An attempt to find cDNA clones for pea pod extensin and pea pod specific protein

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AN ATTEMPT TO FIND cDNA CLONES FOR PEA POD EXTENSIN AND PEA POD SPECIFIC PROTEIN

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ABSTRACT

Three trials have been made for the hybridization reaction between the total RNA extract prepared from the pod of pea (*Pisum sativum* L.) and the probe prepared from a clone for a *Brassica* root protein homologous to carrot extensin, no sign of any hybridization could be seen; this shows that there is no similar sequence between those two.

Another attempt was done to identify a pea pod specific clone from the cDNA library of pea pod by the use of the method of plus and minus screening technique. Two clones (pPP1052 & pPP954) have been selected that hybridized strongly to pea pod RNA but not that of pea leaf. One of them (pPP1052) has been successfully cloned into M13mp18 and sequenced by Sanger method.

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Dedicated to my family for heartily support and encouragement.

ABBREVIATIONS

The abbreviations used thoughout the dissertation were based on those that recommended by the Biochemical Society in the Biochemical Journal "Policy of the Journal and Instruction to Authors", volume 209 (1983), pp 1-27. Notations that had been used but not listed in the list were given below:

bp: base pairs

cDNA: complementary DNA

BSA: bovine serum albumin

GRP: glycine-rich protein

HPRG: hydroxyproline-rich glycoprotein

IPTG: isopropyl β-D-thiogalactoside

PEG: polyethylene glycol

SDS: sodium dodecyl sulphate

SSC: saline sodium citrate

TEMED: N,N,N',N'-tetramethylethylenediamine

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1.1 General Introduction

Pisum sativum L. (2n=14), was the familiar green pea, constitutes one of the four most important seed legumes. It is grown mostly in cool countries, but probably originated in central or western Asia. Originally, peas seem to have been consumed exclusively in the dry or mature stage as in 'split pea' soup in the USA, but in recent centuries varieties grown as sweet or green peas have been given much attention. Most of the pea crop, is now produced commercially for fresh or green peas, which are canned or frozen. The plants are well adapted to cool spring weather or climate with reasonably cool summer. As such, this crop provided an important source of protein for human consumption (Janick et al., 1974).

The fruit of pea is composed of a single ovary. In matured fruit, the seeds are housed in a pod composed of ovary wall. When the enclosed seed is fully developed, the pod is dry and is made up of non-living sclerenchyma cells with lignified or suberized walls. Dehiscence of the seed occurs by separation of the two sutures in the pod (Holman, 1939).

Despite the nutritive value of pea, the crop produced from conventional inbreeding of high yielding varieties is more susceptible to major disease outbreaks. Nonetheless, with the advent of genetic engineering, that provides the ability to change the genetic makeup of the organism, a powerful new mean for investigation of all the aspects of plant function at the molecular level is provided. The full potential of the plant's own defence mechanism against disease can be explored and exploited.

1.2 Importance of Cell Wall

One of the differences that distinguish the cells of plant from those

of animal is the presence of the extraprotoplasmic wall in the cells of plants. The cell wall is a complex structure, and is cell-specific in its composition, so that each particular cell-type in the plant has a wall specifically adapted to its function, and related to the development of the given cell type. The cell walls of different cell types also have a quantitative differences in the composition of polysaccharides and other polymers. For example, epidermal cells have a layer of cutin as part of the wall, limiting the loss of water. Moreover, the cell wall is important in providing support to the whole plant through the collective mechanical strength of the individual cell wall, and in providing barrier against the invasion of disease.

As the cell wall is important in disease resistance and in protection against physical damage, a knowledge of how the cell wall functions is needed for the design of plants with increased resistance to disease and physical damage. Moreover, cell walls constitute a large fraction of the edible plants, and are therefore the major determinant of the character of dietary fibre (Hood, 1988). An idea of the structure of cell walls is needed for an understanding of the nutritional role of fibre.

1.3 Hydroxyproline-rich Proteins

In general, plant cell walls were comprised of cellulose, hemi-cellulose, pectic compounds, lignin, suberin, proteins, and water. For the portion of proteins, cell walls contain enzymes as well as structural proteins, for example, peroxidase in tobacco is associated with the cell wall (Lagrimini et al., 1987). Here, only the structural protein components of the wall are discussed. Lamport & Northcote in 1960 first identified a protein in the primary cell wall which contains a high proportion of hydroxyproline. So far, three classes of soluble hydroxyproline-rich glycoproteins have been found in higher plants. The first type is the

arabinogalactan proteins, which are acidic glycoproteins, composed mainly of serine, alanine, and hydroxyproline, which may function in cell-cell recognition, they are localized primarily in the extracellular matrix and are sometimes associated with the plasma membrane. A second type is the cell wall lectins, found only in the solanaceae family, which contain both a hydroxyproline/serine-rich and a cystein-rich domain and have been shown to increase upon wounding. The third type is the extensins, components of the primary cell wall, that may also become part of the insoluble matrix of the cell wall. In addition to their high content of hydroxyproline, another feature common to these three types of proteins is a high level of serine, and a repeating unit of tetrahydroxyproline-serine. However, up to now, only extensin has been well characterized.

1.3.1 Structure of Extensin

After the first discovery of a hydroxyproline-rich protein in plant, Lamport (1965) later proposed that this protein must be involved in cell extension. This led to the hydroxyproline-rich protein being given the name extensin. In 1967, by using partial alkaline hydrolysis of tomato cell wall, Lamport found that extensin has a polypeptide backbone with hydroxyproline residues containing *O*-glycosidic links to short oligo-arabinosides. He also shown that galactose is an additional sugar component of the glycoprotein (1969), and is attached *O*-glycosidically to serine residues (1973). This finding led to the proposal of the structure shown below for one of repeating units of the glycoprotein.

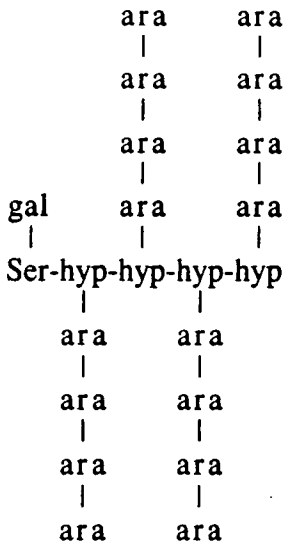

Diagram showing one of the repeating unit of extensin

He further suggested that as extensin peptides are so rich in hydroxyproline, the polypeptide may adopt the polyproline II helix conformation, stabilize by the arabinosides, which probably bond to the peptide backbone through hydrogen-bonds (1977). This results in a rigid rod-shaped molecule.

Liquid HF can be used to break the bonds of polysaccharides (by solvolysis), but not the protein. After the treatment of the cell wall with liquid HF, the cell wall could not be totally solubilized. As a result, Mort and Lamport (1977) proposed that extensin is actually forming a network semi-independent but interacting with that of cellulose. In this way, they contribute to the strength and rigidity of the cell wall.

The way that individual extensin molecule joins together was explained by Fry in 1982, he isolated a tyrosine dimer from cell walls which he called isodityrosine. It consists of two tyrosine units linked together by a diphenyl ether bridge. As a result, an interpeptide link, isodityrosine, is expected to form between pairs of tyrosine residues in extensin molecules that cause them to join together as a network.

1.3.2 Biosynthetic Pathway

The biosynthesis of extensin has been thoroughly investigated. The

general scheme of biosynthesis is given below.

The proline-rich polypeptide backbone is synthesized first (Stuart, Mozer & Varner, 1982), and then undergoes extensive post-translational modifications, namely hydroxylation of proline residues by the action of peptidylproline hydroxylase (Chrispeels, 1970), and glycosylation by arabinosyl transferase in the golgi apparatus (Gardiner & Chrispeels, 1975; Wienecke et al., 1982). The fully formed soluble glycoprotein is then secreted to the cell wall. Before it is insolubilized there, it can be extracted by salt solutions (Brysk & Chrispeels, 1972), and it has been suggested that the glycoprotein is ionically bound to pectin at this stage (Smith, Muldoon & Lamport, 1984). It has been also shown that proline hydroxylation and glycosylation are not required for secretion and insolubilization (Smith, 1981). However, secretion and even cross-linking of unglycosylated extensin does not necessarily mean that the protein is functioning properly in the wall (Stafstrom & Staehelin, 1986).

The glycoprotein is then bound in the cell wall by the formation of interpeptide isodityrosine links (Fry, 1982). The synthesis of this phenolic link between tyrosine residues is catalysed by peroxidase (Cooper & Varner, 1983), and the reaction is inhibited by acidic pH (Cooper & Varner, 1984). This has led to the suggestion that cross-linking can be regulated by auxin-induced hydrogen ion secretion.

When the deposition of labelled extensin in cell walls was studied, the extensin label was distributed quite uniformly across the cell wall but was absent from the expanded middle lamella at the interception of three or more cells, and was reduced in the narrow middle lamella between two cells, similar to the distribution of cellulose (Stafstrom and Staehelin, 1988). This indicated that extensin can not cross the middle lamella separating the walls of adjacent cells. The wall surrounding a given cell is synthesized by that cell alone, and cross-links probably do not form between extensin

molecules synthesised by adjacent cells, implying that the wall strengthening properties of the extensin matrix are confined to each individual cell. In addition, as extensin can be found across the entire wall, the newly synthesized extensin was added to the wall by intus-susception - this was, by the intercalation of new particles among those existing in the wall.

1.3.3 Functions of Extensin

Until recently, the insolubility of extensin has been the major obstacle to its study. Although, for some time, it has been accepted that extensin plays a role in controlling growth and is involved in disease resistance, the inability to extract this glycoprotein from the cell wall has restricted knowledge of its structure. Moreover, because of the presence of many imino acid residues and of many posttranslational modifications, it is very difficult to complete the sequence of extensin by protein chemical methods.

A better understanding of the composition, sequence and secondary conformation of extensin has only emerged since the discovery of soluble extensin precursors. These precursors can be readily eluted from the wall of carrot root explants or tomato cells with a salt solution (Smith et al., 1984; Stuart & Varner, 1980), or solubilied the hydroxyproline-rich protein (HRGP) from a cell homogenate of potato tubers or tobacco callus at a very acidic pH (Leach et al, 1982; Mellon & Helgeson, 1982).

More recently, information on extensin structure has came from studies of its biosynthetic intermediates at a stage even earlier than soluble precursors, mRNA and DNA. Part of the peptide sequence of extensin has been predicted from the RNA, and most recently, the DNA, which codes for this protein.

1.3.3.1 Structural Importances of Extensin

The distribution of extensin amongst different cell types is not uniform; for example, elevated levels of salt-extractable glycoprotein were found during the development of specialized tissues (Wilson and Fry, 1986). This led to the conclusion that extensin is expressed in a tissue-specific and developmental-specific manner.

During soybean seed development, extensin is localized and accumulated in cell walls of the palisade and hourglass cells (which represents the mechanical and protective part of the Leguminous seed). The synthesis of extensin occurs just before the drying and shrinking of the seed coat started, suggesting that extensin may play a role in the mechanical and protective function of the testa towards the embryo (Cassab et al., 1985).

Extensin is also found to be a major component of sclerenchyma cell walls. Cassab and Varner (1986) have suggested that extensin, together with other cell wall components, contributes to the tensile strength of mechanical cells. The sclerenchyma cells are supposed to enable plant organs to withstand various strains, those may result from stretching, bending, weight, and pressure, without undue damage to the thin-walled softer cells, such as parenchyma. In agreement with this hypothesis, very low level of extensin is found in the walls of parenchyma cells, which do not have great tensile strength (Cassab et al., 1985). In the parenchymatous cell walls of soybean seed coats, extensin is almost absent (Cassab & Varner, 1987).

1.3.3.2 Cell Extension Control

The growth of plant cell consists of irreversible extension of the pre-existing wall. The ability of the cell wall to extend is found to depend on the amount of extensin and the state of its cross-links to the other wall components (Wilson & Fry, 1986).

The binding of hydroxyproline-rich glycoprotein to the cell wall has

been proposed to be the cause of reduced growth. In elongating tissue of pea stems the protein content of the wall increased, as well as the content of Hyp and Hyp-arabinosides; these change only slightly once elongation is completed (Klis, 1976). Moreover, Van Holst et al. (1980) showed that the degree of arabinosylation of wall-bound hydroxyproline increases when the rate of cell elongation decreases in bean seedlings. As a result, there is an inverse relation between the rate of elongation and the concentration of the hydroxyproline-rich wall proteins, which suggest that extensin stiffens the wall during growth, thus reducing the rate of elongation. The presence of extensin in the soybean apical hook indicates that while the major increase in wall-bound extensin is detected in the elongating hypocotyl, extensin is also synthesized in the region of the seedling that is undergoing active cell division (Klis, 1976).

Fujii, Suzuki & Kato (1981) suggested that a growth inhibitor is one of the factors that caused differential growth between the upper and lower halves of geotropic Zea roots, causing them to curve downwards. The inhibitor selectively increases the concentration of hydroxyproline-containing proteins rigidly bound to the cell walls in the lower halves of the roots, thereby reducing the growth. Further support for the proposed role of the hydroxyproline-rich glycoprotein in stopping cell elongation was provided by Monro et al. (1974). They used sequential procedures to compare elongating and non-elongating hypocotyl sections and found that there is more bonding of the glycoprotein in the wall of non-elongating tissue.

A current view of extensin's role is that it provides a second network which restricts movement between cellulose microfibrils. To achieve this requirement of a separate network, extensin is secreted to the wall in a soluble form and is then locked in position around the cellulose skeleton by the formation of isodityrosine cross-links by the extensin peroxidase (Everdeen, 1988). It is assumed that the insolublization of extensin in the

wall is an irreversible process. Extension is a major control point for plant hormones like auxin and gibberellin. Isolated cell walls have been shown to extend when placed in acidic pH (Taiz, 1984; Cleland et al., 1987); since auxins induce acid secretion, they may also suppress cross-linking of soluble extensin during wall growth. When growth hormone production ceases, extensin can cross-link to harden the new wall.

Lamport and Epstein (1985) have suggested that the xyloglucan components of the wall may function as reversible bonds or 'latches', which can be broken to permit growth. The xyloglucans are hydrogen-bonded to the cellulose fibrils forming cross links, their turnover can be enhanced by auxin (via hydrogen ion secretion), in effect opening the latches, and allowing the microfibrils to slip through the extensin mesh and permit cell extension. No synthetic process would be necessary in cell extension by this method. By using chemical denaturants and boiling in water to remove the enzymes in walls from frozen-thawed cucumber hypocotyls, creep (which is the extension of the wall when places in tension under acidic conditions) seemed to be inhibited. These results indicated that creep depends on enzymes that are firmly attached to or entangled in the wall, but are denatured under the treatments used (Cosgrove, 1989).

1.3.3.3 Disease Defense

Enhanced extensin levels may play a role in resistance to pathogens either through their function of strengthening the cell wall, perhaps in a lignin-glycoprotein complex, or as bacterial agglutinins.

The level of the hydroxyproline-rich glycoprotein has been shown to undergo a ten-fold increase in melon seedlings infected with the fungus *Colletotrichum lagenarium* (Esquerre-Tugaye & Lamport, 1979; Mazau et al., 1986). Moreover, cell wall Hyp levels increase more rapidly in resistant than in susceptible cultivars of cucumber infected with the fungus

Cladosporium cucumerunum (Hammerschmid, 1984). The increase in extensin is also correlated with resistance to anthracnose, a disease caused by the fungal infection (Esquerre-Tugaye & Toppan, 1976). Extensins have been shown to accumulate in the walls of living, uninfected cells close to sites, where fungal and bacterial growth has been restricted by the plant. Extensin also accumulates in plant papillae, which may present a physical barrier to penetration by fungi (Mazau, 1987).

Esquerre-Tugaye et al. (1979) concluded that the accumulation of glycoprotein acts as a defence mechanism, which is effective if started early in the host. Conversely, inhibiting glycoprotein synthesis in diseased plants results in an accelerated and more intense colonization of the host by the pathogen.

Many plants have been reported to respond to an attack by pathogens with enhanced ethylene production (Chrispeels, 1969). Hence, it has been proposed that ethylene may function as a signal for the plant to enhance or activate its defences against pathogens (Averyhart-Fullard, 1987). In 1982, Toppan, Roby & Esquerre-Tugaye found indirect evidence that ethylene regulates the synthesis of extensin as a defence mechanism. They showed that in the presence of specific inhibitors of ethylene biosynthesis, both ethylene production and ^{14}C-hydroxyprotein deposition in the cell walls of infected tissue is significantly lowered. Furthermore, treatment of healthy tissues with a natural precursor of ethylene stimulats both the production of the hormone and incorporation of ^{14}C-hydroxyproline into cell wall protein.

The increase in cell wall hydroxyproline content observed in aged carrot discs has also been suggested to occur as part of a wound response in the excised tissue (Chrispeels, 1969). It is possible that production of hydroxyproline-rich glycoprotein in each of these stress situations may be controlled by ethylene, as wounding, aging and infection are all known to

cause plants to release large amounts of ethylene (Toppan et al., 1982).

Toppan & Esquerre-Tugaye (1984) found that ethylene levels are raised before the pathogen has invaded the tissues and before the onset of symptoms. They have demonstrated that the production of ethylene is stimulated by glycopeptides or 'elicitors' from the fungal cell walls. They suggested that the early increases in ethylene observed after inoculation may be occurring as a response to cell-surface interaction between host, possible via special receptors, and pathogen. They also found that this interaction is not species specific; elicitors from different fungi can induce ethylene synthesis in non-host plants. Furthermore, these elicitors have been shown to stimulate the synthesis of both ethylene and the hydroxyproline-rich glycoproteins (Esquerre- Tugaye et al., 1985). It has been suggested (Mauch, Hadwiger & Boller, 1984) that ethylene and fungal elicitors are actually separate signals which can both induce the biochemical defence reaction, of which synthesis of hydroxyproline-rich glycoproteins was a part. As shown in soybean cotyledons treated with a fungal elicitor, suppression of ethylene production by aminoethoxyvinyl-glycine (AVG) did not reduce phytoalexin production (Calt et al., 1976), and AVG treatment only slightly reduced the biosynthesis of hydroxyproline-rich cell wall glycoprotein in diseased melon seedlings (Chrispeels, 1969).

The distinct nature of the signals provided by ethylene and the wound response is confirmed by evidence from the accumulation of mRNA species for extensin, ethylene induces two extensin mRNAs (1.8 and 4.0 kb), whereas wounding produces the accumulation of an additional extensin mRNA (1.5 kb) (Ecker & Davis, 1987).

The exact role of extensins in the defence response is not clear, but they may act as structural barriers, and provide matrices for the deposition of lignin.

Further evidence for the possible role of extensin in the defence

mechanism of plants can be found in the structural similarity between this glycoprotein and bacterial agglutinins. Mellon & Helgeson (1982) isolated a glycoprotein agglutinin from tobacco callus tissue cultures. They found that, like extensin, the protein is basic and rich in hydroxyproline, while the carbohydrate is predominantly arabinose. The glycoprotein also has a high content of lysine and histidine with a low proportion of acidic residues. This results in its being positively charged at physiological pH. The avirulent bacterial cells, which lack an extracellular polysaccharide, are negatively charged and hence agglutination can be caused by simple ionic interaction. The agglutinins may not have any race-specific role and, if they have any function in recognition, they may merely be non-specific sensors of foreign invaders.

Using immunofluorescence techniques, Leach, Cantrell & Sequeira (1982) have shown that potato agglutinins are located in the plant cell wall. Smith, Muldoon & Lamport (1984) pointed out the similarities between tomato extensin precursors and these agglutinins, and suggested that structural and agglutination roles are probably similar to each other.

1.3.3.4. Other Roles

A number of other possible functions have been proposed for extensin. For example, Albersheim (1978) indicated that it can act as a carrier protein, with the function of transporting polysaccharides from the cytoplasm to the wall. Kauss & Glaser (1974) pointed out that the glycoprotein can guide the polysaccharides into their 'right' position in the cell wall. An alternative idea involves extensin's acting as a primer for the synthesis of the wall polysaccharides, in a similar mechanism to the polymerization of starch and glycogen (Brown & Kimmins, 1977).

1.4 Other Cell Wall Protein

Many different kinds of cell wall protein have been found, glycine-rich and proline-rich proteins are among those that have been more widely studied.

1.4.1 Glycine-rich Protein

Varner and Cassab in 1986 pointed out that certain plant organs contain little HRGP and instead the cell walls are rich in glycine, raises the possibility that the wall of certain plant organ may have a wall rich in glycine instead of HRGP. Glycine-rich protein has been isolated from pumpkin seed coat (Varner and Cassab, 1986), strawberry fruit (Reedy and Poovaiah, 1987), petunia (Condit and Meagher, 1986), and bean (Keller et al., 1988). All these reports suggest that GRPs occur in a wide variety of plants. The entire amino acid sequence of this GRP can be represented as $(Gly\text{-}X)_n$, where X is frequently glycine.

Moreover, it was found that the antibodies raised against glycine-rich protein (based on a $(Gly\text{-}X)_n$ motif) react with a purified cell wall protein showed that glycine-rich protein were indeed cell wall protein. It is also highly localized in regions closely associated with the vascular system, suggest that the protein has a specific role in the functional specialization of vascular tissue (Keller et al., 1988). It has also been shown that this protein is important in development and wounding response (Condit & Meagher, 1986).

The probe for HRGP can be used to isolate GRP, this shows that gene sequences encoding GRPs and HRGP are quite similar. Their mRNAs are encoded by opposite strands of this similar sequence. This suggests during evolution, one of these sequences has duplicated and inverted to provide a novel protein.

1.4.2 Proline-rich Protein

A cDNA from carrot root was isolated that increased greatly upon wounding (Chen & Varner, 1985). The predicted peptide sequence of the cDNA contained 24 repeat units of Pro-Pro-Val-Xa-Xaa. It has been shown that the level of the protein increases after wounding and during plant development (Tierney et al., 1988).

1.5 Aims of the Project

The aims of this project are:

a. To identify a homologous sequence of extensin from pea pod by the use of a clone for Bassica root protein which is homologous to carrot root extensin. If hybridization can be seemed between the RNA extracted from pea pod, and the labelled extensin probe, the corresponding clone from the cDNA library of pea pod can be picked out and use for further studied.

b. To identify a pea pod specific clone by using the plus and minus screening technique. If a specific clone can be found, it is then characterized by DNA sequencing.

CHAPTER II
MATERIALS AND METHODS

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2.1 Materials

2.1.1 Biological and Chemical Reagents

Reagents, unless otherwise mentioned, were purchased from BDH Chemicals Ltd., Poole, Dorset, UK. and were of AnalaR (analytical) grade or the finest available. The following materials were obtained from the designated sources.

Agarose: Bethesda Research Laboratories, Inc., Cambridge, England.

Acrylamide, N,N'-methylene-bis-acrylamide: Sigma Chemical Co. Ltd., Poole, Dorset, England.

Bacto-agar, Bacto-Yeast extract: Difco Laboratories, Detroit, Michigan, USA.

Calf intestinal alkaline phosphatase: Boehringer Co. Ltd., London.

Dialysis tubing: Medical International Ltd., London.

Guanidinium thiocyanate: Fluke Chemie AG, CH 9470 Bucks.

Lysozyme, spermidine, bovine serum albumin (BSA), Dithiothreitol (DTT), ampicillin (sodium salt), herring sperm DNA, ethidium bromide (EtBr): Sigma Chemical Co., Poole, Dorset, UK.

One-Phor-All Buffer Plus: Pharmacia LKB Biotechnology.

Nitrocellulose filters (BA85, 0.45 mm): Schleicher and Schull, Anderman and Co. Ltd., Surrey, UK.

Random primed DNA labelling kit: Boeringer Mannheim GmbH, West Germany.

Restriction endonucleases: Northumbria Biologicals Ltd, Northumbria, UK. and Bethesda Research Laboratories UK. Ltd., Cambridge, UK.

Sephadex G-50, Ficoll 400: Phamacia Fine Chemicals, Uppsala, Sweden.

Tris (hydroxymethyl) aminomethane (Tris), 5-dibromo-4-chloro-3-3-indoyl-galactoside (X-gal): Boehringer Mannheim Co. (London) Ltd., Lewes, East Sussex, UK.

3 MM paper: Whatman Ltd., Maidstone, Kent, UK.

2.1.2 Bacterial Strains, Plasmids and Bacteriophage

The bacterial strains used throughout the project were derivatives of *E. coli* namely JM83 & TG2.

2.1.3 Buffers and solutions

2.1.3.1 Buffers

6x Type I gel loading buffer: 0.25% bromophenol blue
0.25% xylene cyanol
40.00% sucrose

10x Alec's gel buffer: 96.80 g Tris
7.44 g EDTA

Adjusted to pH 7.7 with glacial acetic acid and made up to 2 litres with distilled water.

10x high-salt buffer: 100 mM NaCl
50 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
1 mM dithiothreitol

10x medium-salt buffer: 50 mM NaCl
10 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
1 mM dithiothreitol

10x MOPS/EDTA: 0.50 M MOPS
0.01 M Na_2EDTA
adjust to pH 7.0 with NaOH

10x TBE buffer: 54.0 g Tris
27.5 g boric acid
20 ml 1 M EDTA (pH 8.0)

Electrophoretic buffer for protein: 3.0 g Tris
14.1 g glycine

1.0 g SDS

It was made up to 1 litre with distilled water.

Electrophoresis buffer for RNA gel: 1x MOPS/EDTA

Extraction buffer: 0.125 M Tris-HCl (pH 6.8)

2% SDS

TE buffer: 10 mM Tris-HCl (pH 8.0)

1 mM EDTA

2.1.3.2 Solutions

Protein gel electrophoresis

Solution I: 30 g acrylamide

0.135 g Bis

100 ml water

Solution II: 30 g acrylamide

0.435 g Bis

100 ml water

RNA gel electrohporesis

Buffer A: 294 µl 10x MOPS/EDTA

706 µl water

89 µl formaldehyde

250 µl formamide

Dyes: 322 µl Buffer A

5 mg XC

5 mg bromocresol green (BCG)

400 mg sucrose

Gel loading buffer: 2 µl formaldehyde

5 µl formamide

7 µl Dyes

Electrophoresis buffer: 1x MOPS/EDTA

Miniprep of Plasmid DNA

YT-amp medium: 2.00 g trypticase peptone

1.25 g yeast extract

1.25 g NaCl

It was made up to 250 ml with distilled water, and then was taken to autoclave. 50 μg/ml ampicillin was added to the solution afterward.

Solution I: 2 mg/ml lysozyme in 50 mM glucose

10 mM EDTA

25 mM Tris-HCl (pH 8.0)

Solution II: 0.2 M NaOH

1% SDS

Solution III: 3 M sodium acetate (pH 4.8)

M13 cloning

$CaCl_2$ solution (50 mM): 7.4 g $CaCl_2 \cdot 2H_2O$ in 1 l of distilled water

PEG/NaCl solution: 20.0 g polyethylene glycol 6000

14.0 g NaCl

for 100 ml

IPTG 100mm: 11.9 mg for 0.5 ml

X-gal 2% in dimethylformamide: 10 mg for 0.5 ml

M9 salts (1x): 6.0 g Na_2HPO_4

3.0 g KH_2PO_4

1.0 g NH_4Cl

0.5 g NaCl

for 1 l

Glucose minimal medium plates:

M9 salt with 15 g agar	1 litre
1 M $MgSO_4$	1 ml
1 M thiamine HCl	1 ml
0.1 M $CaCl_2$	1 ml
20% glucose	10 ml

2x TY medium:

Bacto tryptone	16 g
Bacto yeast extract	10 g
NaCl	5 gper litre

H plates:

Bacto tryptone	10 g
NaCl	8 g
Agar	15 g per litre

H top agar:

Bacto tryptone	10 g
NaCl	8 g
Agar	8 g per litre

2.2 Methods

2.2.1 Restriction Analysis

Type-2 restriction endonucleases were used to digest the DNA molecules. Usually EcoRI was used as most of the inserted fragments in the plasmid was through the EcoRI site.

Generally, the enzymes were added at a concentration of 2-5 u/μg of DNA. The reaction mixture was incubated at 37°C for 3 h. For digestion of mini-prepared plasmid DNA, 25 μg/ml of RNase were included in the reaction mixture to remove the tRNA present. spermidine was also included to enhance the effficiency of digestion.

RNase that was free of DNase contamination was prepared by adding the RNase A to distilled water at a concentration of 10 mg/ml, it was then boiled in a water bath for 15 min, and allowed to cool down slowly to room temperature. It could be used immediately or stored at -20°C.

2.2.2 Gel Electrophoresis of DNA

2.2.2.1 Normal Gel

1.4 g of agarose was added to 180 ml distilled water, the mixture was then boiled to dissolve all the agarose. It was allowed to cool to about 70°C, 20 ml 10x Alec's gel buffer and 10 µl ethidium bromide (10 mg/ml) were added. Then it was poured onto the gel frame, and allowed to cool down and solidify.

The gel was placed on the gel tank and covered with 1x Alec's gel buffer containing 500 µg/l ethidium bromide. After 5 µl of the 6x gel loading buffer was added to the samples, the samples were loaded to gel slots. Electrophoresis was performed at 100 V for 4 h or 30 V for overnight (about 15 h).

2.2.2.2 Minigel

0.35 g agarose was added to 45 ml distilled water and heated until the solution started to boil. The solution was then allowed to cool down to 70°C. 5 ml 10x TBE buffer and 5 µl ethidium bromide (10 mg/ml) were added. The solution was then poured into the gel frame in the minigel apparatus, and allowed to set. 50 ml 1x TBE buffer with 1 µl ethidium bromide (10 mg/ml) was poured into the electrophoretic tank. The gel was ran for 50 mA for 1 h.

2.2.3 Transformation

Firstly, 500 µl of overnight JM83 culture was added to 100 ml YT medium and incubated at 37°C with shaking. The OD_{660} of the culture was measured until it has reached 0.5 (about 4-5 h); cells were cooled on ice for 10 min and centrifuged to pellet the cells. The cells were resuspended in 40 ml of 0.1 M $CaCl_2$ and left on ice for 1 h. The cells were then centrifuged, resuspended in 2 ml of 0.1 M $CaCl_2$, and stored on ice. 100 µl of cells were taken from the tube and added to the sample of DNA to be transformed. The

mixture was stood on ice for 20 min, it was subsequently heat shock at 42°C for 2 min. 1 ml of YT medium was added and incubated at 37°C for 1 h to allow the transformed plasmid to express. 100 µl aliquots of the transformed cells were plated on YT-amp-X-gal plates, which were incubated at 37°C overnight. The remainder of the transformation mixture was stored at 4°C.

2.2.4 Separation of Protein

2.2.4.1 Direct Protein Extraction

The plant material was taken freshly and put into liquid nitrogen. It was then dried in vacuum. The resulting material was grounded in mortar and pestle into powder form, and was poured into an Eppendorf tube. 1 ml extraction buffer was added. The tube was then mixed in a rotating disc at 4°C overnight. Afterwards, the tube was centrifuged at 1000 g for 5 min, and the supernatant was collected. 10% sucrose was then added, and the resulting solution was loaded directly onto the gel.

2.2.4.2 Protein Gel Electrophoresis

A gel slab was prepared by fitting the spacers between the two glass plates. The spacer was lightly greased to ensure the gel slab was water-tight, bulldog clips was used to clamp the gel slab all together. The gel slab was placed vertically.

The separation gel was prepared as follow: 8.33 ml solution I, 7.5 ml 1 M Tris-HCl (pH 8.8), 3.3 ml distilled water and 0.5 ml 1% ammonium persulphate were added to a Buchner flask, the mixture was degased by evacuation on a water pump, 0.2 ml 10% SDS and 7.33 µl TEMED were added. The mixture was mixed and poured into the gel slab immediately up to a level approximately 2.5 cm below the cutout. Water was then added gently to a depth of 2-3 mm. It was left to polymerise for at least 30 min.

The stacking gel was made by mixing 1.5 ml solution II, 1.25 ml 1 M Tris-HCl (pH 6.8), 6.9 ml water and 0.25 ml 1% ammonium persulphate in a

Buchner flask, degased as before. 0.1 ml 10% SDS and 5 µl TEMED was then added. The solution was mixed and poured immediately to fill the gel slab, and the well-forming comb then gently slid into the cutout. It was left to polymerize. The comb, and the spacer at the bottom of the gel could be removed. The slab was clamped onto the electrophoretic tank. 500 ml electrophoretic buffer was added to the top and bottom reservoirs. Any bubbles trapped under the gel was removed using a syringe with a bent needle. The sample could be loaded. The gel was run at 15 mA for 1 h and then 25 mA for 4 h.

2.2.5 Total RNA Extraction Using Modified Guanidinium Thiocyanate Method

Guanidinium thiocyanate is among the most effective protein denaturants and was first introduced as a deproteinization agent for isolation of RNA by Cox (1968). It dissolves protein readily and releases nucleic acids from nucleoproteins, as their cellular structures disintegrated and the secondary structure lost.

A method modified from Chomczynski (1987) was adopted here. In general, sample was first frozen immediately in liquid nitrogen after it was removed from the plant to prevent deterioration. 1 g was weighed and put into a 35 ml polycarbonate tube. The samples were homogenised (at room temperature) with 4.5 ml of solution D (4 M guanidinium thiocyanate, 25 mM Na citrate, pH 7; 0.5% N-lauroyl sarcosine, 0.1 M 2-mercaptoethanol) in a Polytron at speed 10 for 20 s. The Polytron was rinsed with further 0.5 ml solution D. The homogenate was centrifuged for 10 min at 10 K, and the clear supernatant was collected and poured into a 15 ml cornex tube. 5 ml phenol and 1 ml chloroform:isoamyl alcohol mixture (24:1) were added, and the mixture was centrifuged for 20 min at 10 K. The aqueous (upper) layer was collected and made up to 0.2 M Na acetate by adding 2 M Na acetate stock. The RNA was precipitated with 2 volume of cold ethanol for 1 h at -20^{o}C. The pellet was

repeat pg

Buchner flask, degased as before. 0.1 ml 10% SDS and 5 µl TEMED was then added. The solution was mixed and poured immediately to fill the gel slab, and the well-forming comb then gently slid into the cutout. It was left to polymerize. The comb, and the spacer at the bottom of the gel could be removed. The slab was clamped onto the electrophoretic tank. 500 ml electrophoretic buffer was added to the top and bottom reservoirs. Any bubbles trapped under the gel was removed using a syringe with a bent needle. The sample could be loaded. The gel was run at 15 mA for 1 h and then 25 mA for 4 h.

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collected by centrifuging for 20 min at 10 kg, and was resuspended in 1.5 ml solution D and reprecipitated as before. The precipitate was finally washed with 70% ethanol, vacuum dried and resuspended in 1 ml DEPC treated water.

2.2.6 Quantitation of DNA and RNA

Spectrophotometric method was used to measure the amount of DNA or RNA in a preparation (Maniatis et al., 1982).

When the sample was purified, a Philips PU 8700 Series UV/Visible Spectrophotometer and 1-cm path length quartz cells were used to measure the absorbance. The sample was scanned from wavelenght 260 nm to 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample.

2.2.7 Formaldehyde Gel Electrophoresis of RNA

Formaldehyde gel electrohporesis is frequently used to separate small molecular weight RNAs under denaturing conditions (Miller, 1988).

2.2.7.1 Sample Preparation

1 µl of the RNA sample was placed in DEPC-treated Eppendorf tube; large volume was reduced by ethanol precipitation if necessary. Sequentially, 4.4 µl Buffer A, 11.6 µl of formaldehyde/ formamide were added. The mixture was heated at 70°C for 10 min and chilled on ice. 1.5 µl gel loading buffer was added to the mixture before it was loaded onto the gel.

2.2.7.2 Gel Preparation and Electrophoresis

A 1.5% agarose gel was prepared by dissolving 1.4 g hot agarose in 67 ml water, the mixture was then heated to about 70°C, 9.3 ml 10x MOPS/EDTA and 17 ml formaldehyde were added in fume hood. The gel was allowed to set for 1 h. The gel was subjected to pre-electrophoresis at 60 V for 30 min before loading the samples. The gel was electrophoresed at 100 V for 4 h , or at 30 V overnight.

2.2.7.3 Staining, Destaining

After electrophoresis, the gel was stained for 5 min in distilled water with 5 µg/ml ethidium bromide. It was destained in distilled water for 2 h at 4°C. The gel was finally viewed under UV light.

2.2.8 Northern Blotting

After electrophoresis, the gel (without prior treatment) was placed over 4 sheets of Whatman 3MM paper saturated with 20x SSC in a tank. A nitrocellulose filter was prepared by first wet with water, and then equilibrated with 20x SSC for 5 min. The filter was then laid over the gel, making sure that no air bubbles were trapped between the gel and the filter. It was then covered with 4 sheets of Whatman 3MM paper and 3 layers of absorbive pads. Finally, a glass plate was placed on the pads and a 500 g weight was placed on the top. Transfer of RNA was complete in 12-15 h. The gel has not been treated with alkali to reduce the size of the RNA in the gel, because treatment of the gel with alkali and neutralization with salt buffers substantially reduces the efficiency of transfer of RNA from the gel to the nitrocellulose paper, particularly for larger RNAs. Moreover, presoaking the gel in 20x SSC or staining the gel with ethidium bromide reduces transfer. The most efficient transfer was obtained if the gel was in low salt and the transfer buffer is high salt.

The blot was then baked in an vacuum oven for 1 h at 80°C. Baking was required for retention of the RNA on the nitrocellulose.

2.2.9 Staining RNA After Transfer to Nitrocellulose Filters

This method was used to check the size of the RNA transferred to nitrocellulose and the efficiency of its transfer. A lane was cut off from the filter, either after baking, or after hybridization and exposure to

X-ray film.

Firstly, the filter was put into the 5% acetic acid for 15 min at room temperature and then transferred to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 5-10 min. The filter was soon rinsed in distilled water for 5-10 min.

2.2.10 Miniprep of Plasmid DNA

10 ml of YT-amp medium was inoculated with a single bacterial colony containing the desired plasmid, and the medium was incubated at 37°C overnight with vigorous shaking. The culture was centrifuged, and most of the supernatant was removed to leave 0.5 - 1.0 ml. It was resuspended and transferred to an 1.5 ml Eppendorf tube and recentrifuged. As much supernatant as possible was removed to leave a bacterial pellet. 200 µl of solution I was added to resuspend the pellet and the mixture was kept on ice for 30 min. 600 µl of solution II was added, mixed gently and kept on ice for 5 min. Subsequently, 450 µl of solution III was added and inverted a few times while the DNA clot was forming. The mixture was kept on ice for 60 min, and centrifuged for 5 min afterward. From the supernatant 1100 µl was removed to a new Eppendorf tube, to this 500 µl cold isopropanol was added, and the tube was kept at -20°C for at least 30 min. After centrifugation for 5 min, the supernatant was removed and the pellet was redissolved in 200 µl of 0.1 M sodium acetate/0.05 M Tris-HCl (pH 6.0), then repre- cipitated by 500 µl cold ethanol, left at -20°C for at least 30 min. It was centrifuged again, and the pellet obtained was washed with 1 ml cold ethanol. The pellet was then vacuum dry and redissolved in 50 µl of sterile water.

2.2.11 Recover of DNA From Agarose Gel

2.2.11.1 Preparation of Dialysis Tubing

Dialysis tubing was first cut into pieces with length about 10 cm. The

resulting tubings were boiled in a large volume of 2% sodium bicarbonate and 1 mM EDTA for 10 min, and then boiled again in 0.001 M EDTA for 10 min. The tubings could then be used immediately, or could be kept at 4°C for one month. The tubings should be rinsed with distilled water before using (Maniatis, 1982).

2.2.11.2 Electroelution

After gel electrophoresis of DNA, the gel was viewed under UV, the desired band was then located and was cut out from the gel using a sharp scalpel. The dialysis tubing that had been prepared before was then filled with the electrophoresis buffer that has been used for gel electrophoresis of the DNA, except ethidium bromide was not included in the buffer. The gel slice was put into the dialysis tubing and tied with a clip at both end. No gas bubbles should be trapped. The whole tubing was placed in a minigel electrophoretic tank filled with electrophoretic buffer. Electric current was passed through the tubing at 100 V for 1/2 h, the polarity was then reversed for 30 s to cause the DNA to become detached from the wall of dialysis tubing. The whole setup was viewed under UV to ensure all the DNA had eluted out from the gel slice and stayed in the buffer. The dialysis tubing could remove from the tank and opened up, and the buffer surrounding the gel slice was then removed carefully by using a pasteur pipette. The DNA could be recovered from the buffer by phenol extraction (Maniatis, 1982).

2.2.12 Radiolabelling DNA Using Random Oligonucleotides as Primers

The method could produce DNA labelled to high activity and was devised by Feinberg (1984). The principle was based on the hybridization of a mixture of random oligonucleotides to the DNA to be labelled.

The DNA (about 25 ng) using for labelling was adjusted to 31 µl, denatured by boiling in a water bath for 3 min, and stored briefly (10-60 min) at 37°C until needed. Labelling reaction was set up by adding the

following solutions in order:

water, if needed to make a final volume of 50 µl	
OLB	10 µl
BSA (10 mg/ml)	2 µl
DNA	31 µl
^{32}P-dCTP (50 µCi)	5 µl
Klenow enzyme (2 u)	2 µl

The mixture was incubated at room temperature for 2.5 h, and then stopped by addition of 200 µl of 20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.25% SDS. The unincorporated label was removed by gel filtration on Sephadex G-50.

Sephadex was prepared by swelling in the buffer (150 mM NaCl, 10 mM EDTA, 0.1% SDS, 10 mM Tris-HCl, pH 7.5) overnight, and was packed into a 5 ml Dispocolumn. The labelling reaction mixture was loaded and the column was washed with the column buffer. 6 ml of column effluent was collected into 12 Eppendorf tubes with 0.5 ml each. 2 µl from each tube was taken and mixed with 4 ml of scintillant (Ecoscint A) in a plastic counting vial. Protocol no. 7 was used for ^{32}P counting and the whole process was done automatically by a Packard PL Tri-Carb Liquid Scintillation Counter. The first peak of eluted radioactivity was the labelled DNA and was recovered by phenol extraction and ethanol precipitation. It was then added to the hybridization solution and was used as probe.

Random primed DNA labelling kit from Boehringer Mannheim was also used to prepare radioactive probes. At first, the DNA (the volume adjusted to 9 µl) was denatured by heating for 5-8 min at 100°C and subsequent cooling on ice. The following were added.

3 µl dATP, dGTP, dTTP mixture (prepared by making a 1+1+1 mixture)

2 µl reaction mixture

5 µl = 50 µCi [^{32}P] dCTP

1 µl Klenow enzyme

The reaction mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 2 µl EDTA (0.2M, pH 8.0). Finally, the reaction mixture was loaded into the Sephadex G-50 column and separated as above.

2.2.13 Hybridization

The prehybridization buffer contained 50% (vol/vol) form- amide, 5x SSC, heat denatured herring sperm DNA at 100 µg/ml, and 0.02% each BSA, Ficoll, and Polyvinylpyrrolidone. The RNA blots were prehybridized for 4 h at 42°C. The hybridization buffer was the same as prehybidization buffer, except with the probe added. The labelled probes are denatured at 100°C for 10 min, cooled, and added to the hybridization buffer, and the blots were hybridized for about 24 h at 42°C. The RNA blots were subsequently washed to a hybridization stringency of 0.1x SSC /0.1% SDS at 50°C.

2.2.14 Autoradiography

The nitrocellulose filter to be autoradiographed was attached to a Whatman 3 MM paper. Radioactive ink was dotted at several locations on the 3 MM paper in a random pattern for easier distinguishing the orientation after the film was exposed. The filter was put in a plastic bag when the ink was blotted dry. It was then placed in a Kodak X-omatic cassette with intensify screen.

The whole cassette was taken to a dark room with only safty light on. A X-ray film (Fuji) was pre-flashed once by a ordinary camera flash. The flashed side of the film was placed opposite to the filter. The cassette was stored at -80°C for several days depending on the strength of the radioactivity on the filter.

Before the film was developed, it should be warmed up to room temperature for at least 30 min. The film was removed from the cassette and

put into the developer for 10 min with occasional turning. It was then rinsed in water and placed into the fixer for 2 min. Finally, the film was rinsed in tap water for 30 min. The film was hung to dry and examined under light.

2.2.15 M13 cloning

2.2.15.1 Phosphatase Treatment of Linear, Plasmid Vector DNA

During ligaton, DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only when one nucleotide possessed a 3'-hydroxyl group and the other contained a 5'-phosphate group. Recircularization of plasmid DNA can be minimized by removing the 5'-phosphate groups from both end of the linear DNA with calf intestinal phosphatase (Maniatis, 1985). As a result, foreign DNA segment with 5'-phosphate groups can be ligated efficently to the dephosphated plasmid DNA to give an open circular molecule with two nicks. Because nicked circular DNA transforms more efficently than linear plasmid DNA, most of the transformants will contain recombinant plasmids.

The following ligation reaction was set up:

4 µl M13 (Yanisch-Perron et al., 1985)

2 µl high salt buffer

2 µl EcoRI

12 µl water

Incubated for 1 h at 37°C, then 1 µl calf intestinal phosphatase (CIP) was added and incuabated at 37°C for 10 min. Afterwards, 2 µl of 500 mM EGTA was added, and incubated at 65°C for 1 h. Lastly, 30 µl TE buffer was then added to the mixture and the dephosphated plasmid DNA was recovered by phenol extraction.

2.2.15.2 Ligation

After the recovery of fragment from the gel by electroelution, it was

added to the dedephosphated vector with ATP and T_4 ligase, they were incubated at 12-14°C for 4 h.

2.2.15.3 Transformation

The *E. coli* was made competent by $CaCl_2$ treatment. Firstly, a single colony was picked from the glucose/minimal medium plate. It was growth overnight in 10 ml 2x TY medium at 37°C with continuous shaking. 2 ml overnight culture was taken and added to 40 ml 2x TY medium in a 250 ml flask, it was kept at 37°C for 2 h until OD_{550} was equal to 0.3. Moreover, 1 drop of overnight culture was added to 20 ml of 2x TY medium to provide log phase cells, which was needed for plating out stage.

The cells were spinned down from 40 ml culture at 3000 g for 5 min. They were then resuspend in 20 ml of sterilized 50 mM $CaCl_2$ and kept in ice for 20 min. The cells were again spinned down at 3000 g for 2 min and resuspended in 4 ml cold 50 mM $CaCl_2$. They were ready to use at that time or might be kept for several hours to increase the efficiency of transformation.

Approriate amount of DNA from ligation was added to 300 µl of competent cells. The mixture was incubated in ice for 40 min and heat-shock at 42°C for 45 s, shaking should be avoided. It was placed on ice for 5 min.

To the transformation mixture, 200 µl of log phase *E. coli* cells, 40 µl IPTG, and 40 µl 2% X-gal in dimethylformamide were added. 3- 4 ml molten H top agar was subsequently added and kept at 45°C. The mixture was mixed by rolling, and was poured immediately onto a prewarmed (37°C) H plate, it could then be leave at ambient temperature to set. It was then inverted and incubated at 37°C overnight.

2.2.15.4 Isolation of Single and Double-stranded DNA

The transformed plaque (clear plaque) could be picked out from the plate by using a sterile toothpick, and inoculated in 5 ml 2x TY medium with 50 ul of a fresh overnight culture of TG2 cells. A control (blue plaque) was

also picked.

The culture was incubated for 5 h at 37°C with shaking. it was poured into five 1.5 ml Eppendorf tubes and centrifuged for 5 min. The preciptates were kept for miniprep to testify whether there were inserts or not..

The supernatants were transferred to five fresh tubes, one tube was kept as a phage stock. For the remaining four tubes, 200 µl PEG/NaCl (20% w/v polyethylene glycol 6000, 2.5 M sodium chloride) was added to each tubes. It was shaken and left at room temperature for 15 min, centrifuged for 5 min and discarded the supernatant. All PEG was then removed by using a drawn out Pasteur pipette after recentrifuged for 2 min.

100 µl of TE buffer was added to resuspend the pellet. It was then undergone phenol extraction and ethanol precipitation.

All four tubes were finally resuspended in 100 µl TE buffer by transferred from tube to tube. It was centrifuged again to remove any protein precipitate remained, the supernatant was transferred to a new tube. The amount of yield was determined by measure the OD at 260 nm, 1 OD was equivalent to 40 µg/ml of single-stranded DNA.

CHAPTER III
RESULTS

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RESULTS

3.1 Preliminary Investigations

3.1.1 Restriction Analysis

4 µl (0.25 µg/µl) of lambda DNA was digested with 10 u EcoRI (5u/µl) in high salt buffer and 10 u HindIII (5u/µl) in medium salt buffer. After restriction, the mixture was separated by gel electrophoresis and viewed under UV light. The result was shown in Fig. 3.1. A calibration graph was drawn from Table 3.1 by plotting the log of the sizes of restriction fragments against distance migrated (Fig. 3.2).

3.1.2 Transformation

3.25 ng and 13 ng of pUC18 were used to transform JM83 strain of *E. coli*. The cells were made competent by the use of $CaCl_2$. Heat shock was used to increase the efficiency of transformation. The transformed bacteria was then grown on YT-Amp-X-gal plates. Positive transformants would be blue in color. The result was shown in Table 3.2.

$$\text{Efficiency} = \frac{\text{Number of transformed cells}}{\text{Amount of DNA (µg)}}$$

Amount of DNA (ng)	Number of colony	Efficiency
0.00 (control)	0	0
3.25	530	1.63×10^5
13.00	991	7.62×10^4

Table 3.2 Efficiency of transformation for *E. coli* strain JM83

3.1.3 Protein Electrophoresis

Protein was extracted from 0.12 g leaf, 0.17 g green pod, 0.08 g purple pod, 0.02 g purple seed, and 0.02 g green seed by the method of direct extraction of protein. A 12% slab gel was done with markers included (Fig. 3.3).

3.2 Attempt to Use a Homologous Extensin Probe to Identify Extensin mRNA in Pea Pod RNA

A clone for a *Brassica* root protein homologous to carrot extensin that has been previous identified was used. It was supplied as an insert in pUC18 as the plasmid vector, and was ligated to the vector at the EcoRI site.

3.2.1 Preparation of Plasmid

In order to prepare plasmid DNA, an overnight culture (10 ml) of the clone in YT-amp medium was used. Miniprep was done and the amount of DNA obtained was shown in Fig. 3.4.

20 µl of plasmid DNA was restricted with EcoRI to isolate the inserted fragment. The components of the restriction were as follow:

Components	Amount (µl)
EcoRI	2
Sample	10
High salt buffer (10x)	2
Distilled water	6

Table 3.3 Components of the restriction of a clone for a Brassica protein homologous to carrot root extensin

Several attempts were made to restrict the plasmids, but only partial or even no restriction could be obtained. Finally, a complete restriction was obtained by reducing the amount of enzyme used and increasing the incubation time of the digestion. As the enzyme was dissolved in glycerol that would inhibit the enzyme in high concentration (Maniatis et al., 1982); moreover, the plasmid made from miniprep contained impurities that would inhibit enzymatic activity (Draper et al., 1988), a longer incubation time was needed for complete restriction (Fig. 3.5). A calibration curve was drawn (Fig. 3.6) from Table 3.4. The insert was found to be 603 bp.

In order to test whether there was homologous sequence to extensin

present in pea pod, a northern blotting of the total RNA of pea pod was carried out.

3.2.2 Pea Pod Total RNA Extraction Practices

1 g of pods was used as starting material. Modified guanidinium thiocyanate method was employed.

After the pea pod total RNA was extracted and resuspended in 500 µl of sterile water, 5 µl was taken out and quantified by spectrophotometric method (Fig. 3.7). The total amount of RNA extracted was 51.77 µg.

In order to confirm whether RNA was present or not, formaldehyde gel electrophoresis was used. 45 µl (5.8 µg) of sample was added to each wells. RNA from *E. coli* was used as a marker. Two ribosomal RNA bands together with the smears of mRNA and tRNA were clearly shown on the gel (Fig. 3.8).

3.2.3 Northern Blotting

The total RNA present on the gel was then transferred to a nitrocellulose filter by northern blotting. The blot was later stained by methylene blue to ensure a successful transfer has taken place (Fig. 3.9).

3.2.4 Hybridization

The radioactive probe was then used to hybridize to the nitrocellulose filter. At the first attempt, the autoradiograph obtained had a high background but some degree of hybridization seemed present (Fig. 3.10). As a result, another northern blot of pea pod was used for hybridization. However, only a very low degree of hybridization was obtained (Fig. 3.11). One more trial has done but showed no improvement (Fig. 3.12). It was concluded that the homologous probe was not suitable to detect extensin in pea pod RNA.

3.3 Attempt to Find Pea Pod Specific Clones

3.3.1 Total RNA from Pea Pod, Leaf and Cotyledon

Total RNA was extracted from pea pod, leaf and cotyledon by the

modified guanidinium thiocyanate method. The spectrophotometric method was used to access the amount of total RNA extracted (Fig. 3.13-3.15). The amount of total RNA extracted was follow:

Tissue	Amount of tissue used (g)	Amount of RNA extracted (µg)	% of RNA against tissue used
pod	1.07	476.25	0.045
leaf	0.78	273.75	0.035
seed	0.28	33.75	0.012

Table 3.5 Amount of total RNA extracted from pea pod, leaf and cotyledon.

3.3.2 Transfer the Total RNA to Nitrocellulose Filter

Northern blotting was done to transfer the total RNA of pea pod, leaf and cotyledon from the gel to the filter.

3.3.3 Probe Preparation

12 abundant clones from pea purple pod cDNA library were obtained. The plasmids from the clones were extracted by miniprep of overnight cultures. 50 µl of plasmid was obtained from each clones. 10 µl from each clones was then restricted by using 10 u EcoRI (5u/µl) and separated by gel electrophoresis (Fig. 3.16). A calibration curve for finding the insert size was drawn (Fig. 3.17), and the resulting insert size for each of the clones was then determined (Table 3.6). A southern blot was prepared from the gel, but as the probe for ribosomal RNA of pea was not available, the southern blot has not been used.

Insert fragments from pPP523 and pPP1017 with sizes 927 bp and 1308 bp respectively were then isolated and used for random primer labelling (Table 3.7). The probes produced were used to screen the northern blot made from pea leaf. However, no hybridization was given (Fig. 3.18). This might be caused by inadequate radioactivity of the probes. Another labelling reaction was done (Table 3.8) and the probe for pPP523 was used to hybridize to northern blot of pea pod and pea leaf (Fig. 3.19). Again, no hybridization was obtained.

Four more clones (pPP240, pPP354, pPP954, and pPP1052) were once again

selected. 50 µl of plasmid DNA from of them were recovered from miniprep of 10 ml overnight culture. 40 µl was taken and restricted with EcoRI, the restricted mixture was separated by gel electrophoresis. The DNA from the corresponding bands on the gel was recovered by electroelution. It was used for random primer labelling (Table 3.9-3.12).

The probes prepared from these clones were then used to hybridize to the northern blot of pea pod (Fig. 3.20) and pea leaf (Fig. 3.21). After that, autoradiographs were developed with the same length of exposure time (1 week).

From the autoradiographs, this could be shown that all the clones hybridized to the total RNA of pea pod, but only two of them (pPP354 and pPP240) hybridized to the total RNA of pea leaf.

3.3.4 M13 Cloning

In order to characterize the clones (pPP1052 and pPP954) that seemed to be pea pod specific clones, the best method was to clone the fragments into M13mp18 and determined the DNA sequences of the insert fragments.

3.3.4.1 Phosphatase Treatment of Linear, Plasmid Vector DNA

4 µl (1 µg) of M13 was used, and was restricted with 10 u (2 µl) of EcoRI. It was then treated with calf intestinal phosphatase (1 µl) to remove the 5'-phosphate groups from both ends of the linearized DNA. The vector was recovered by phenol extraction and redissolved in 5 µl of water.

3.3.4.2 Isolation of Insert Fragments from Clones

Again, miniprep, gel electrophoresis and electroelution were done to isolate the fragments from the clones. 5 µl was obtained from pPP1052 and pPP954.

3.3.4.3 Ligation

Three ligation reactions were set up with the compositions as followed:

Components	Reaction no. 1	2	3
Vector	1	1	1
Fragment from pPP1052	1	2	-
Fragment from pPP954	-	-	1
1/10 ATP	1	1	1
10x ligation buffer	1	1	1
water	5	4	4
T_4 ligase	1	1	1

Table showing the components of the ligation reaction.

3.3.4.4 Transformation

After the ligation, 3 µl and 7 µl was taken from each ligation mixtures and added directly to the competent cells of TG2. These were then grown on H plates at 37°C overnight, totally, six plates were prepared.

Only four transformants could be picked up from all the plates, one control was also taken for characterization as shown below:

Tube no.	Source of transformant
1	3 µl of ligation reaction 1
2	7 µl of ligation reaction 1
3	7 µl of ligation reaction 2
4	7 µl of ligation reaction 3
5	control (blue plaque)

Table showing the sources that plaques were picked for further single- and double-stranded M13 extraction.

They were then grown in 2x TY medium for 5 h. Single-stranded and double-stranded M13mp18 were then extracted from these cultures by the method 2.2.15.4.

Minipreps have been done to the bacteria containing the double-stranded M13mp18, 30 µl was obtained. 10 µl was then restricted with EcoRI (10 u) and separated in a minigel (Fig. 3.22). No restriction could be seen.

BalI (10 u) and PstI (10 u) were used to restrict the remaining 20 µl of vector (Fig. 3.23). A calibration curve was drawn (Fig. 3.24) to determine the size of restricted fragments. Although partial restriction was seemed but it could be shown that there was insert to the M13 vector for Tube no. 1, 2, and 3 when comparing the control of tube no. 5, and the

calculated insert size was quite agreeable with that before.

Single-stranded M13mp18 was then prepared from supernatants of tube number 3. 6 μl was recovered that 1 μl was used for spectrophotometry. Finally, 4 μg in 5 μl was obtained and used for DNA sequencing. The sequence obtained was shown in Fig. 3.25.

Fig. 3.1 Gel electrophoresis of lambda DNA restricted with HindIII & EcoRI.

EcoRI			HindIII		
fragment size (bp)	log bp	distance migrated (cm)	fragment size (bp)	log bp	distance migrated (cm)
21226	4.33	2.25	23130	4.36	2.25
7421	3.87	4.50	9416	3.97	3.75
5804	3.76	5.40	6682	3.82	4.95
5643	3.75	5.70	4361	3.64	7.05
4878	3.69	6.30	2322	3.37	10.50
3530	3.55	7.95	2027	3.31	11.40

Table 3.1 Fragment sizes of lambda DNA after restricted with EcoRI and HindIII alternatively.

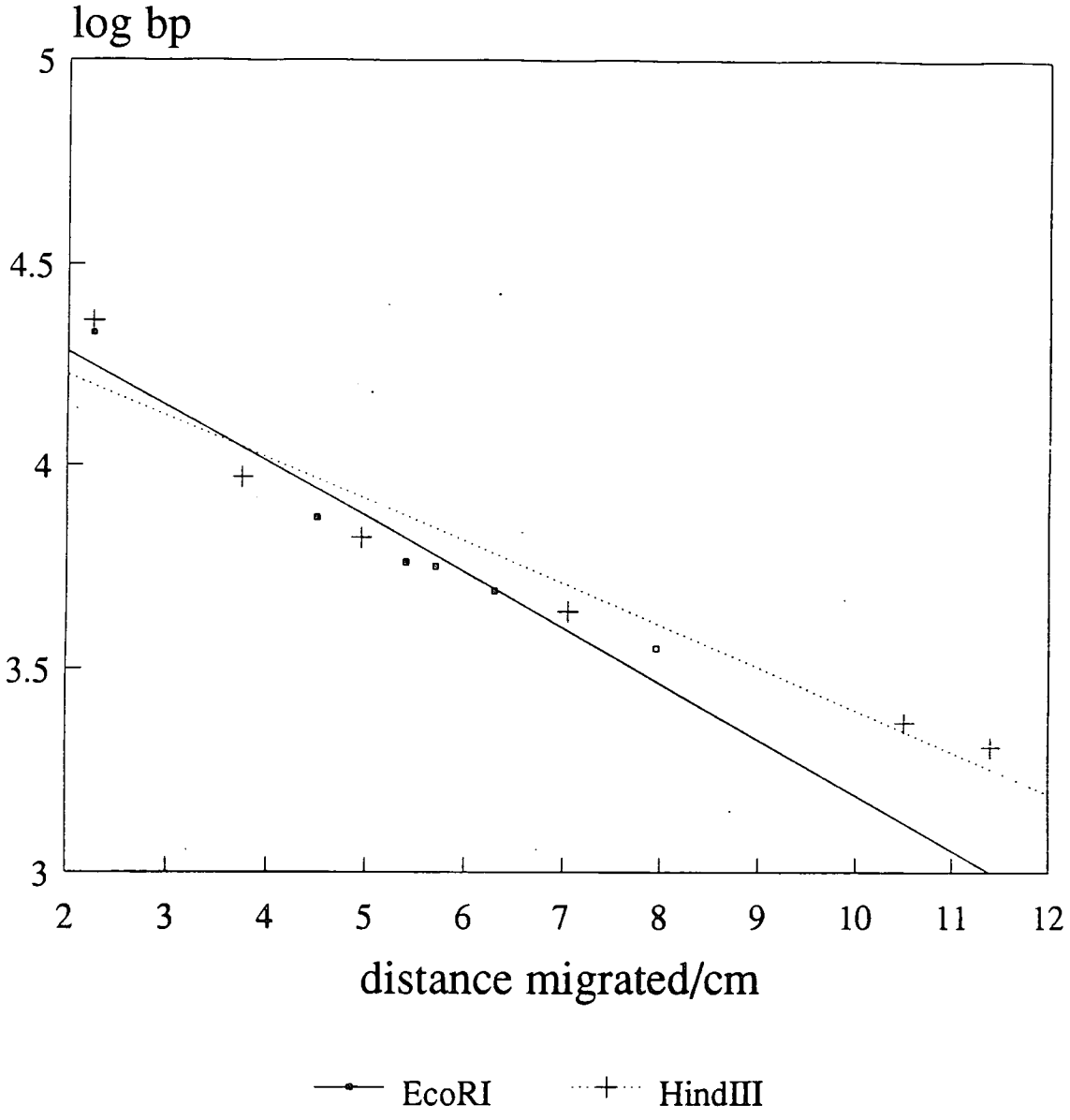

Fig. 3.2 A calibration curve for log the size of fragments of lambda DNA with HindIII and EcoRI against distance.

Fig. 3.3 Gel electrophoresis of protein extracted from pea purple and green pod, purple and green seed, leaf. With molecular markers included.

Lane number	Components	Molecular weight
1	abumin	-----
2	chymotrysinogen	25700
3	cytochrome	11700
4	trypsin	20100
5	abumin (egg)	43000
6	green seed	
7	purple seed	
8	leaf	
9	green pod	
10	purple pod	
11	purple pod	

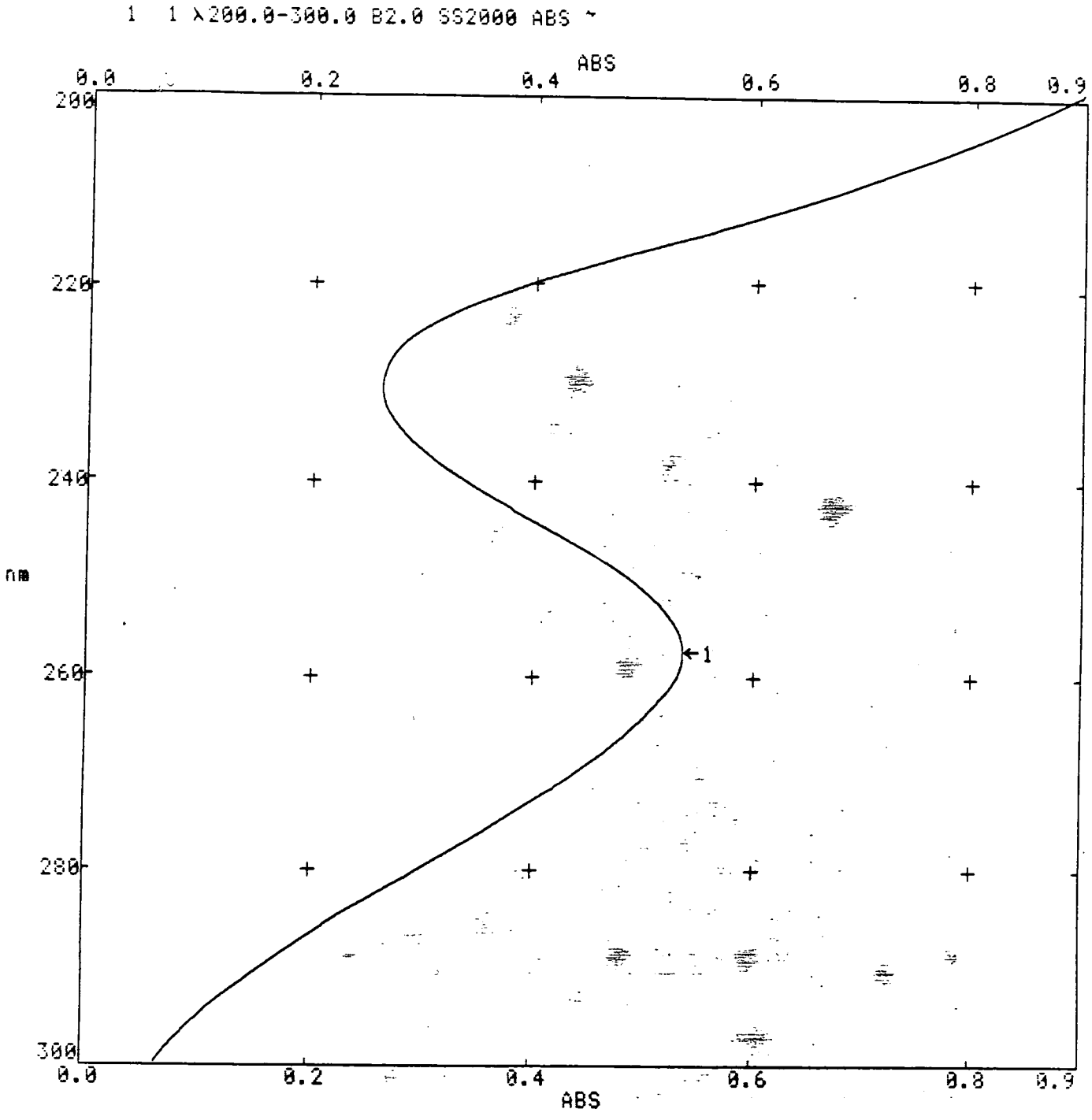

Fig. 3.4 UV absorption spectrum of the plasmid containing insert encoding Brassica protein homologous to carrot extensin.

The absorbance was 0.536 at 257 nm. Because an OD of 1 corresponded to about 50 µg/µl for dsDNA (Maniatis et al., 1982), the total amount of DNA extracted was:

0.536 x 50 x 3

= 75 µg/10 µl (10 µl of sample was added to 3 ml of water)

Total amount of DNA left

= 75 x 4 µg = 300 µg

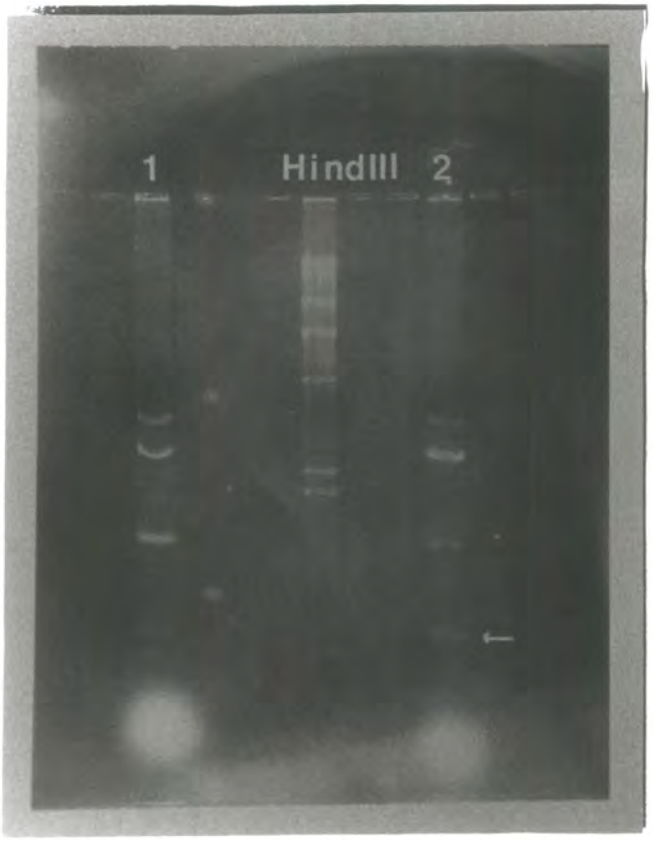

Fig. 3.5 Plasmid containing insert encoding Brassica protein homologous to carrot extensin.

HindIII		
fragment size (bp)	log bp	distance migrated (cm)
23130	4.36	7.50
9416	3.97	12.50
6682	3.82	16.50
4361	3.64	22.50
2322	3.37	34.00
2027	3.31	36.50

Table 3.2 Fragment size of lambda DNA after restricted with HindIII

Distance migrated of the insert = 54.5 cm
The calculated insert size of the fragment = 603 bp

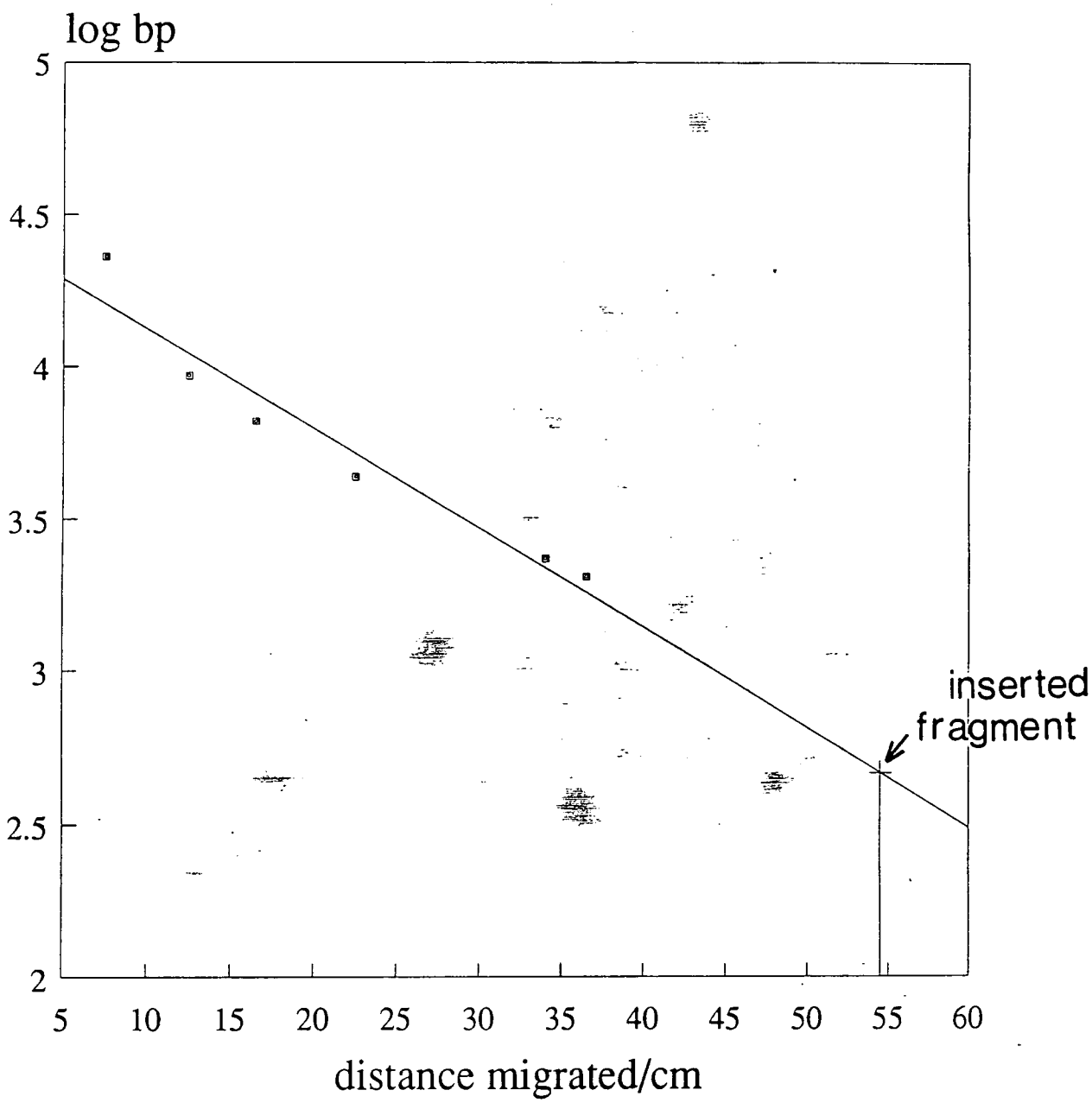

Fig. 3.6 A calibration curve for log the size of fragments of lambda DNA cut with HindIII against distance migrated.

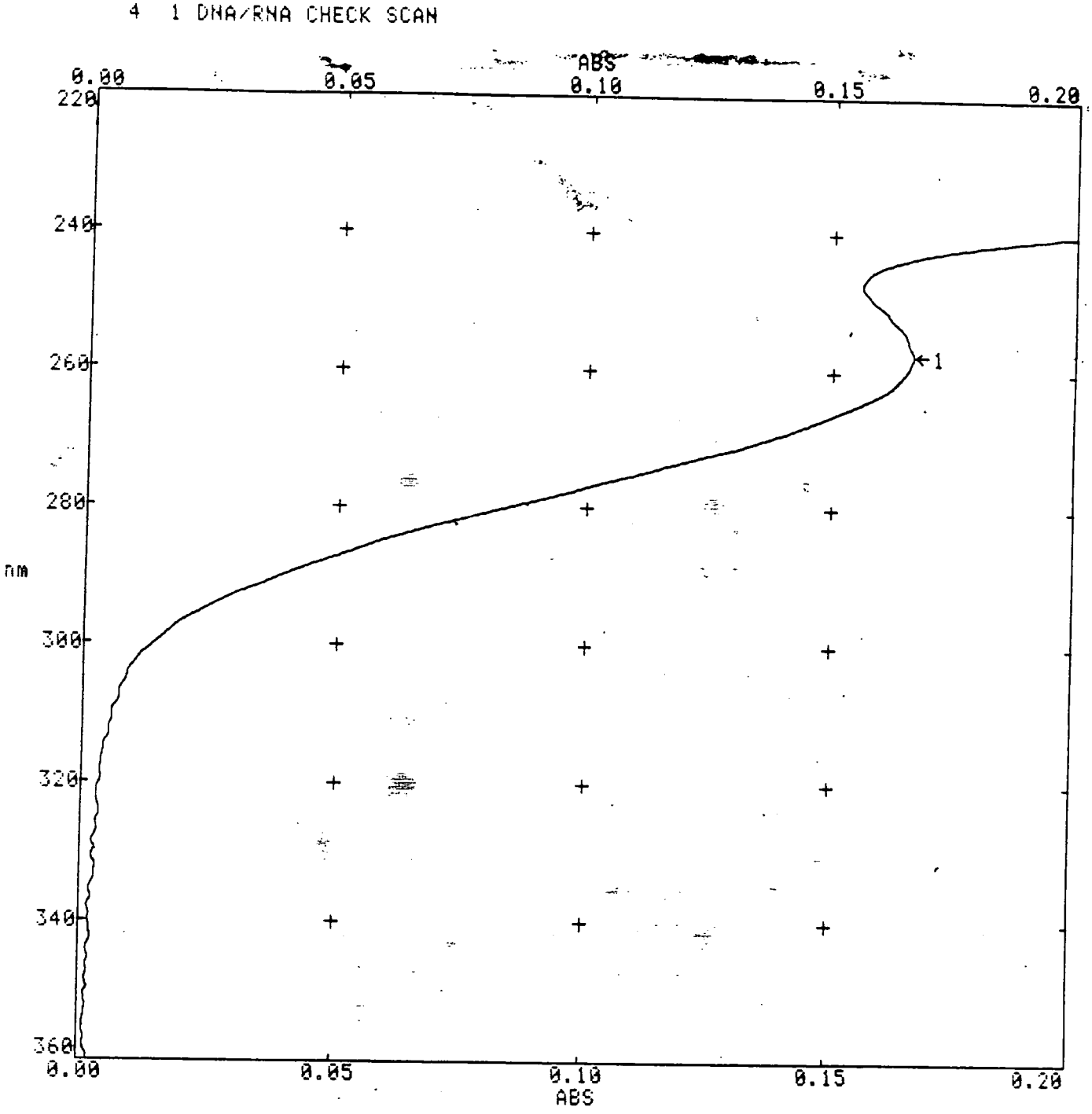

Fig. 3.7 UV absorption spectrum of the RNA from pea pod.

The absorbance was 0.167 at 257.6 nm. Because an OD of 1corresponded to about 25 µg/µl for ssRNA (Maniatis et al., 1982), the total amount of DNA extracted was:

0.167 x 25 x 3.1

= 12.9 µg/100 µl (100 µl of sample was added to 3 ml of water)

Total amount of RNA left

= 12.9 x 4 µg = 51.6 µg

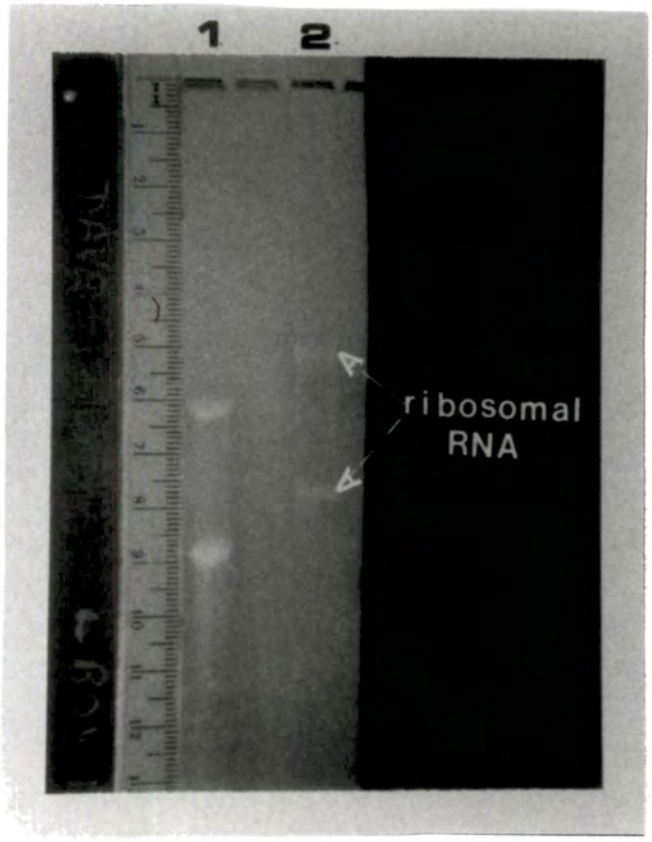

Fig. 3.8 Gel electrophoresis of the total RNA of pea pod, RNA from *E. coli* was used as a marker on Lane 1.

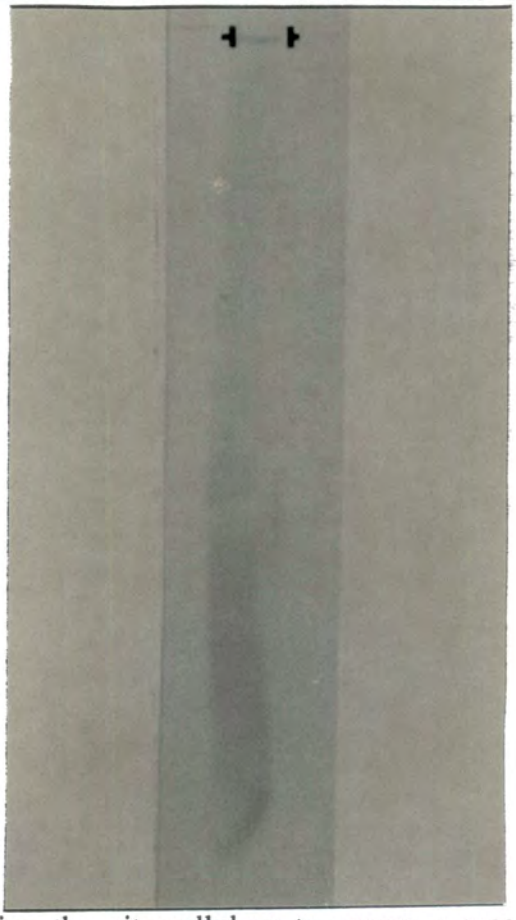

Fig. 3.9 Staining the nitrocellulose to ensure a successful transfer of pea pod total RNA.

Fig. 3.10 First attempt to prepare a autoradiograph to find the homologous extensin clone in pea pod. Bands were seemed to present.

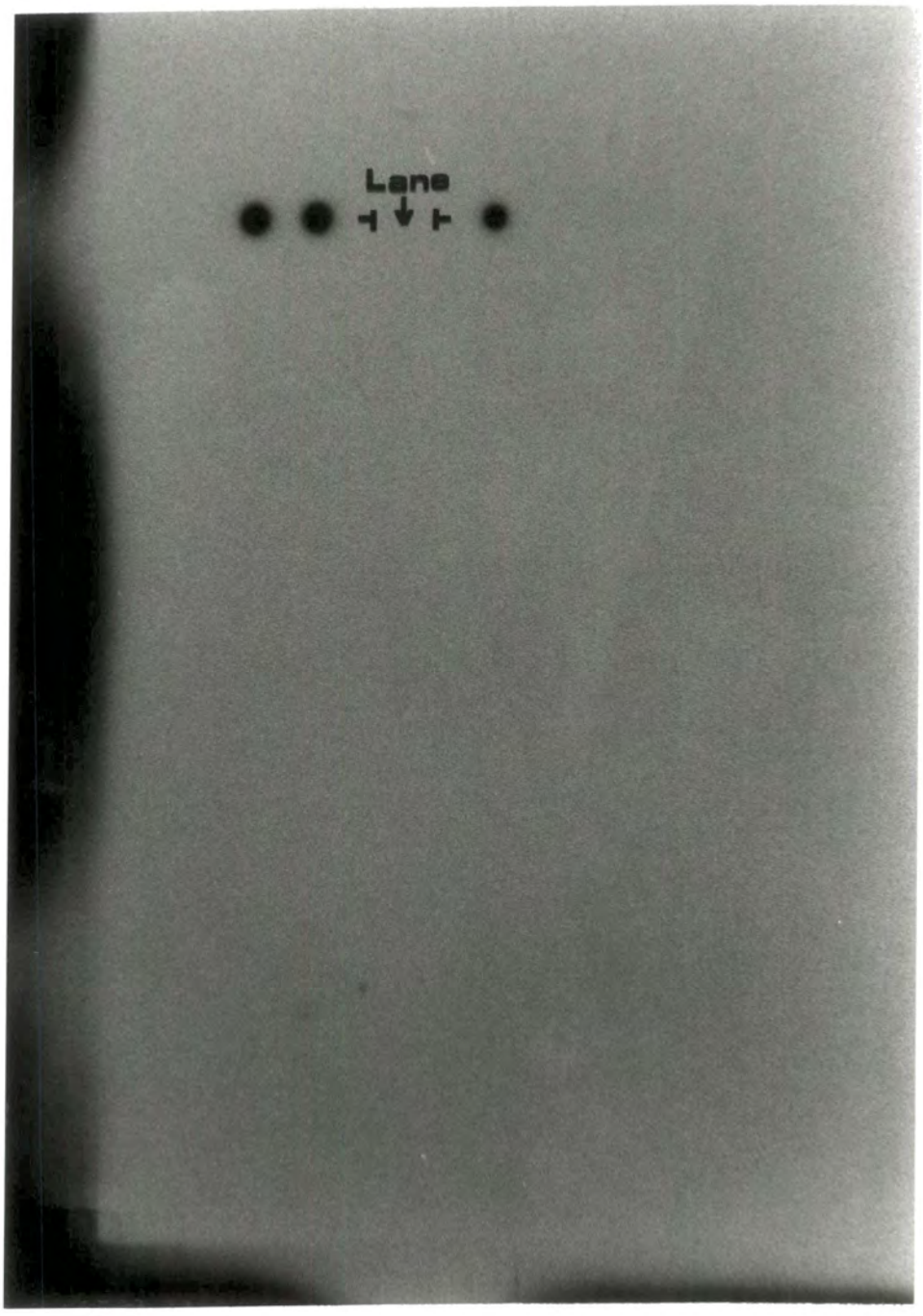

Fig. 3.11 Second attempt to prepare a autoradiograph to find the homologous extensin clone in pea pod. No band could be seen.

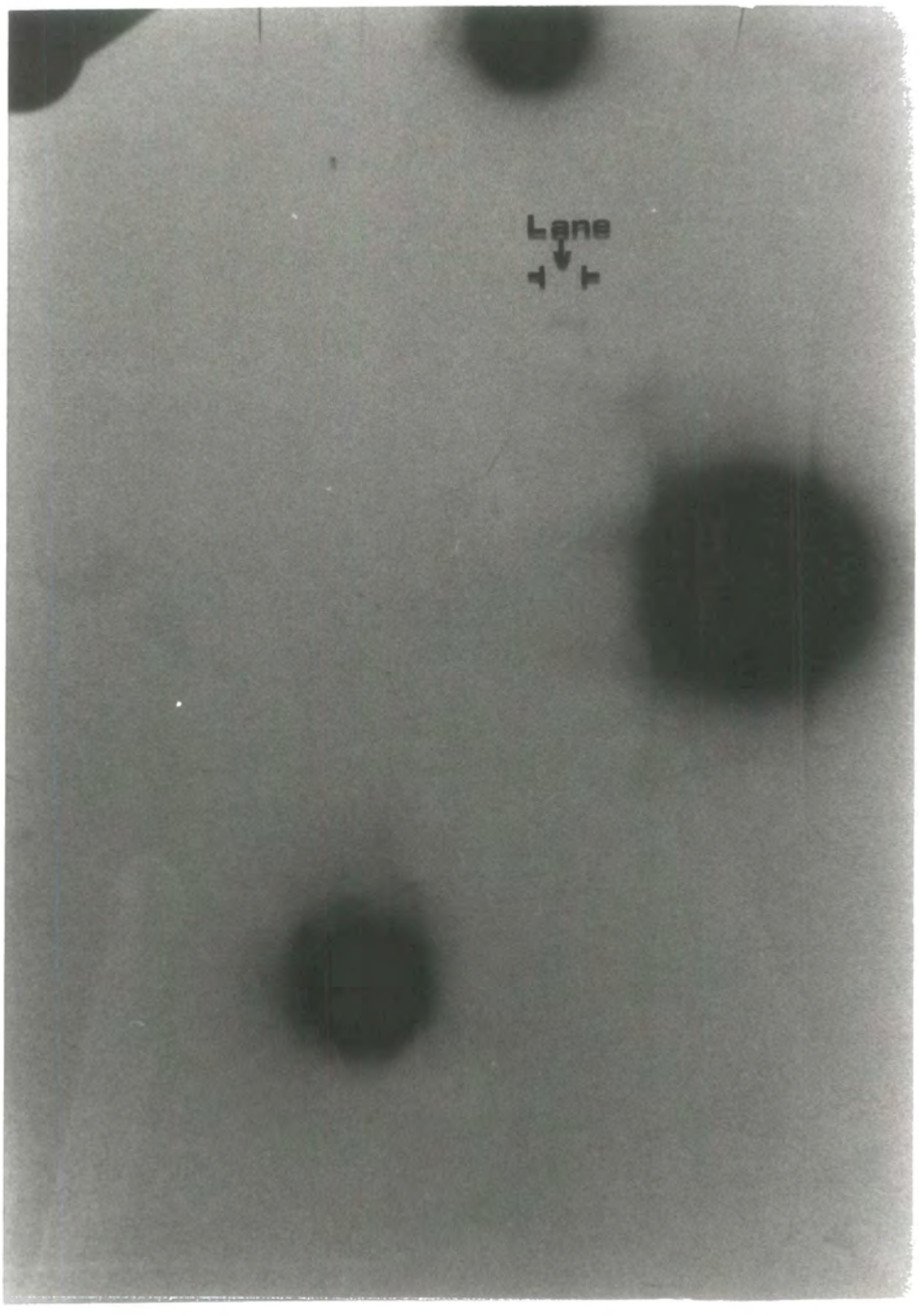

Fig. 3.12 Final attempt to prepare a autoradiograph to find the homologous extensin clone in pea pod. No band could be seen.

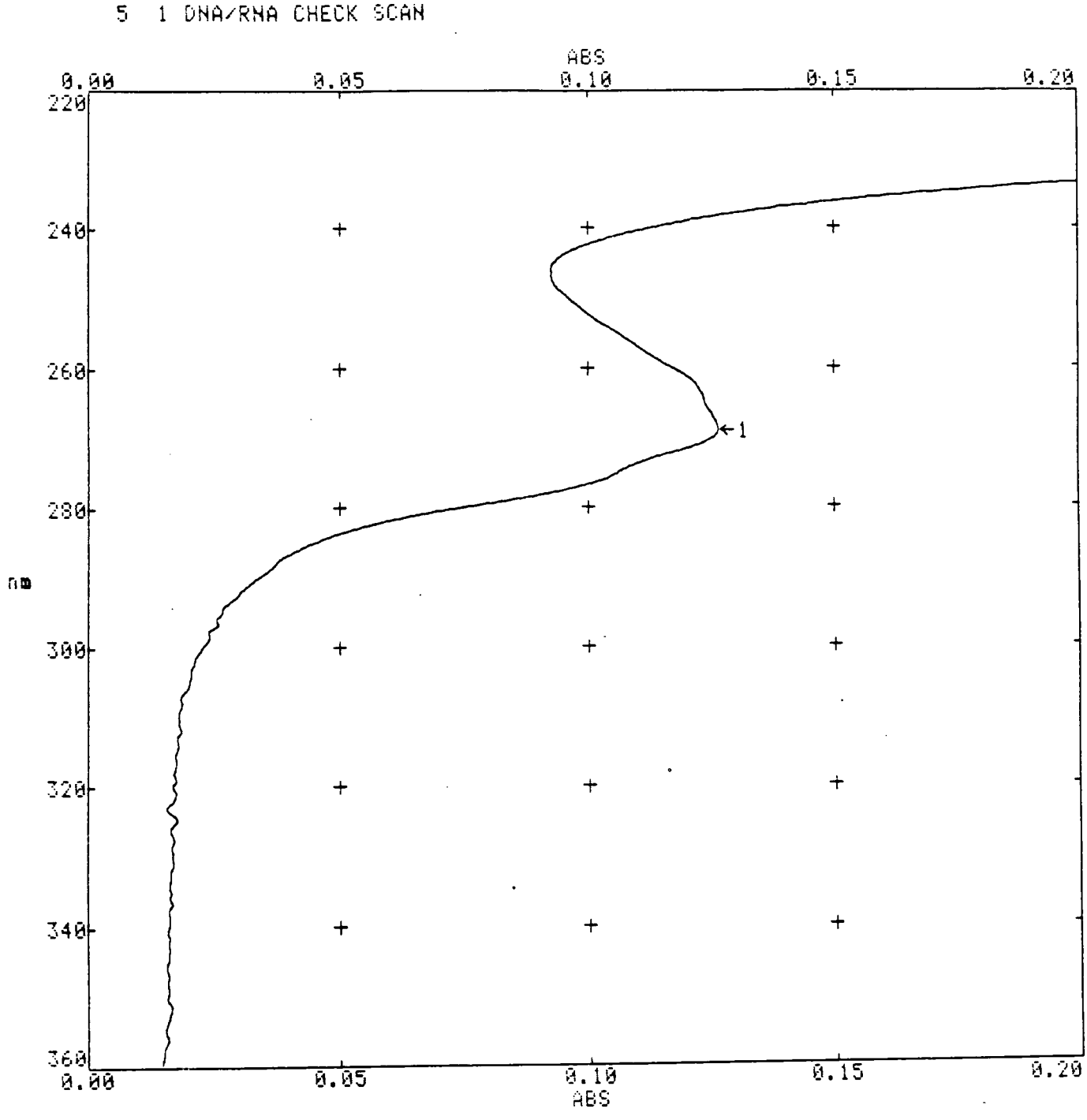

Fig. 3.13 UV absorption spectrum of the total RNA from pea pod.

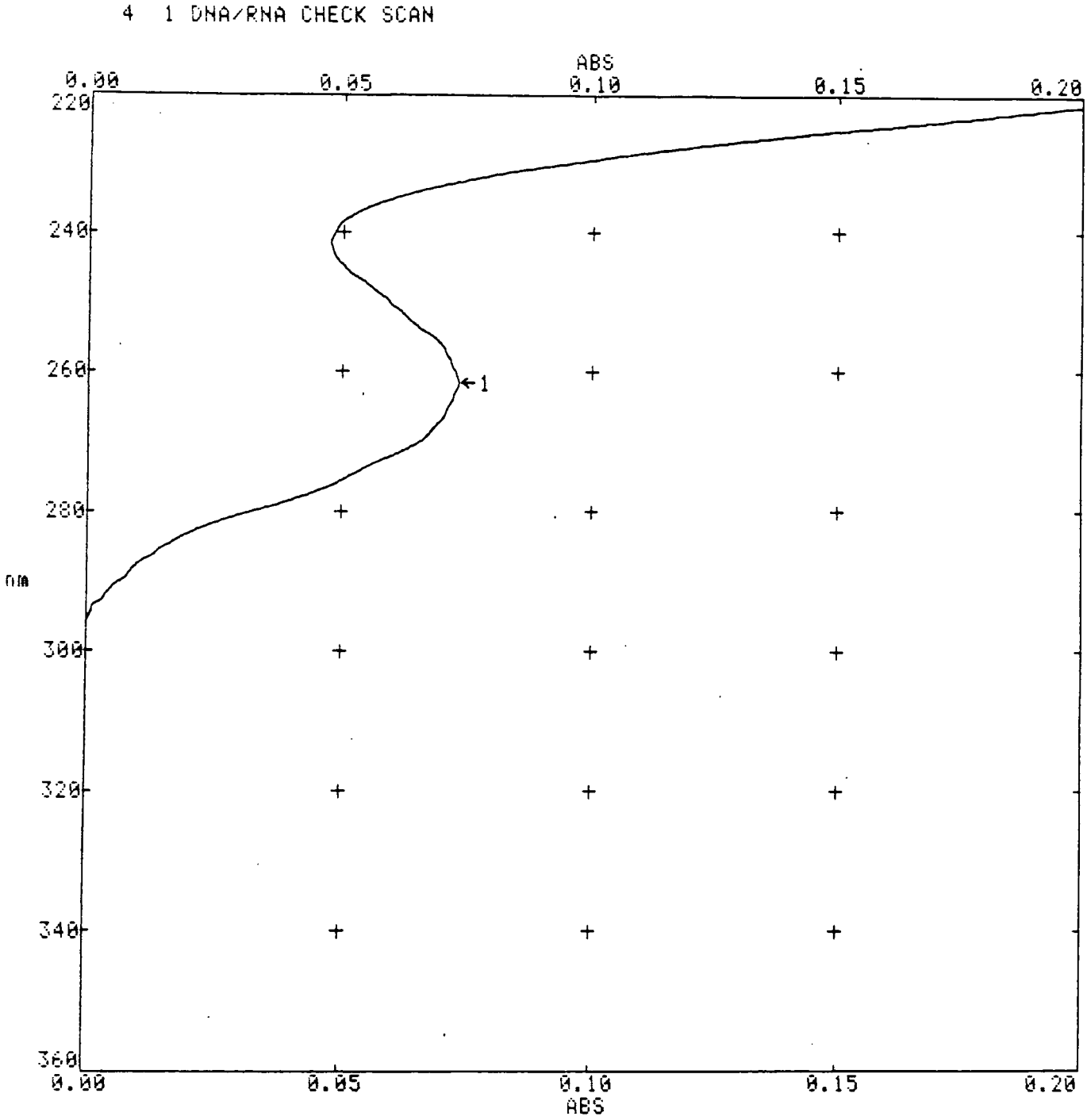

Fig. 3.14 UV absorption spectrum of the total RNA from pea leaf.

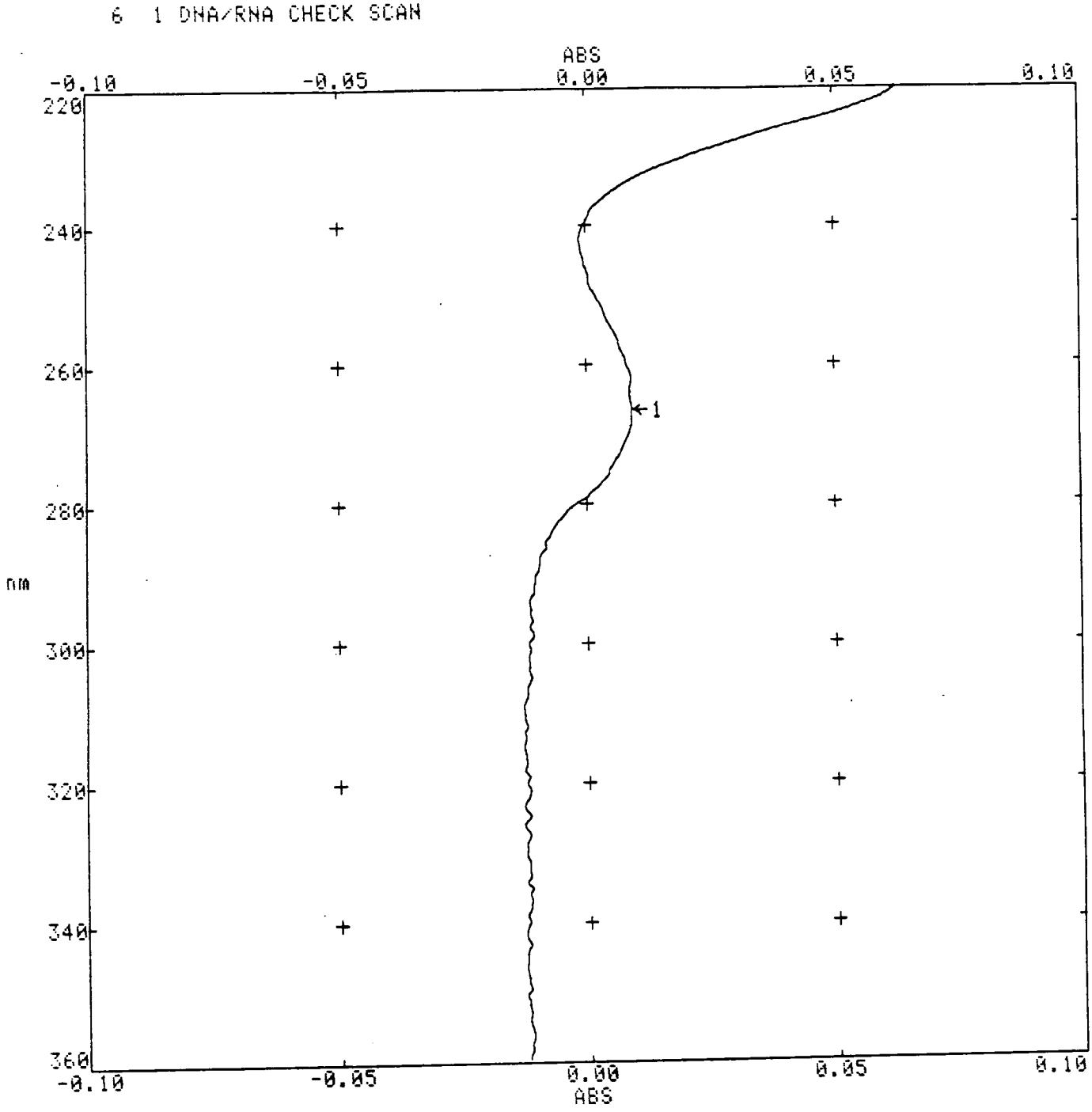

Fig. 3.15 UV absorption spectrum of the total RNA from pea seed.

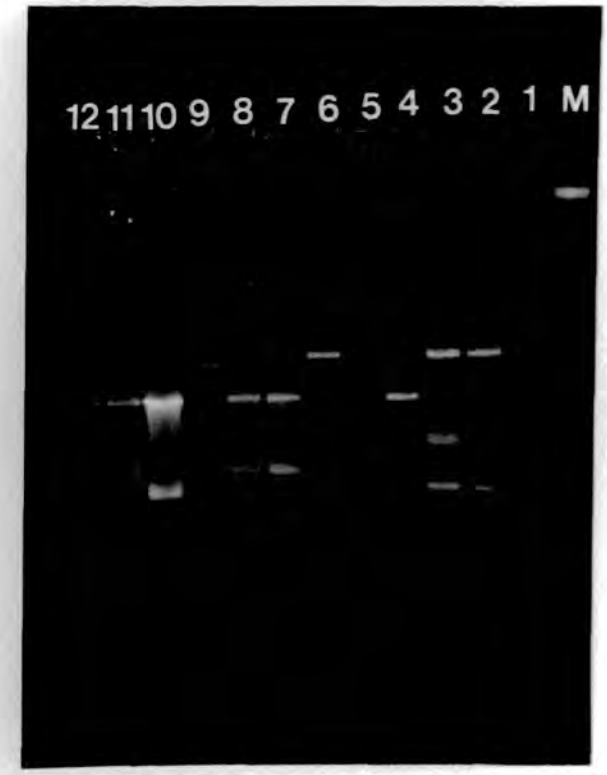

Fig. 3.16 12 abundant cDNA clones from purple pea pod restricted with EcoRI. Lambda DNA restricted with HindIII and EcoRI was used as a marker.

number	clone number	distance migrated (cm)	log bp	bp
1	585	14.3	3.23	1681
2	240	15.2	3.15	1437
3	354	14.8	3.18	1530
4	523	17.5	2.98	927
5	694	16.7	3.04	1084
6	954	14.7	3.20	1579
7	1052	17.5	2.97	927
8	1017	15.7	3.12	1308
9	1103	----	----	----
10	615	20.3	2.74	544
11	777	15.5	3.17	1483
12	496	----	----	----

Table 3.6 Insert sizes of pea purple pod abundant clone after restricted with EcoRI

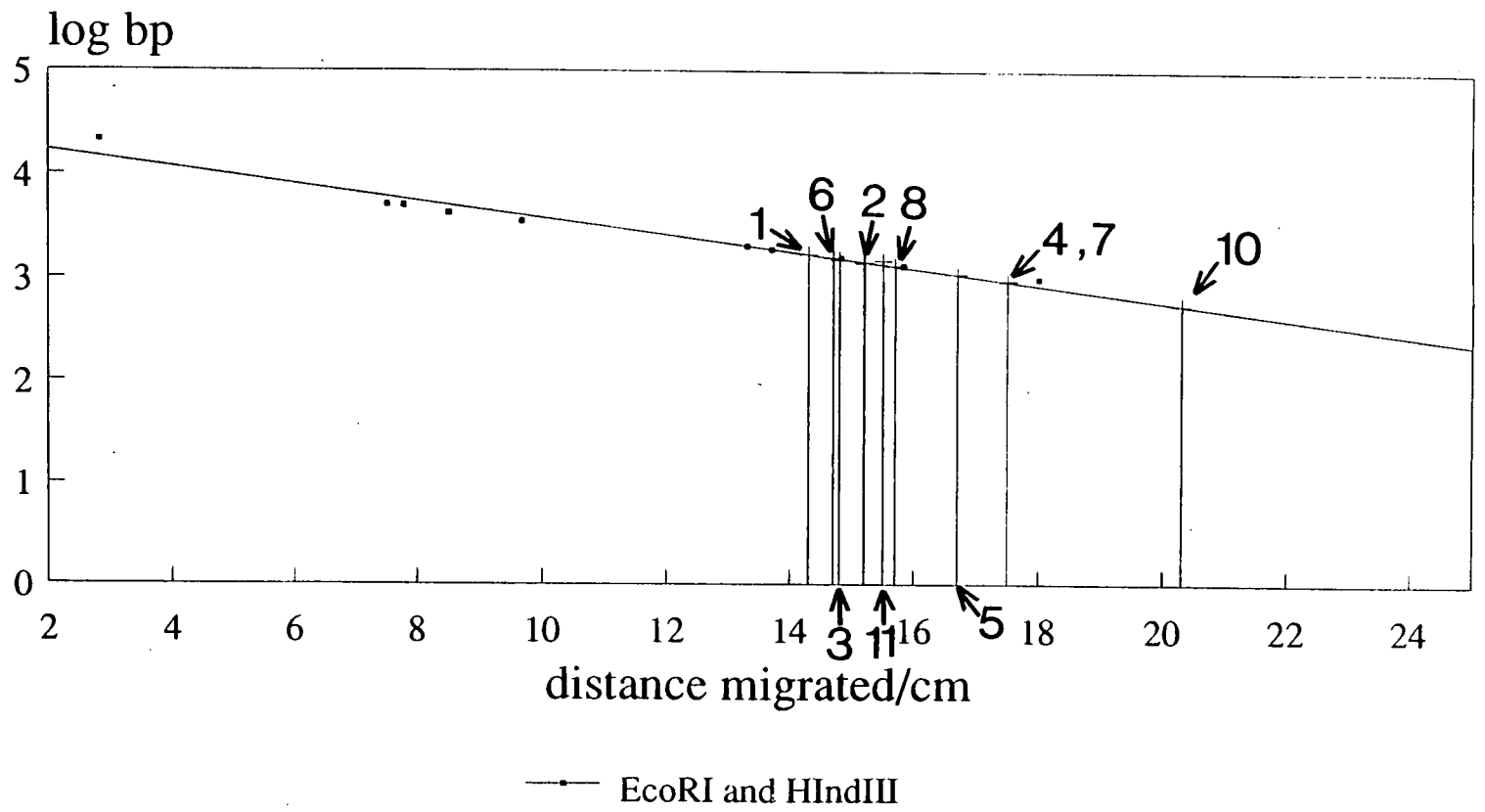

Fig 3.17 A calibration curve for log the size of fragments of lambda DNA with EcoRI and HindIII.

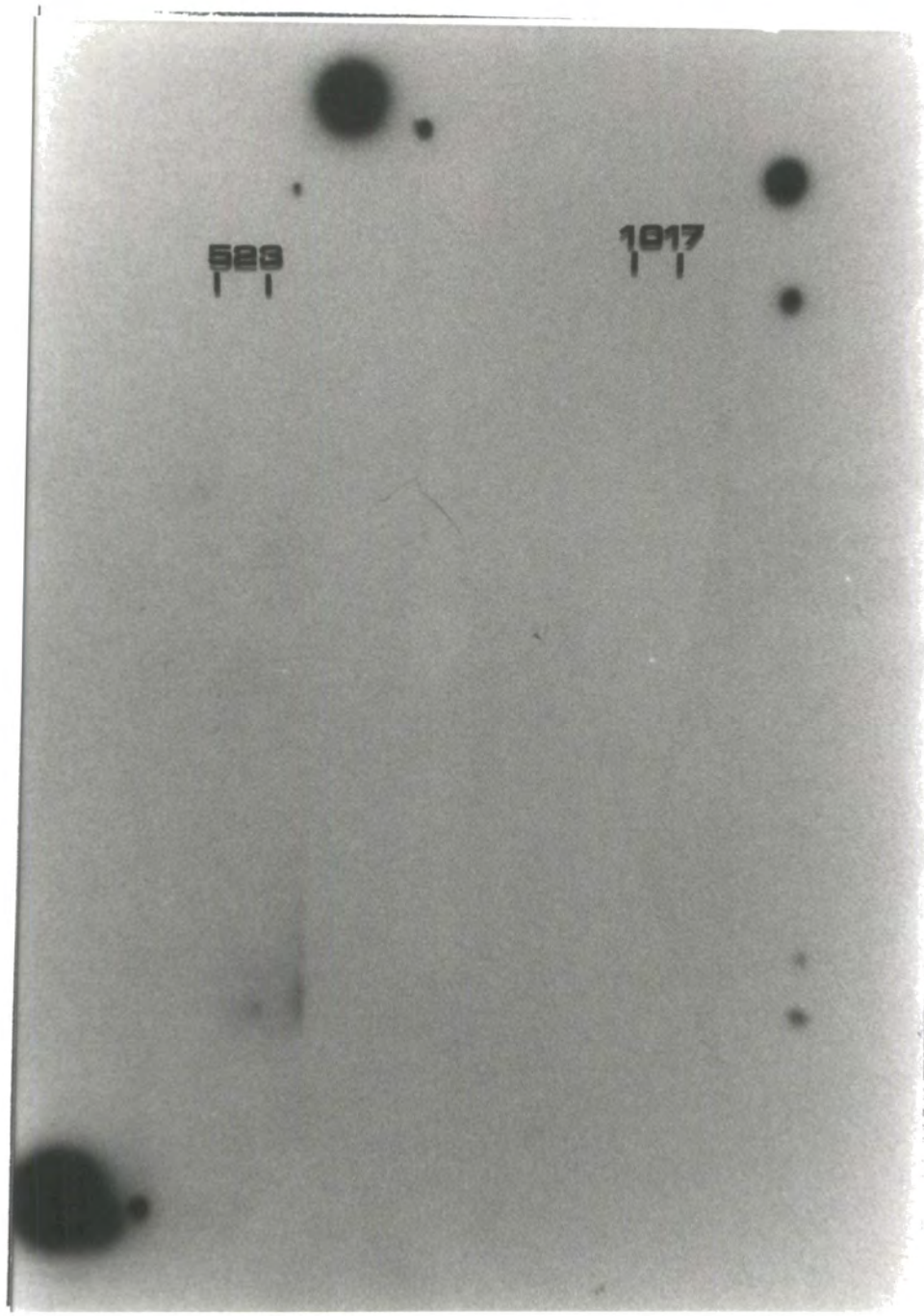

Fig. 3.18 Autoradiograph for the hybridization of labelled probes pPP523 and pPP1017 to the northern blot of leaf.

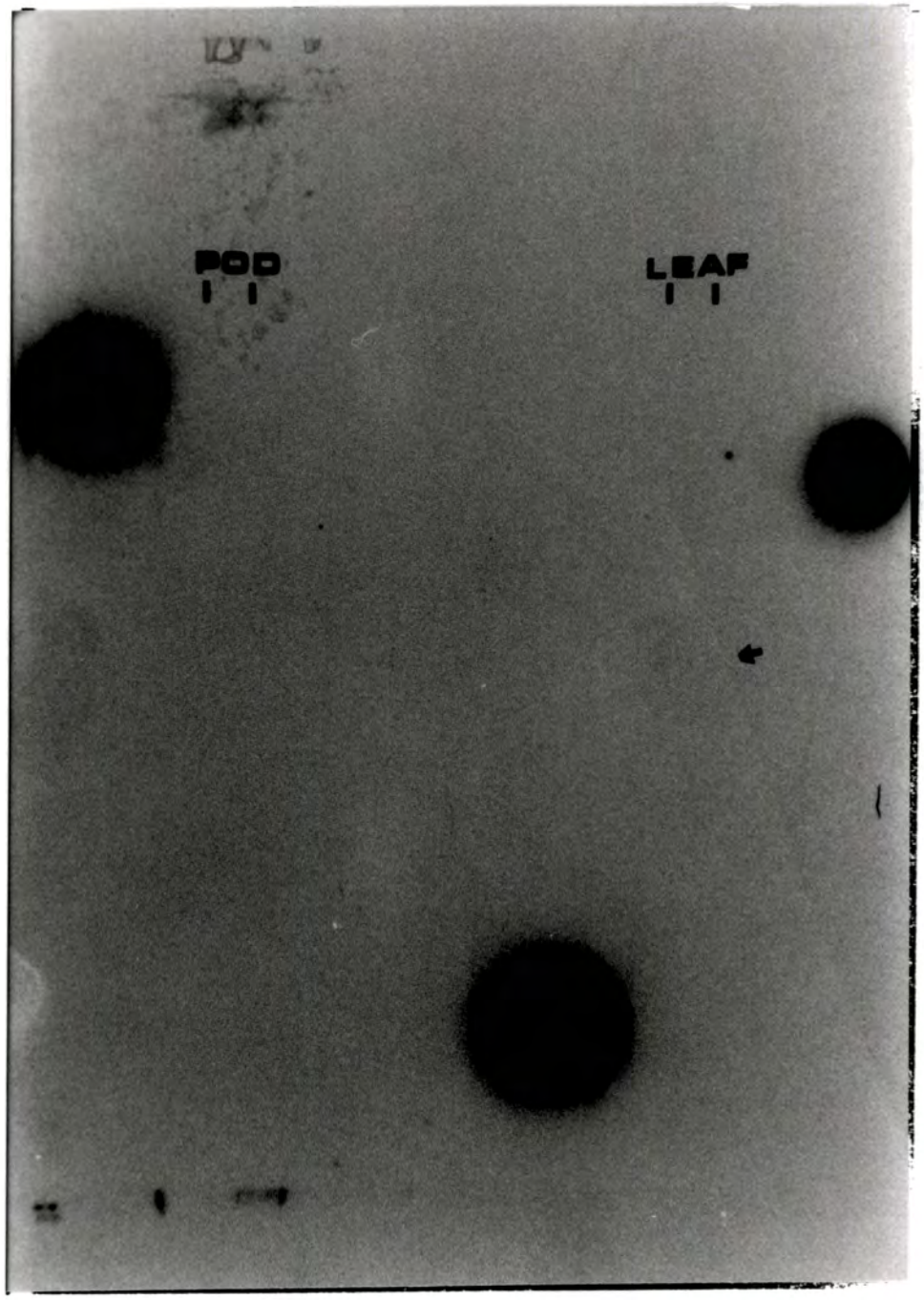

Fig. 3.19 Autoradiograph for the hybridization of labelled probe pPP523 to the northern blot of leaf and pod.

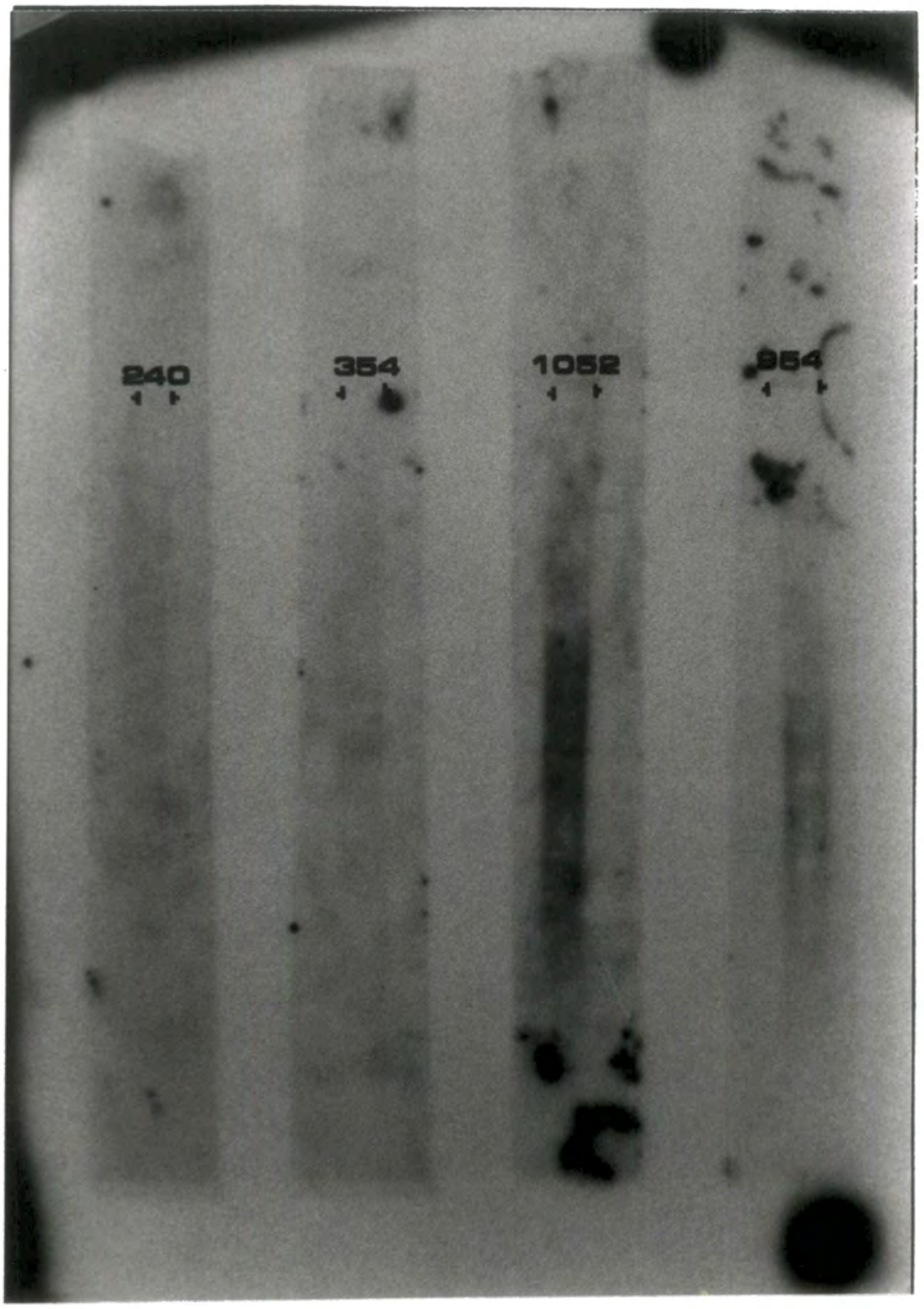

Fig. 3.20 Autoradiograph for the hybridization of labelled probes to the northern blot of pod.

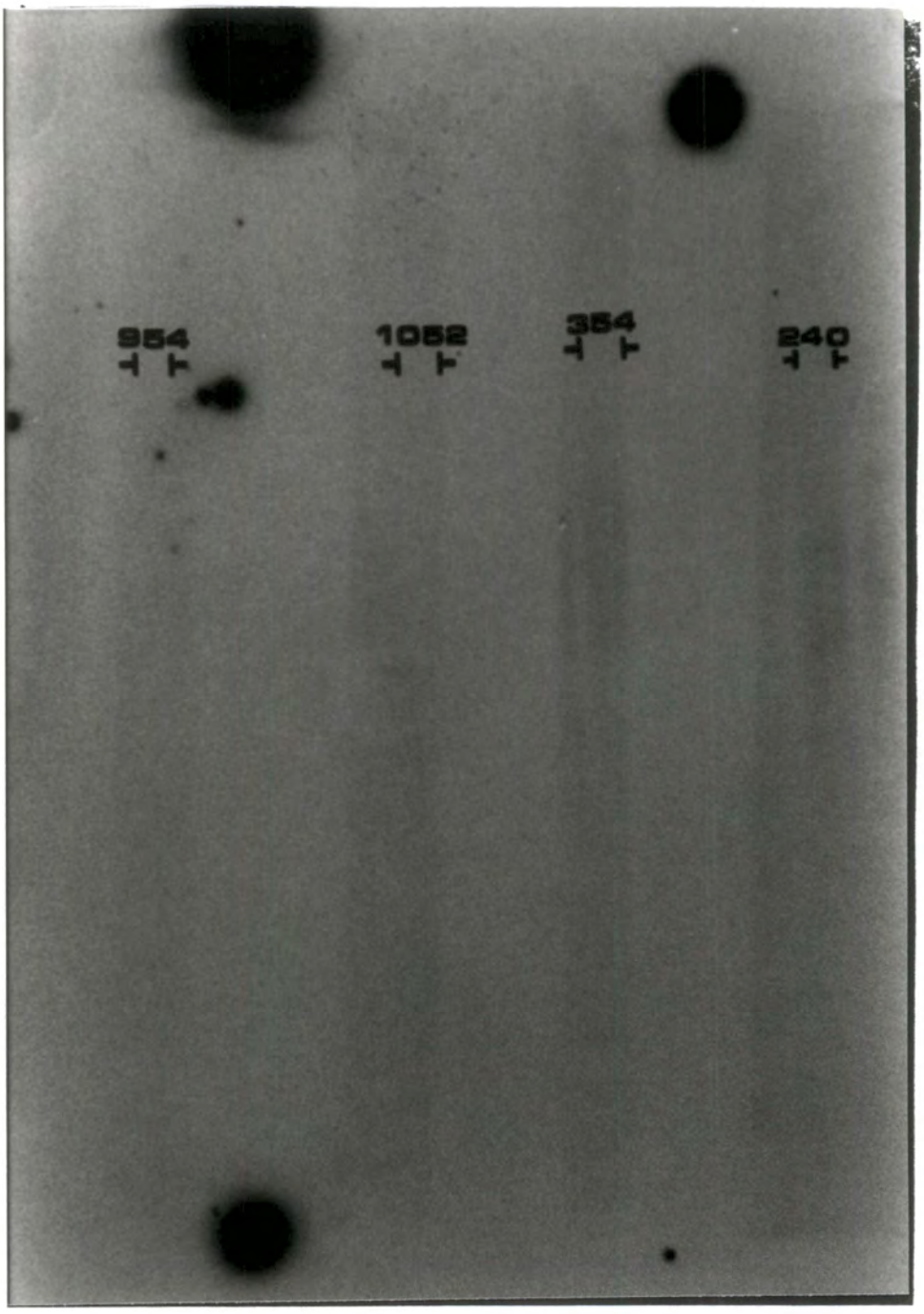

Fig. 3.21 Autoradiograph for the hybridization of labelled probes to the northern blot of leaf.

```
Protocol #: 7        Name:32P 1min  cpm              26-Jun-89   17:10
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 5899.00  2.60 4891.00 1185.5 557.
   2    1.00 7655.00  2.28 6312.00 1149.0 548.
   3    1.00 1086.00  6.06  896.00 1131.6 547.
   4    1.00 1271.00  5.60 1157.00 1284.3 543.
```

Table 3.7 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP523 & pPP1017. Tubes in slots one and two were supposed to be the desired fraction coming from pPP523, tubes in slots three and four were these coming from pPP1017. Each sample contained 3 µl of effluent from Sephadex G-50.

```
Protocol #: 7        Name:32P 1min  cpm              07-Jul-89   18:54
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    25.00 39.99     8.00 232.61 563.
   2    1.00 1369.00  5.40  896.00 823.11 562.
   3    1.00 2140.00  4.32 1536.00 876.92 565.
   4    1.00  459.00  9.33  322.00 812.42 556.
   5    1.00 1212.00  5.74  962.00 1125.4 562.
   6    1.00 9167.00  2.08 7666.00 1156.2 560.
   7    1.00 46061.0  0.93 36089.0 987.30 561.
(1 missing vial)
   9    1.00    17.00 48.50     7.00 945.21 565.
  10    1.00   159.00 15.86   120.00 921.74 561.
  11    1.00 5490.00  2.69 4144.00 931.49 558.
  12    1.00 1220.00  5.72  944.00 978.49 562.
  13    1.00 38655.0  1.01 32514.0 1100.3 560.
  14    1.00 82405.0  0.69 65738.0 963.90 569.
  15    1.00 41120.0  0.98 33745.0 1005.9 557.
```

Table 3.8 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP523 and pPP1017. Tubes in slots 2 and 3 were expected to be the peak fraction coming from pPP1017, tubes in slots 11 and 12 were these coming from pPP523.

Protocol #: 7 Name:32P 1min cpm 17-Jul-89 17:01
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	265.00	12.28	73.00	281.84	522.
2	1.00	140.00	16.90	60.00	346.99	522.
3	1.00	354.00	10.62	228.00	738.05	518.
4	1.00	85964.0	0.68	68513.0	999.06	524.
5	1.00	71668.0	0.74	55506.0	918.32	525.
6	1.00	7288.00	2.34	5924.00	1049.4	520.
7	1.00	1116.00	5.98	848.00	946.68	523.
8	1.00	9141.00	2.09	7692.00	1072.5	516.
9	1.00	16769.0	1.54	11336.0	748.94	518.
10	1.00	1798.00	4.71	1206.00	749.04	522.
11	1.00	623.00	8.01	392.00	313.62	518.
12	1.00	77.00	22.79	22.00	37.660	526.
13	1.00	3189.00	3.54	833.00	138.39	516.

Table 3.9 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP954. Tubes in slots 4 and 5 were expected to be the desired fraction.

Protocol #: 7 Name:32P 1min cpm 17-Jul-89 17:34
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	122.00	18.10	102.00	1049.3	520.
2	1.00	26.00	39.22	13.00	630.16	524.
3	1.00	91831.0	0.65	81722.0	1214.2	529.
4	1.00	21595.0	1.36	18059.0	1094.4	522.
5	1.00	16775.0	1.54	13284.0	1067.8	521.
6	1.00	14619.0	1.65	12418.0	1076.6	520.
7	1.00	21600.0	1.36	19480.0	1220.1	522.
8	1.00	12371.0	1.79	11269.0	1252.6	522.
9	1.00	3977.00	3.17	3584.00	1251.7	523.
10	1.00	439.00	9.54	331.00	911.11	522.
11	1.00	497.00	8.97	373.00	925.04	523.
12	1.00	86.00	21.56	64.00	798.26	522.

Table 3.10 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP1052. Tubes in slots 3 and 4 were supposed to be the labelled fraction.

Protocol #: 7 Name:32P 1min cpm 17-Jul-89

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	23.00	41.70	15.00	869.01	523.
2	1.00	35687.0	1.05	28108.0	1051.7	526.
3	1.00	119059.	0.57	102829.	1154.1	526.
4	1.00	5326.00	2.74	3454.00	741.12	523.
5	1.00	16409.0	1.56	13951.0	1133.7	526.
6	1.00	14900.0	1.63	12306.0	982.46	525.
7	1.00	1861.00	4.63	1526.00	907.34	518.
8	1.00	231.00	13.15	200.00	1170.9	522.
9	1.00	127.00	17.74	95.00	1029.0	519.
10	1.00	45.00	29.81	32.00	817.17	517.
11	1.00	45.00	29.81	27.00	640.39	524.

Table 3.11 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP354. Tubes in slots 2 and 3 were expected to be the labelled pPP354.

Protocol #: 7 Name:32P 1min cpm 18-Jul-89

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	28.00	37.79	13.00	599.22	516.
2	1.00	37.00	32.87	21.00	653.91	520.
3	1.00	69809.0	0.75	62360.0	1155.0	529.
4	1.00	38272.0	0.87	67032.0	891.05	521.
5	1.00	6629.00	2.45	4877.00	897.42	520.
6	1.00	7794.00	2.00	7370.00	849.97	515.
7	1.00	8508.00	2.16	7349.00	1101.7	519.
8	1.00	1402.00	5.34	1212.00	1128.2	515.
9	1.00	250.00	12.64	199.00	1016.1	520.
10	1.00	137.00	17.08	89.00	671.51	511.
11	1.00	80.00	22.36	64.00	951.38	518.

Table 3.12 Liquid scintillation counts in count per minute (CPM) for separation of radiolabelled pPP240. Tubes in slots 3 and 4 were expected to be the desired fraction.

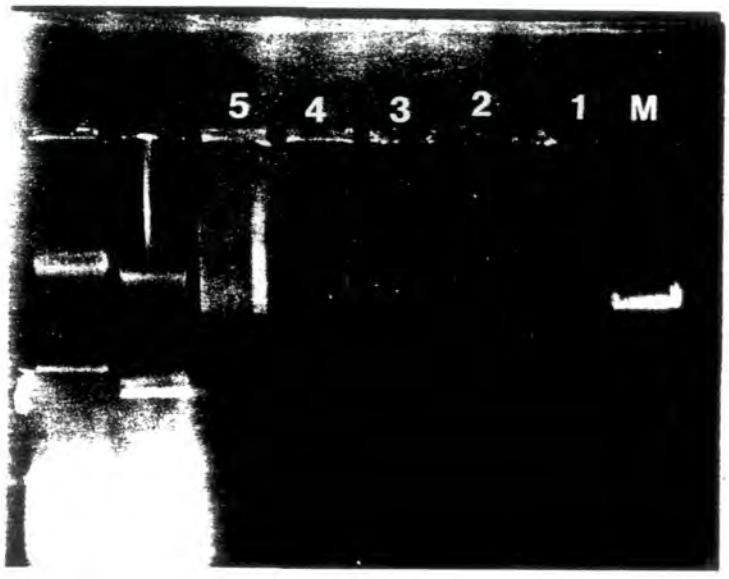

Fig. 3.22 Restriction of transformants of M13mp18 by EcoRI. Lane 5 was the control. M was the fragments of λ DNA restricted with EcoRI and HindIII.

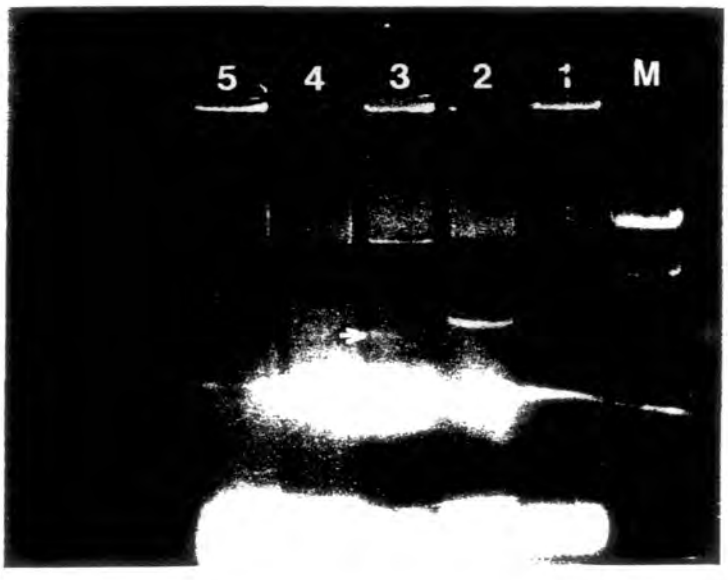

Fig. 3.23 Restriction of transformants by BalI and PstI. Lane 5 was the control. M was the marker.

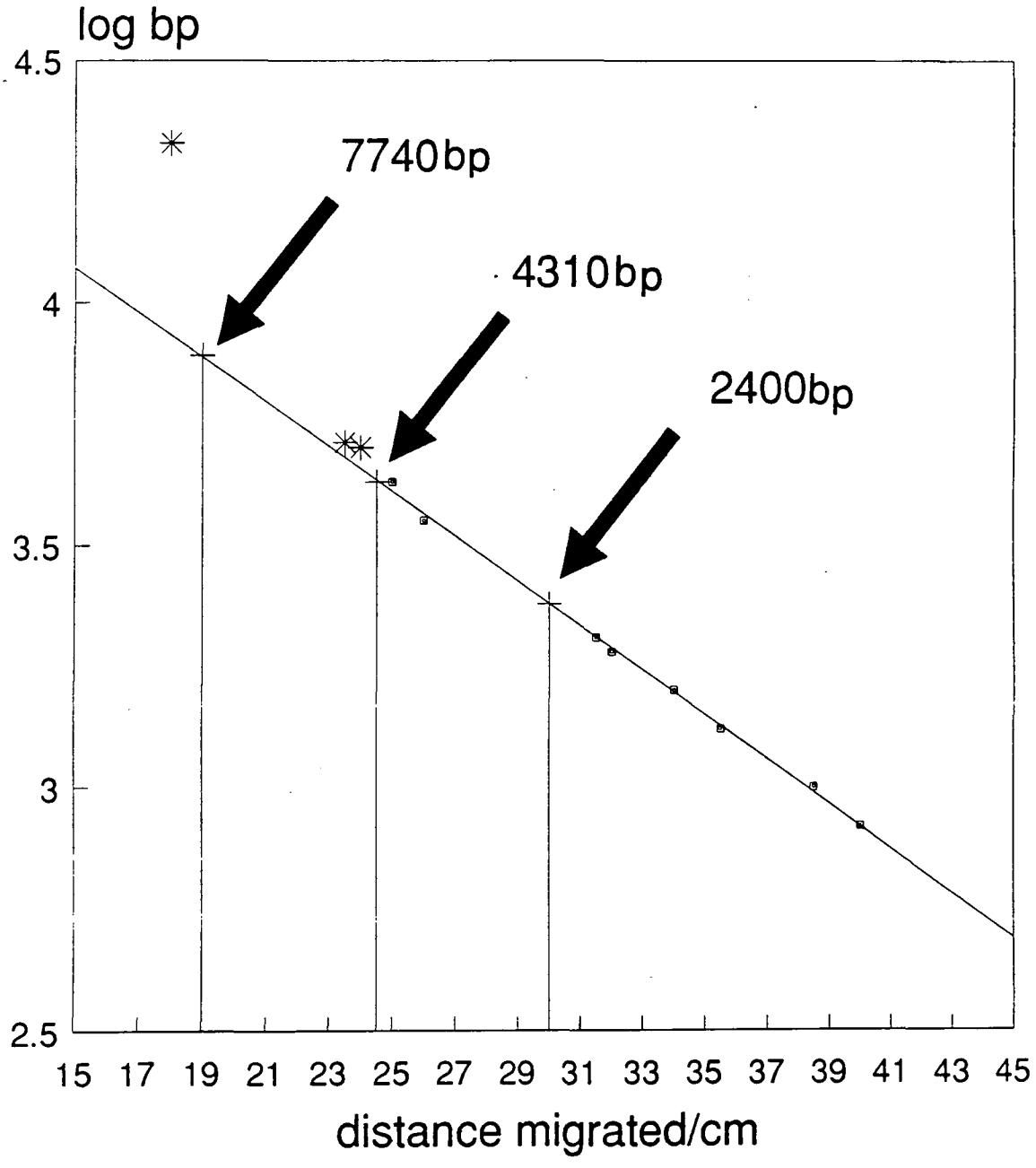

Fig. 3.24 A calibration curve for lambda restricted with HindIII and EcoRI.

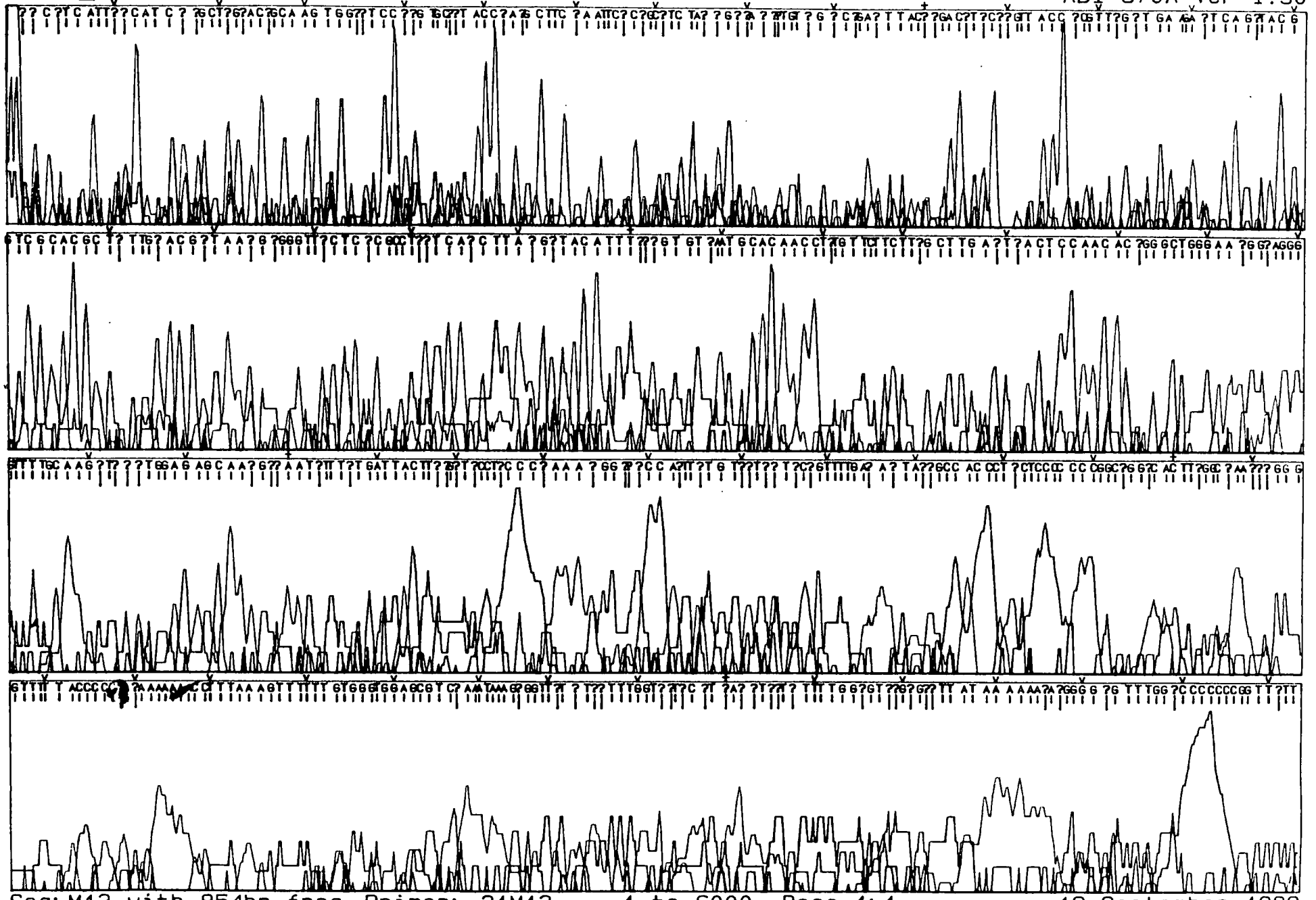

fig 3.25 DNA sequence of pPP1052

CHAPTER IV
DISCUSSION

CHAPTER IV
DISCUSSION

4.1 Preliminary Investigations

4.1.1 Restriction Analysis

During the project, some elementary works were done, as most of the experiments for molecular biology are very much depend on techniques. One of the most important techniques is the restriction analysis.

After restriction of lambda DNA with HindIII and EcoRI, sharp bands could be seen on the gel as shown in Fig. 3.1.

4.1.2 Transformation

In order to make a complete cDNA library, a high transformation efficiency was needed. JM83 strain of *E. coli* was used. The method of transformation by calcium chloride was employed. For maximum transformation efficiency, it was very important that the bacterial culture was in the logarithmic phase of growth.

After the transformation, the transformation efficiency for pUC18 was calculated. It has been found that with 13 ng of DNA added, the transformation efficiency was seen to be lower than that with 3.25 ng.

4.1.3 Protein Electrophoresis

Protein gel electrophoresis was done at the very beginning of the project to act as an elementary study. Protein was eluted from the plant tissues by the method of direct protein extraction (Method 2.2.4.1). The protein from pea pod was seemed to be quite different from that of pea leaf. As leaf tissue has shown to contain only a trace amount of Hyp (Cassab et al., 1985), which was am important component of extensin. However, pod tissue were expected to relatively rich in hydroxyproline (Van Etten et al., 1961). Moreover, pod was an important organ that provided protection for the

seeds inside, level of extensin should be higher as extensin has been known to involve in disease resistance of plants.

On the other hand, the difference between green pod and purple pod was only on the gene controlling the color of the pod, the proteins were seemed to have little differences.

4.2 Attempt to Use a Homologous Extensin Probe to Identify Extensin mRNA in Pea Pod RNA

In 1985, Chen & Varner isolated a partial cDNA clone from wounded carrot root mRNA. The clone was then used to isolate genomic clones of carrot, which was in turn used to identify the tomato extensin genomic clone (Showalter et al., 1985).

In both the carrot and tomato extensins, the two most prominent repeat sequences are Ser-$(Hyp)_4$ and Val-Tyr-Lys. The former is the site of the attachment of the oligoarabinosyl and galactosyl side chains, while the latter is the major non-glycosylated region that formed both intra- and intermolecular cross-links.

Moreover, hydroxyproline-rich proteins in soybean cell wall (Averyhart, 1988) and soybean cell culture (Hong et al., 1986) was also identified, both have the same pentameric repeat Pro-Pro-Val-Tyr-Lys.

It can be noticed that from the sequences of extensins identified so far, all of the prolines in the pentameric repeats are hydroxylated, the repeats are not continguous and are separated either by Ser-Hyp-Hyp or by Tyr-Lys (Smith et al., 1986).

From these experiments, it can be noticed that extensin probes from other species are used to identify the corresponding gene in other species, so it is reasonable to use the existing extensin probe to screen the cDNA library of other species to obtain a homologous extensin probe for that species. The corresponding gene regulation and expression can then be

investigated.

Moreover, as the protein sequence of the extensin containing an repeat sequence of Ser-$(Hyp)_4$, synthetic oligonucleotide can also be synthesized and used to screen the cDNA library. Those methods were more convenience than classical method to identify extensin.

Unfortunately, as shown from all the results of the attempt to localize a related mRNA species in total RNA preparation of pea pod, it was reasonable to deduce that pea pod contains sequences that has no homologous to carrot root extensin.

4.3 Attempt to Identify a Pea Pod Specific Clone

As shown before, the differences between the protein of pea pod and pea leaf are quite great, plus or minus screening technique can be used to identify cDNA clones from mRNA molecules present in one cell type but not the other (Old & Primrose, 1985).

Total RNA extractions by the modified guanidinium thiocyanate method were done on pea leaf, pod and cotyledon. From the amount of RNA collected, it was shown that the percentage of RNA extracted from pea cotyledon (0.012%) was lower than those from pea pod (0.035%) and pea leaf (0.045%). It is concluded that this method is not effective in isolating total RNA from pea cotyledon.

At the beginning, 2 of the abundant pea pod clones were selected that have short insert sizes. However, after several trials have been, no binding of the probes to the total RNA of pea pod and pea leaf could be seen. The possible explanations for those results are not enough radioactivity of the probes, or those clones have no similar sequences in pea pod or pea leaf.

As a result, four more clones were selected, they were labelled with a higher radioactivity and were used to hybridize to pea pod. At this time, hybridization can be seen for all of the clones, some (pPP954 & pPP1052) are

hybridized more strongly than the others (pPP240 & pPP354). On the other hand, no band can be seen from these hybridizations, the greatest possibility is that there are some degradations of the RNA during the gel electrophoresis process, further trials were needed to confirm the result.

The four labelled probes were also used to hybridize to the total RNA of pea leaf with the same conditions as before, two clones (pPP954 & pPP1052) were seen to hybridize strongly to pea pod but not pea leaf. As a result, it might be possible that those two clones were specific pea pod clones.

Insert fragments from pPP954 and pPP1052 were extracted from pUC18 and tried to ligate to M13mp18 for DNA sequencing.

After the ligation reaction and transformation, pPP1052 had three transformants and pPP954 had only one, but numerous blue plaques could be seen on the plates, this shown that the dephosphation of the linearized DNA was not so effective that some phosphate groups were not removed. Self-ligation of the linearized DNA occurred that caused a drop in the amount of recombinants. Moreover, the sizes of the fragments (927 & 1579 bp) were too big to fit in the phage that cause a lower in transformation efficiency

For the transformants picked, double-stranded M13mp18 was isolated from the cell pellet by the method 2.2.15.4.

At first, no restriction could be seen when restricted with EcoRI, this might due to not enough incubation time for the restriction enzyme (2 h), or there was a mutation in the sequence of the site of EcoRI that the enzyme could not recognize. But as the migration distance for the control was long than that of transformants, it was sure that there were inserts in those.

As a result, two restriction enzymes were used. One (Pst1) cut the phage closed to the site for EcoRI (position 6269), while the other (BalI) cut at position 5082. As the size of the whole phage was 7250, the

restricted fragment with insert from pPP1052 should be about

6269 bp - 5082 bp + 927 bp = 2114 bp,

while the restricted phage should be

7250 bp - (6269 bp - 5082 bp) = 6063 bp.

From the calibration curve obtained (Fig. 3.24), the migration distances of bands were quite similar to that expected. The band with 7740 bp would be the unrestricted M13mp18, the band with 4310 bp was the restricted remain of M13mp18, and the band with 2400 bp would be the restricted fragment with insert from pPP1052. For the expected transformant of pPP954, no M13mp18 could be extracted from the bacteria, the most possible explanation was that a colony rather than a plaque was taken from the plate.

As a result, only the single-stranded M13mp18 of pPP1052 could be isolated and taken to sequence using Sanger's dideoxynucleotide-sequencing method (1979).

Summary

1. During the extraction of plasmid from the clone encoding the *Brassica* root protein homologous to carrot root extensin by the method of miniprep, the plasmid obtained was not pure that would need a much longer incubation time for restriction with EcoRI before a complete restriction could be obtained.

2. Proteins were extracted from pea pod and pea leaf by the method of direct protein extraction, and was subsequently separated on a 12% polyacryamide gel. It was found that the proteins from pea pod and pea leaf looked quite different from each other, as leaf tissue has shown to contain only a trace amount of hyp, while pod were expected to rich in hydroxyproline.

3. Modified guanidinium thiocyanate method could be used to isolated sufficient amount of RNA from pea leaf (0.045% of tissue used) and pod (0.035% of tissue used), but not very effective for pea cotyledon (0.012% of tissue used).

4. Three trials have been done trying to find whether there was any homologous sequences to carrot root extensin was present in the total RNA of pea pod or not. No hybridization could be seen between the labelled probe and the northern blotting of total RNA of pea pod. It was reasonable to conclude that no homologous sequence of carrot root extensin was present in the total RNA extract of pea pod.

5. Abundant pea purple pod clones were picked up from the cDNA library of pea purple pod and screened by their degree of hybridization to the total RNA of pea pod and pea leaf. Two clones were selected that shown hybridization strongly to the total RNA of pea pod but not pea leaf. They were then taken and tried to clone into M13mp18 for DNA sequencing by Sanger method. By restriction analysis of the transformants, only one clone, pPP1052, could get a correct restriction pattern. Single-stranded M13mp18 was then purified from the culture and the DNA sequence was determined.

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