Errors might arise from these processes.

The joining of the blunt-ended newly synthesized cDNAs with the blunt ends of the adaptors utilized T_4 DNA ligase. The latter has the ability to link DNA molecules possessing blunt termini (Sgaramella et al., 1970). However, this "flush-end" reaction was several orders of magnitude less efficient than "cohesive-end" joining (Sugino et al., 1977). If the concentration of ATP in the ligation buffer was high, accumulation of 5'-adenylated intermediates (Sgaramella and Khorana 1972) with consequent low yield of recombinant molecules would happen. In addition, the T_4 enzyme could carry out additional reactions such as the sealing of gaps in duplex DNA (Nilsson and Magnusson 1982) and its low substrate specificity could lead to the generation of unexpected recombinants.

Besides, DNA polymerase I was able to manipulate the DNA sequence at a junction generated during the end-to-end joining of two DNA fragments. With the

presence of deoxynucleoside triphosphates (dNTPs), particular ends could be filled-in to different extents (Donoghue and Hunter 1982). Restricted termini with DNA polymerase I in the presence of dNTPs could ligate to generate site(s) of different specificity in which no longer recognized by a specific restriction enzyme. Since both T_4 DNA ligase and DNA polymerase I were used during the cDNA synthesis, incomplete inactivation or inefficient removal of dNTPs, DNA polymerase I as well as T_4 DNA ligase by phenol/chloroform and spun column purification might be the root of the problem. As well as the inefficient blunt-end joining between adaptor molecules and cDNAs, some cDNAs could be left without ligating with adaptors. Extremities of the EcoRI restricted vectors which have been filled-in by DNA polymerase I treatment could be ligated to the flush-end termini of cDNAs to generate a different restriction recognition sequence which was not able to be identified by EcoRI. Moreover, 20-50 fold molar excess of adaptor molecules employed to link with target cDNA molecules might not be completely removed by gel filtration, these remaining adaptors could compete with them in subsequent steps. This enhance the potential of defined sequence oligonucleotides to introduce specific alteration into a DNA sequence totally devoid of restriction sites and the chance could not be underestimated.

Recombination systems of both host and vector origin might produce sequence arrangements during initial plating or amplification. Apparent sequence rearrangements due to reverse transcriptase artefacts have also been reported (O' Hare et al., 1979).

In some cases, loops at the 3' ends of the first strands could remain due to the tendency of reverse transcriptase to aid the formation of second strands dur-

ing cDNA synthesis. These loops could be used by the DNA polymerase I as primers for second strands synthesis, resulting in molecules that were covalently closed at the ends corresponding to the 5' ends of the original mRNAs (Gubler 1987). Since the RNase H replacement strategy for second strand synthesis did not involve any nuclease step, such hairpin molecules could not accept adaptors at both ends and consequently could not be cloned into the vectors. Moreover, the exonuclease functions of DNA polymerase I has always been overlooked. Meanwhile, reverse transcriptase associated with RNase H which could cleave RNA in heteroduplex molecules also equipped with degradative activities. These enzymes could displace both DNA and RNA from a template during synthesis of the second strand. To complicate matters further, certain properties of the enzymes such as lack of possessivity, and properties of the template, such as secondary structure and sequences that were difficult for enzyme to traverse, contributed to the production of incomplete chains; the cDNAs remain nicked or gapped. The end result was a plethore of both single-stranded and double-stranded fragments which could interfere with the cloning of full-length molecules.

Another possibility that created the odd restriction results could be the relaxed specificity and site preference of EcoRI. It has been recognized for many years that restriction enzymes would relax their specificity under certain conditions. EcoRI cleaved GAATTC at pH 7.3 and 100mM NaCl in the presence of 5mM $MgCl_2$ but raising the pH or lowering the NaCl concentration (Polinsky et al., 1975) or substituting Mn^{2+} for Mg^{2+} (Hsu and Berg 1978) or adding organic solvents (Malyguine et al., 1980) tended to reduce their specificity. Inhibition of enzymatic activity and sites which differed in sequence at one or two positions from the

canonical sequences were cleaved (Woodbury et al., 1980). Thus restriction errors caused by the use of poor quality restriction buffer might have occurred. An increase in the number of bands seen on the gel after EcoRI restriction could be an evidence of such "star" activity. On the other hand, methylation of DNA sequences on restriction sites that prevent cleavage by enzymes might occurred after transformation. Although eukaryotes were not generally thought to possess restriction-modification systems, certain yeasts have been shown to possess site-specific endodeoxyribonucleases (Watabe et al., 1981) and site-specific methylation was to be anticipated. This could occur in the competent cells used in the project (Lathe et al., 1983, table 6).

Restriction enzymes almost certainly binded non-specifically to DNA as well as to their recognition sequence (Woodhead and Malcolm 1980a) and this could usually results in inhibition of activity. This related to the molecular properties of restriction endonucleases which were important to their site-specific cleavage capabilities. The positively charged chains of lysine and arginine seemed to be important for the hydrolytic activity and/or DNA binding in EcoRI (Woodhead and Malcolm 1980a). The activity of EcoRI depended on a uniquely reactive carboxyl side chain (Woodhead and Malcolm 1980b) and this was interesting in view of the strong interaction between guanine and carboxyl groups (Lancelot and Helene 1977). Therefore, a molecular alteration on side chain could affect the performance of restriction enzyme and its specificity.

All these factors contributed to the low yielding of cDNA and created cloning problem. Subsequently, sequence rearrangement or alteration as well as the efficiency of transformation might be affected so that unexpectable restriction pat-

terns were shown.

4.8 Isolation of Full-Length cDNA Clones Using pea Root-Specific Probes

Three root-specific genes used to isolate the full-length pea root cDNA clones were mentioned in section 1.3.5 and 2.2.7. These genes were probably first clones isolated from a library screen which were partial cDNA clone. Once such particular cDNA clones have been isolated, they in turn could be used as probes to screen the library for longer cDNA clones. Using this rescreening stepwise approach, full-length cDNA which contained the entire coding region from a large cDNA library could be isolated. This principle was employed to isolate full-length cDNA which contained pea root-specific gene so that subsequent analysis could be carried on (see section 1.7).

After the partial root-specific cDNA clones were isolated and hybridized with pea root cDNAs, autoradiographs were taken and corresponding colonies were identified. Plasmid DNA minipreparations were done and later restricted with EcoRI (Fig. 3.41 and 3.42). Similarly, the restriction patterns shown were unexpectable as those which had done before. Possible reasons have been discussed in section 4.7.

The intensity of dark spots on autoradiographs (not shown here) were lower than the one done before using pea root cDNA as probe (synthesized in the project). This might due to the depreciation of master filters resolution after several preparation. This caused the probes hybridized weaker to the replica filters so produced. In addition, storage in the refrigerator might result in the plates

becoming contaminated, a process that would destroyed the library.

Since the probes used were excised from plasmid recombinants with a restriction endonuclease EcoRI and gel purified to remove the plasmid sequences, absolute separation between plasmids vector and the DNA inserts might not be attained. The inserts would be contaminated with plasmids sequences which would also be radiolabelled. As the pea root cDNA library constructed with similar pUC19 plasmid vectors was screened with such probes, all the colonies in the library would hybridize to the probe or to the contaminants or to both. A large irrelevant colony might hybridized better, by virtue of the large amount of the desired recombinant plasmid and hence contributed to errors.

Probe pPR179 hybridized much stronger to the corresponding cDNA clone than the other two probes pPR287(A) and pPR340. From Fig. 3.41, only sample no. 1 contained the pUC19 vector and a DNA insert while sample no. 5 was unrestricted vector with no insert in. Restriction patterns after electrophoresis probed by pPR287(A) and pPR340 inserts were shown in Fig. 3.42. Sample no. 4 contained no cDNA insert but only EcoRI restricted pUC19 vector. Sample no. 8 and 10 were purely unrestricted/partially restricted pUC19. All the remainings were virtually a mixture of cDNA alone and/or ligated with DNA fragment (pUC19 vector?). Contaminant DNAs ligated with the inserts were not impossible.

Conclusively, the enzymatic conversion of mRNA into double-stranded cDNAs, their insertion into appropriate vectors, transformation and subsequent screening were in sum a difficult and inefficient process. Hundreds of criteria and factors were affecting the formation of a representative library as well as the isolation of

specific clone(s) which we were interested in. Careful manipulation during the processes, use of high quality materials and choice of suitable protocols to follow were all of paramount important. These parameters should often be balanced in order to obtain satisfactory results though they were not guaranteed unless one has got his promising results on hands.

SUMMARY

1. A complete pea root cDNA library was constructed by using poly(A)$^+$ RNA purified from pea roots total RNA.

2. Guanidinium/Cesium chloride extraction method was proved to be effective in isolating total pea root RNA from the tissues while hot SDS/proteinase K method was not suitable.

3. Oligo-d(T) cellulose affinity chromatography for pea root poly(A)$^+$ RNA purification was not satisfactory. Other methods, like poly (U)-sepharose chromatography, were suggested.

4. After pea root cDNA synthesis and cloning into dephosphorylated pUC19 vectors, *E. coli.* competent cells transformation, colony hybridization and autoradiography, pea root-abundant cDNA clones were isolated.

5. Selected cDNA-pUC19 recombinant clones were restricted with restriction endonuclease(s) (e.g. EcoRI, BamHI) to assess the cDNA inserts size.

6. Pea root-specific partial DNAs were also purified from plasmid recombinants which have been constructed previously and radiolabelled to function as nucleic acid probes for full-length cDNA clones isolation from the library. Restriction patterns shown after EcoRI digestion revealed that some artefactual cDNAs were synthesized.

7. Possible reasons included: errors during the processes of enzymatic cDNA synthesis; the ligation among cDNAs, EcoRI adaptors, and pUC19 plasmid vectors; relaxed specificity of restriction endonuclease as well as methylation.

– END –

LIST OF REFERENCES

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1. Amersham Nucleic Acids Hybridization Bulletin. (1985). Amersham International plc.

2. Arrand, J. E. (1986). Preparation of nucleic acid probes. In *Nucleic Acid Hybridization: A Practical Approach*, Hames, B. D. and S. J. Higgins (eds.), pp. 17-44, IRL Press, Oxford.

3. Auffray, C. and F. Rougeon. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumour RNA. *Eur. J. Biochem.* **107,** 303-314.

4. Aviv, H. and P. Leder. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA.* **69,** 1408-1412.

5. Bahl, C. P. and R. Wu. (1978). Cloned seventeen-nucleotide-long synthetic lactose operator is biologically active. *Gene* **3,** 123-134.

6. Bahl, C. P., R. Wu, R. Brousseau, A. K. Sood, H. M. Hsiung and S. Narang. (1978). Chemical synthesis of versatile adaptors for molecular cloning. *Biochem. Biophys. Res. Comm.* **81,** 695-703.

7. Barton, K. A. and W. J. Brill. (1983). Prospects in plant genetic engineering. *Science* **219,** 671-676.

8. Bingham, J. (1981). The achievements of conventional plant breeding. *Philo.*

Trans. R. Soc. Lond.[*Biol.*] **293,** 441-455.

9. Birnboim, H. C. and J. Doly. (1979), A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7,** 1513-1523.

10. Bolivar, F., R. L., Rodriguez, P. J. Greene, M. V. Betloch, H. L. Heynecker, M. W. Boyer, J. H. Crosa, S. Falkow. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2,** 95-113.

11. Bostian, K. A., R. C. Lee, and H. O. Harvolson. (1979). Preparative fractionation of nucleic acids by agarose gel electrophoresis. *Anal. Biochem.* **95,** 174-182.

12. Brawerman, G., J. Mendecki, and S.Y.Lee. (1972). A procedure for the isolation of mammalian messenger ribonucleic acid. *Biochemistry* **11,** 637-641.

13. Brigati, D. J., D. Myerson, J. J. Leary, B. Spalholz, S. Z. Travis, C. K. Y. Fong,G. D. Hsiung and D. C. Ward. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridization probes. *Virology* **126,** 32-50.

14. Broome, S. and W. Gilbert. (1978). Immunological screening method to detect specific translation products. *Proc. Natl. Acad. Sci. USA.* **75,** 2746-2749.

15. Brown, J. W. S., D. R. Ersland and T. C. Hall. (1982). Molecular aspects of storage protein synthesis during seed development. In *The Physiology and Biochemistry of Seed Development, Dormancy and Germination.* Khan, A. A. (ed.), pp. 3-42. Elsevier, The Hague.

16. Buell, G. N., M. P. Wickens, F. Payvar and R. T. Schimke. (1978). Synthesis of full-length cDNAs from four partially purified oviduct mRNAs. *J. Biol. Chem.* **253,** 2471-2482.

17. Burr; H. K. (1975). In *Protein Nutritional Quality of Foods and Feeds, Vol. I,* Friedman, M. (ed.), pp. 119-34. Marcel Dekker, New York.

18. Cathala, G., J. -F. Savouret, B. Mendez, B. L. West, M. Karin, J. A. Martial and J. D. Baxter. (1983). *DNA* **2,** 329.

19. Catterall, J. F., J. P. Stein, E. C. Lau, S. L. C. Woo, A. Dugaiczyk, M. L. Mace, A. R. Means and O'Malley. (1979). The chick ovomucoid gene contains at least six intervening sequences. *Nature* **278,** 323-327.

20. Chaconas, G., J. H. van de Sande. (1980). 5'-^{32}P labelling of RNA and DNA restriction fragments. *Methods in Enzymology* **65,** 75-85.

21. Chang, A. C. Y., J. H. Nunberg, R. K. Kaufman, H. A. Ehrlich, R. T. Schimke and S. N. Cohen. (1978). Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase. *Nature* **275,** 617-624.

22. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald and W. J. Rutter. (1979). Isolation of biologically active ribonucleic acid from source enriched in ribonuclease. *Biochemistry* **18,** 5294-5298.

23. Cocking, E. C. and M .R. Davey. (1987). Gene transfer in cereals. *Science* **236,** 1259-1262.

24. Cocking, E. C., M. R. Davey, D. Pental and J. B. Powe. (1981). Aspects in

plant genetic manipulation. *Nature* **293,** 265-269.

25. Cox, R. A. (1968). The use of guanidinium chloride in the isolation of nucleic acids. *Methods in Enzymology.* **12B,** 120-128.

26. Craig, R. K., L. Hall, D. Parker and P. N. Campbell. (1981). The construction identification and partial characterization of plasmids containing guinea-pig milk protein complementary DNA sequences. *Biochem. J.* **194,** 989-998.

27. Craig, R. K., P. A. Brown, O. S. Harrison, D. McIlreavy and P. N. Campbell. (1976). Isolation and characterization of messenger ribonucleic acids from lactating mammary gland and identification of caseins and pre-α-lactalbumin as translation products in heterologous cell-free systems. *Biochem. J.* **160,** 57-74.

28. Dahl, H. H., R. A. Fravell and F. G. Grosveld. (1981). The use of genomic libraries for the isolation and study of eukaryotic genes. In *Genetic Engineering Vol. 2.*, Williamson, R. (ed.). pp. 49-127. Academic Press. New York.

29. Davies, R. D. (1976). Peas. In *Evolution of Crop Plants* Simmonds, E. D. (ed.), pp. 172-174. Longman Group Limited, U.K.

30. Davis, R. W., D. Botstein, J. R. Roth. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Habour, New York.

31. Day, P. R., J. A. Barret and M. S. Wolfe. (1983). The Evolution of the Host-Parasite Interaction. In *Genetic Engineering of Plants.* Kosuge, T., T. P. Meredith and E. Hollaender. (eds.), pp. 419-30. Plenum, New York.

32. Delauney, A. J. (1984). Cloning and characterization of cDNAs encoding the major pea storage proteins, and expression of vicilin in *E. coli*. Ph.D. Thesis. University of Durham.

33. Denhardt, D. (1966). *Biochem. Biophy. Res. Commun.* **23,** 641-645.

34. Derbyshire, E., D. J. Wright and D. Boulter. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochem.* **15,** 3-24.

35. Donoghue, D. J. and D. Hunter. (1982). A generalized method of subcloning DNA fragments by restriction site reconstruction: Application to sequencing the amino-terminal coding region of Gazdar murine sacroma virus. *Nucl. Acids Res.* **10,** 2549-2564.

36. Downie, J. A., Q. S. Ma, B. Wells, C. D. Knight, G. Hombrecher and A.W. B. Johnstone. (1984). The Nodulation Genes of *Rhizobium leguminosarum.* In *Advances in Nitrogen Fixation Research.* Veeger, C. and W. E. Newton. (eds.), pp. 678. Martinus Nijhoff, The Hague.

37. Eden, L., L. Heslinga, R. Klok, A. M. Ledeboer, J. Maat, M. Y. Toonen, C. Visser and C. T. Verrips. (1982). Cloning of cDNA encoding the sweet plant protein thaumatin and its expression in *E. coli*. *Gene* **18,** 1-12.

38. Edmonds, M., M. H. Vaughn, Jr. and H. Nakazato. (1971). Polyadenylic acid sequences in the heterlogous nuclear RNA and rapidly-labelled polyribosomal RNA of HeLa cells: Possible evidence for a percursor relationship. *Proc. Natl. Acad. Sci. USA.* **68,** 1336-1340.

39. Efstratiadis, A. and L. VillaKomaroff. (1979). Cloning of double-stranded

cDNA. In *Genetic Engineering Vol. 1.* Setlow, J. K. and A. Hollaender. (eds.), pp. 15-36. Plenum Press, New York.

40. Efstratiadis, A., F. C. Kafatos, A. M. Maxam and T. Maniatis. (1976). Enzymatic in vitro synthesis of globin genes. *Cell* **7,** 279-288.

41. Eggum, B. O. and R. M. Beames. (1983). In *Seed Proteins: Biochemistry, Genetics, Nutritive Value.* Gottschalk, W. and H. P. Muller (eds.), pp. 499-531. Martinus Nijhoff, The Hague.

42. Ehrenberg, H., I. Fedorcsak, F. Solymosy. (1976). Diethyl pyrocarbonate in nucleic acid research. *Prog. Nucl. Acids Res. Mol. Biol.* **16,** 189-262.

43. Engler, M. J. and C. C. Richardson. (1982). DNA ligases. In *The Enzymes, Vol. XV.* P. D. Boyer. (eds.), pp. 3-29. Academic Press, New York.

44. Evans, I. M., R. R. D. Croy, P. Brown and D. Boulter (1980). Synthesis of complementary DNAs to partially purified mRNAs coding for the storage proteins of *Pisum sativum. Biochim. Biophy. Acta* **610,** 81-95.

45. Feinberg, A. P. and B. Vogelstein. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6-13.

46. Feinberg, A. P. and B. Vogelstein. (1984). Addenum. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137,** 266-267.

47. Ferretti, L., V. Sgaramella (1981). Temperature dependence of the joining by

T_4 DNA ligase of termini produced by type II restriction endonucleases. *Nucl. Acids Res.* **9,** 85-93.

48. Forde, B. G. (1983). Molecular cloning of cDNA: Bacterial transformation and screening of transformants. In *Techniques in Molecular Biology.* Walker, J. M. and W. Gasstra. (eds.), pp. 221-238. Croom Helm, London.

49. Gardner, R. C., A. J. Howarth, J. Messing and R. J. Shepard. (1982). *DNA* **1,** 109.

50. Gatehouse, L. N. (1985). Construction of a cDNA library encoding pea seed proteins. M. Sc. Dissertation. University of Durham.

51. Gatehouse, J. A., R. R. D. Croy and D. Boulter (1984). The synthesis and structure of pea storage proteins. In *Critical Reviews in Plant Science, Vol. I,* pp. 287-314. CRC Press, Boca Raton, Fla.

52. Gatehouse, A. M. R. (1984a). In *Developments in Food Proteins, Vol. 13.* Hudson, B. J. F. (ed.), pp. 245-94. Elsevier Applied Science Publishers, London.

53. Gatehouse, A. M. R., J. A. Gatehouse., P. Dobie., A. M. Kilminster and D. Boulter. (1979). Biochemical basis of insect resistance in *Vigna unguiculata. J. Sci. Food Agric.* **30,** 948-958.

54. Gillespie, D. and D. Spiegelman. (1965). A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12,** 829-842.

55. Gillespie, D. (1968). The formation and detection of DNA-RNA hybrids.

Methods in Enzymology **12B,** 641-668.

56. Glisin, V., R. Crkvenjakov and C. Byus. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13,** 2633-2637.

57. Goodman, R. M., H, Hanptli., A. Crossway., V. C. Knauf. (1987). Gene transfer in crop improvement. *Science* **236,** 48-54.

58. Green, M. R., T. Maniatis and D. A. Melton. (1983). Human β-globin pre-mRNA synthesized in vitro is accurately spliced in *Xenopus* oocyte nuclei. *Cell* **32,** 681-694.

59. Grunstein, M. and D. Hogness. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA.* **72,** 3961-3965.

60. Gubler, U. and B. J. Hoffman. (1983). A simple and very efficient method for generating cDNA libraries . *Gene* **25,** 263-269.

61. Gubler, U. (1987). Second-strand cDNA synthesis: mRNA fragments as primers. *Methods in Enzymology* **152,** 330-334.

62. Gutschick, V. P. (1980). Energy Flows in the Nitrogen Cycle, Especially in Fixation. In *Nitrogen Fixation, Vol. I.* Newton, W. E. and W. H. Orme-Johnson. (eds.), pp. 17-27. University Park Press, Baltimore.

63. Hall, T. C., Y. Ma, B. U. Buchbinder, J. W. Pyne, S. M. Sun and S. A. Bliss. (1978). Messenger RNA for G1 protein of French bean seeds: Cell-free translation and product characterization. *Proc. Natl. Acad. Sci. USA.* **75,**

3196-3200.

64. Hanahan, D. and M. Meselson. (1980). Plasmid screening at high colony density. *Gene* **10,** 63-67.

65. Hanahan, D. (1983). Studies on transforming *E. coli.* with plasimds. *J. Mol. Biol.* **166,** 557-580.

66. Hanahan, D. and M. Meselson. (1983). Plasmid screening at high colony density. *Methods in Enzymology* **100,** 333-341.

67. Hanahan, D. (1986). Techniques for transformation of *E. coli.* In *DNA Cloning - A Practical Approach.* Glover, D. M., ed. Vol. 1, pp. 109-135. IRL Press, Oxford.

68. Heidecker, G. and J. Messing. (1983). Sequence analysis of zeins cDNAs obtained by an efficient mRNA cloning method. *Nuclei Acids Res.* **11,** 4891-4905.

69. Helling, R. B., H. M. Goodman and H. W. Boyer. (1974). Analysis of R. *E. coli* fragments of DNA from lamboid bacteriophage and ofter viruses by agarose gel electrophoresis. *J. Virol.* **14,** 1235-1244.

70. Heynecker, H. L., J. Shine, H. Goodman, H. W. Boyer, J. Rosenberg, R. E. Dickerson, S. A. Narang, K. Itakura, S. Y. Lin and A. Riggs. (1976). Synthetic *lac* operator DNA is functional in vivo. *Nature* **263,** 748-752,

71. Higgins, T. J. V. (1984). Synthesis and regulation of major proteins in seeds. *Ann. Rev. Plant Physiol.* **35,** 191-221.

72. Higgs, D. R., S. E. Y. Goodbourn, J. Lamb, J. B. Clegg, D. J. Weatherall and N. J. Proudfoot. (1983). α-thalassaemia caused by a polyadenylation signal mutation. *Nature* **306,** 398-400.

73. Higuchi, R., G. V. Paddock, R. Wall and W. Salser. (1976). A general method for cloning eukaryotic structual gene sequences. *Proc. Natl. Acad. Sci. USA.* **73,** 3146-3150.

74. Holmes, D. S. and M. Quigley. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114,** 193-197.

75. Howells, R. D., D. L. Kilpatrick, R. Bhatt, J. J. Monahan, M. Poonian and S. Udenfriend. (1984). Molecular cloning and sequence determination of rat preproenkephalin cDNA: Sensitive probe for studying transcriptional changes in rat tissues. *Proc. Natl. Acad. Sci. USA.* **81,** 7651-7655.

76. Hsu, M. T. and P. Berg. (1978). *Biochemistry* **17,** 131.

77. Ish-Horowicz, D. and J. F. Burke. (1981). Rapid and efficient cosmid vector cloning. *Nucl. Acids Res.* **9,** 2989-2998.

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

79. Jackson, D. A., R. H. Symons and P. Berg. (1972). Biochemical method for inserting new genetic information into DNA of Simian Virus 40: Circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **69,** 2904-2909.

80. Jones, M. G. K. (1986). Applications of Genetic Engineering to Agriculture. In *Plant Genetic Engineering.* Dodds, J. H. (ed.), pp. 269-295. Cambridge University Press, Cambridge.

81. Kaufmann, Y., C. Milcarek, H. Berissi and S. Penman. (1977). HeLa cell poly(A)-mRNA codes for a subset of poly(A)$^+$ mRNA-directed proteins with an actin as a major product. *Proc. Natl. Acad. Sci. USA.* **74,** 4801-4805.

82. Kimmel, A. R. and S. L. Berger. (1987). Preparation of cDNA and the generation of cDNA libraries. it Methods in Enzymology, **152,** 307-315.

83. Kreuzaler, F., H. Ragg, E. Fautz, D. N. Kuhn and K. Hahlbrock. (1983). UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinium hortense. Proc. Natl. Acad. Sci. USA.* **80,** 2591-2593.

84. Krug, M. S. and S. L. Berger. (1987). Determination of the molar concentration of messenger RNA. *Methods in Enzymology* **152,** 262-266.

85. Kurtz, D. T. and C. F. Nicodemus (1981). Cloning of $\alpha 2\mu$ globulin DNA using a high efficiency technique for the cloning of trace messenger RNAs. *Gene* **13,** 145-152.

86. Lancelot, G. and C. Helene. (1977). Selective recognition of nucleic acids by proteins: The specificity of guanine interaction with carboxylate ions. *Proc. Natl. Acad. Sci. USA.* **74,** 4872-4875.

87. Land, H., M. Grey, H. Hanser, W. Lindenmaier and G. Schutz. (1981). 5'-terminal sequences of eukaryotic mRNA can be cloned with high efficiency. *Nucl. Acids Res.* **9,** 2251-2266.

88. Langer, P. R., A. A. Waldrop and D. C. Ward. (1981a). Enzymatic synthesis of biotin-labelled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA.* **78,** 6633-6637.

89. Larkins, B. A. (1983). Genetic engineering of seed storage proteins. In *Genetic Engineering of Plants.* Kosuge, T., C. P. Meredith and A. Hollaender. (eds.), pp. 93-118. Plenum Press, New York.

90. Lathe, R. F., J. P. Lecocq and R. Everett. (1983). DNA engineering: The use of enzymes, chemicals and oligonucleotides to restructure DNA sequences in vitro. *Genetic Engineering, Vol. 4.* Williamson, R. (ed.), pp. 1-56. Academic Press, New York.

91. Leary, J. J., D. J. Brigati and D. C. Ward. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proc. Natl. Acad. Sci. USA.* **80,** 4045-4049.

92. Leis, J. P., I. Berkower, J. Hurwitz. (1973). Mechanism of action of ribonuclease H isolated from avian myeloblastosis virus and *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **70,** 466-470.

93. Lindberg, U. and T. Persson. (1974). Messenger RNA. Isolation with poly(U) agarose. *Methods in Enzymology* **34,** 496-499.

94. Lobban, P. E. and A. D. Kaiser. (1973). Enzymatic end-to-end joining of DNA molecules. *J. Mol. Biol.* **78,** 453-472.

95. Malyguine, E., P. Vannier and P. Yot. (1980). *Gene* **8,** 163.

96. Maniatis, T., E. F. Fritsch and J. Sambrook. (1982). *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.

97. Mantell, S. H., J. A. Matthews and R. A. McKee. (1985). Cultural Tools and Techniques. In *Principles of Plant Biotechnology: An Introduction to Genetic Engineering in Plants*. pp. 89-129. Blackwell Scientific Publications, Oxford.

98. Martin, P. A. W. and D. H. Dean (1981). In *Microbial Control of Pests and Plant Diseases*. Burgess, H. P. (ed.). Academic Press, London.

99. McDonell, M. W., M. N. Simon and F. W. Studier. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* **110,** 119-146.

100. McMaster, G. and G. Carmichael. (1977). Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA*. **74,** 4835-4838.

101. Meinkoth, J. and G. Wahl. (1984). Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138,** 267-284.

102. Michelson, A. M. and S. H. Orkin. (1982). Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase. Implications for the cloning of cDNA. *J. Biol. Chem.* **256,** 1473-1482.

103. Miller, K. (1987). RNA gel electrophoresis. *Mol. Bio. Res. and Dev. Bulletin, Vol. 9, No. 3.* pp. 14-15. Bethesda Research Laboratories.

104. Montell, C., E. F. Fisher, M. H. Caruthers and A. J. Berk. (1983). Inhibition

of RNA cleavage but not polyadenylation by a point mutation in mRNA 3' consensus sequence AAUAAA. *Nature* **305,** 600-605.

105. Myers, J. C., F. Ramirez, D. L. Kacian, M. Flood and S.Spiegelman. (1980). A simple purification of avian myeloblastosis virus reverse transcriptase for full-length transcription of 35S RNA. *Anal Biochem* **101,** 88- .

106. Nilsson, S. V. and G. Magnusson. (1982). Sealing of gaps in duplex DNA by T_4 DNA ligase. *Nucl. Acids. Res.* **10,** 1425-1473.

107. Norrander, J., T. Kempe and J. Messing. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26,** 101-106.

108. Nudel, U., H. Soreq, U. Z. Littauer, G. Marbaix, G. Huez, M. Leclerq, E. Hubert and H. Chantrenne. (1976). Globin mRNA species containing poly(A) segments of different length. Their functional stability in *Xenopus* oocytes. *Eur. J. Biochem.* **64,** 115-122.

109. Nyegaard, A. D. and B. D. Hall. (1963). *Biochem. Biophy. Res. Commun.* **12,** 98.

110. Nyegaard, A. D. and B. D. Hall. (1964). Formation and properties of RNA-DNA complexes. *J. Mol. Biol.* **9,** 125-142.

111. Ogden, R. C. and D. A. Adams. (1987). Electrophoresis in agarose and acrylamide gels. *Methods in Enzymology.* **152,** 61-87.

112. O'Hare, K., R. Breathnach, C. Benoist and P. Chambon. (1979). *Nucl. Acids*

Res. **7**, 321.

113. Okayama, H. and P. Berg. (1982). High-efficiency cloning of full-length cDNA. *Mol. Cell Biol.* **2**, 161-170.

114. Old, R. W. and S. B. Primrose. (1986). Cloning in plant cells. In *Principle of Gene Manipulation: An Introduction to Genetic Engineering.* pp. 203-230. Blackwell Scientific Publications, London.

115. Palatnik, C. M., R. V. Storti, A. K. Capone and A. Jacboson. (1980). Messenger RNA stability in *Dictyostelium discoidenum*: Does poly(A) have a regulatory role? *J. Mol. Biol.* **141**, 99-118.

116. Palatnik, C. M. R. V. Storti and A. Jacboson. (1979). Fractionation and functional analysis of newly synthesized and decaying messenger RNAs from vegetative cells of *Dictyostelium discoidenum. J. Mol. Biol.* **128**, 371-395.

117. Payne, P. I. (1983). In *Seed Proteins.* Daussant, J., J. Mosse and J. Vaughan. (eds.). pp. 223-53. Academic Press, New York.

118. Payne, P. I. and Rhodes, A. P. (1982). In *Encyclopedia of Plant Physiology, Vol. 14A, Nuclei Acids and Proteins in Plants. I. Structure, Biochemistry and Physiology of Proteins.* Boulter, D. and B. Parthier (eds.), pp. 346-65. Springer-Verlag, Berlin.

119. Pharmacia cDNA Synthesis Kit In- structions. (1985). Pharmacia LKB Biotechnology AB.

120. Polinsky, B., B. Greene, D. E. Garfin, B. J. McCarty, H. M. Goodman and H.

W. Boyer. (1975). Specificity of substrate recognition by the EcoRI restriction endonuclease. *Proc. Natl. Acad. Sci. USA.* **72,** 3310-3314.

121. Proudfoot, N. J. (1982). The end of the messenger. *Nature* **298,** 516-517.

122. Pusztai, A., R. R. D. Croy, G. Grant, J. C. Stewart. (1983). In *Seed Proteins.* Daussant, J., J. Mosse and J. Vaughan (eds.), pp. 53-82. Academic Press, New York.

123. Ratzkin, B. and J. Carbon. (1977). Functional expression of cloned yeast DNA in *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **74,** 487-491.

124. Retzel, E. F., M. S. Collet and A. J. Faras. (1980). Enzymatic synthesis of deoxyribonucleic acid by the avian retrovirus reverse transcriptase in vitro: Optimum conditions required for transcription of large ribonucleic acid templates. *Biochemistry* **19,** 513-517.

125. Rosenthal, E. T., T. R. Tansey and J. V. Ruderman. (1983). Sequence-specific adenylations and deadenylations accompany changes in the translation of maternal messenger RNA after fertilization of *Spisula* oocytes. *J. Mol. Biol.* **166.** 309-328.

126. Rothstein, R. J., L. F. Lau, C. P. Bahl, S. A. Narang and R. Wu. (1979). Synthetic adaptors for cloning DNA. *Methods in Enzymology* **68,** 98-109.

127. Schneider, C., M. J. Owen, D. Banville, J. G. Williams. (1984). Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature* **311,** 675-678.

128. Schoffl, F. and J. L. Key. (1983). Identification of a multigene family for small heat shock proteins in soybean and physical characterization of one individual coding region. *Pl. Mol. Biol.* **2,** 269-278.

129. Sela, M., C. B. Anfinsen and W. F. Harrington. (1957). The correlation of ribonuclease activity with specific aspects of teritary structure. *Biochim. Biophys Acta* **26,** 506-510.

130. Sgaramella, V. and H. G. Khorana. (1972). Total synthesis of the structual gene for an alinine transfer RNA from yeast; enzymic joining of the chemically synthesized polydeoxynucleotides to form the DNA duplex representing nucleotide sequence 1 to 20. *J. Mol. Biol.* **72,** 427-444.

131. Sgaramella, V., J. H. Van de Sande and H. G. Khorana. (1970). Studies on polynucleotides, C. A novel joining reaction catalyzed by the T_4-polynucleotide ligase. *Proc. Natl. Acad. Sci. USA.* **67,** 1468-1475.

132. Sharp, P. A., B. Sugden and J. Sambrook. (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose. *Biochemistry* **12,** 3055-3058.

133. Shaw, C. H. (1984). Plant biotechnology. Genetic engineering of crop plants: a strategy for the future, and the present. *Chemistry and Industry* **December,** 817-824.

134. Sonenshein, G. E., T. E. Geoghegan and G. Brawerman. (1976). A major species of mammalian messenger RNA lacking a polyadenylate segment. *Proc. Natl. Acad. Sci. USA.* **73,** 3088-3092.

135. Sorenson, J. C. (1984). The structure and expression of nuclear genes in higher plants. *Adv. Genet.* **22,** 109-144.

136. Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503-518.

137. Sugino, A., H. M. Goodman, H. L. Heynecker, J. Shine, H. W. Boyer and N. R. Cozzarelli. (1977). Interaction of bacteriophage T_4 RNA and DNA ligases in joining of duplex DNA at base-paird ends. *J. Biol. Chem.* **252,** 3987-3994.

138. Sutcliffe, J. G. (1978). pBR322 restriction map marked from the DNA sequence: Accurate DNA size markers up to 4361 nucleotide pairs long. *Nucl. Acids Res.* **5,** 2721-2728.

139. Taylor, J. M. (1979). The isolaion of eukaryotic messenger RNA. *Ann. Rev. Biochem.* **48,** 681-717.

140. Thayer, R. E. (1979). *Anal. Biochem.* **98,** 60.

141. Thorne, H. V. (1966).Electrophoretic separation of polyoma virus DNA from host cell DNA. *Virology* **29,** 234-238.

142. Thorne, H. V. (1967). Electrophoretic characterization and fractionation of polyoma virus DNA. *J. Mol. Biol.* **24,** 203-212.

143. Tilghman, S. M., P. J. Curtis, D. C. Tiemeier, P. Leder and C. Weissmann. (1978). The intervening sequence of a mouse β-globin gene is transcribed within the 15S β-globin mRNA precursor. *Proc. Natl. Acad. Sci. USA.* **75,** 1309-1313.

144. Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rutter, H. M. Goodman. (1977). Rat insulin genes: construction of plasmids containing the coding sequences. *Science* **196,** 1313-1319.

145. Van Loon, L. C. (1980). Regulation of Changes in Proteins and Enzymes Associated with Active Defense Against Virus Infection. In *Active Defense Mechanisms in Plants.* Wood, R. K. S. (ed.), pp. 247-73. Plenum Press, New York.

146. Vieira, J. and J. Messing. (1982). The pUC plasmids, an M-13mp7- derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19,** 259-273.

147. VillaKomaroff, L., A. Efstratiadis, S. Broome, P. Lomedico, R. Tizard, S. P. Naber, W. L. Chick and W. Gilbert. (1978). A bacterial clone synthesizing proinsulin. *Proc. Natl. Acad. Sci. USA.* **75,** 3727-3731.

148. Wahl, G. M., S. L. Berger and A. R. Kimmel. (1987). Molecular hybridization of immobilized nucleic acids: Theroratical concepts and practical considerations. *Methods in Enzymology* **152,** 399-407.

149. Watabe, H. O., T. Shibata, T. Ando. (1981). Site-specific endo-deoxyribonucleases in eukaryotes: endonucleases of yeasts, *Saccharomyces* and *Pichia. J. Biochem* **90,** 1623-1632.

150. Watson, C. J. and J. F. Jackson. (1985). An Alternative Procedure for the Synthesis of Double-stranded cDNA for Cloning in Phage and PLasmid Vectors. In *DNA Cloning: A Practical Approach, Vol. 1.* Glover, D. M. (ed.), pp.

79-88. IRL Press, London.

151. Weiss, B., A. Thompson and C. C. Richard- son (1968). Enzymatic breakage and joining of deoxyribonucleic acids. *J. Biol. Chem.* **243,** 4556-4563.

152. White, R. F. and J.F. Antoniw (1983). Direct control of virus disease. *Crop Protection* **2,** 259-271.

153. Wickens, M. P., G. N. Buell and R. T. Schimke. (1980). Synthesis of double-stranded DNA complementary to lysozyme, ovomucoid, and ovalbumin mRNAs. *J. Biol. Chem.* **253,** 2483-2495.

154. Williams, J. G. (1981).The preparation and screening of a cDNA clone bank. In *Genetic Engineering, Vol. 1.* Williamsom, R. (ed.), pp. 1-59. Academic Press, New York.

155. Woodbury, C. P., O. Hagenbuchle and P. H. Von Hippel. (1980). DNA site recognition and reduced specificity of the EcoRI endonuclease. *J. Biol. Chem.* **255,** 11534-11546.

156. Woodhead, J. L. and A. D. B. Malcolm (1980a). *Nucl. Acids Res.* **8,** 389-402.

157. Woodhead, J. L., N. Bhave and A. D. B. Malcolm. (1980b). Cation dependence of restriction endonuclease EcoRI activity. *Eur. J. Biochem.* **115,** 293-296.

158. Wu, R., C. P. Bahl and S. A. Narang (1978). Synthetic oligodeoxynucleotides for analyses of DNA structure and function. *Prog. Nucl. Acids Rec. Mol. Biol.* **21,** 101-141.

159. Yang, R. C. -A., J. Lis, and R. Wu. (1979). Elution of DNA from agarose gels after electrophoresis. *Methods in Enzymology* **68,** 176-183.

160. Young, R. A. and R. W. Davis. (1983). Efficient isolation of genes by using antibody probes. *Proc Natl. Acad. Sci. USA*. **80,** 1194-1198.