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Isolation of extC, an extensin gene from Brassica napus

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Isolation of *extC*, an extensin gene from *Brassica napus*

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Abstract

A gene coding for the cell wall hydroxyproline-rich glycoprotein extensin was isolated by constructing a size fractionated genomic library of oilseed rape (*Brassica napus*) in the plasmid vector pSK^+. The library was screened with the coding sequence of the *extA* extensin gene. The isolated gene (*extC*) contains a coding sequence of 780 nucleotides specifying a protein of 259 amino acid residues. The protein contains the Ser-Pro-Pro-Pro-Pro repeat motif, which is characteristic of extensins. Southern blots of *Hin*d III-digested oilseed rape genomic DNA hybridised against the *extC* probe revealed six hybridising fragments of sizes 2.5, 3.1, 3.4, 3.8, 7.7 and 10.7 kb. This suggests that *extC* is a member of an extensin multigene family present in the oilseed rape genome which is distinct from the *extA* gene family. Expression studies showed that the *extC* gene was not expressed in the leaves, petioles, stems and roots of healthy plants. Application of a wounding stimulus and treatment of leaves by incubation in abscisic acid (ABA), sodium salicylate and methyl jasmonate (MeJ) solutions also failed to induce expression of the gene. In contrast, the *extA* gene was induced by treatment of leaves with ABA, sodium salicylate and MeJ. Significant levels of *extA* transcripts were detected 12 h after leaves were treated with ABA, and stayed at a constant level for 36 h before increasing at 48 h. Treatment of leaves with sodium salicylate induced the accumulation of *extA* transcripts after 12 h and continued to increase until 48 h. In MeJ treated leaves, significant levels of *extA* transcripts were detected at 12 h, increased and reached a maximum at 24 h before decreasing at 36 h. These results suggest that the *extC* gene is regulated differently from the *extA* gene. *extC* may be transcriptionally activated by non-wounding stresses or may be expressed at developmental stages and organ and tissue types which have not been identified.

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Introduction

1.1 Cell wall

The cell wall is one of the most characteristic structures of plant cells. In the absence of cell movement the definition of the new wall is an essential step in plant morphogenesis, and wall elongation is the main mechanism of plant growth. Plant cells communicate with the environment and with other cells through structures in the wall. The cell wall is also an essential factor in protecting the plant against the attack of pathogens and pests, and in helping to alleviate stress conditions (José and Puigdomenech, 1993).

The dynamics of the cell wall have an obvious essential role in cell development. The components of the wall must interact with the elements that define the timing and the spatial distribution of synthesis and deposition of new materials in the growing cell in particular the cytoskeleton (José and Puigdomenech, 1993). This interaction is complex, and feedback effects have been shown. For instance, carbohydrate oligomers have been shown to act upon morphogenetic pathways (Ebenhard *et al.*, 1989; Marfa *et al.*, 1991), indicating that the control enzymes related to the metabolism of the cell wall components may play an essential role in the regulation of plant developmental processes.

The cell wall is a complex entity with unique characteristics related to the developmental stage of a given plant cell type. Most commonly, each cell within a tissue has its own wall. Moreover, the function of each cell is largely determined by its wall. A plant can not long exist unless its body is firmly knitted together and its organs possess collective mechanical strength. The many variations observed in plant cells reflect both quantitative and qualitative changes in their walls. The thickness and constituents of the cell walls are different (Cassab and Varner, 1988).

Plant cell walls are semi-rigid structures, which surround the cytoplasm membrane of the cell. In a plant tissue, the wall of each cell merges with the

walls of adjacent cells, giving the tissue physical coherence and strength. The morphology of a given plant tissue is thus determined by the morphology of the cell walls within it (Albersheim, 1976). In growing plant tissues, however, the cell walls are not simply rigid. The wall must expand as the cells grow, and new components must be added into the existing wall structure (Cleland, 1971). In addition, the walls must change in size, shape and chemical composition as the cells of the tissues differentiate.

All plant cell walls are made up of distinct layers. All layers have the same basic structure, as they are composed of microfibrils embedded in a polysaccharide matrix. However, they may differ from one another in several respects. The most important of the differences are (i) their thickness, (ii) the ratio of microfibrillar components to the matrix component, (iii) the orientation of the microfibrills within the matrix relative to the long axis of the cell, (iv) the nature of the matrix polysaccharides, (v) the degree of lignification and (vi) the water content (Goodwin and Mercer, 1988).

In general, two types of cell walls are present in plants: primary cell walls and secondary cell walls. Primary cell walls are the walls of growing cells (McNeil *et al.*, 1984). Primary cell walls are very thin and their functions are to control the rate of cell growth and to form the basic structural backbone of growing plant cells and tissues. The secondary cell walls are relatively thicker than the primary walls due to deposition of new layers of new cell wall materials after elongation and expansion of the cells has essentially ceased (Nicklas, 1992).

In higher plants, cell walls are composed of three layers called the middle lamella, the primary wall and the secondary wall. The middle lamella forms an intercellular layer between the primary walls of adjacent cells. It is the first layer to be formed when a cell divides and is therefore the initial partition between the newly formed daughter cells. The primary wall is formed upon the middle lamella from each side by daughter cells. The primary walls continue to grow and to increase in area and thickness while the daughter cells grow in size. They therefore expand to accommodate the increasing size of the daughter cells. Towards the end of their growth, deposition of the

secondary wall on top of the primary wall begins and continues until after growth has stopped. The extent to which the secondary wall thickens depends upon the type of cell into which the daughter cells have differentiated (Goodwin and Mercer, 1988).

1.2 The primary cell wall model

More than 300 years ago, cell biologists believed that the cell wall was a rigid box surrounding the protoplast. With advances in the determination of polymer structure using modern techniques and instrumentation, the view of the cell wall has altered from that of a static structure to one in which the wall represents a virtual extension of the cytoplasm.

In 1973, the primary cell wall model was published by Keegstra *et al.* (Figure 1.1). This model was based on the interconnections of the macromolecular components in the walls of suspension-cultured sycamore cells. In this model, Keegstra *et al.* (1973) concluded that all components of the wall were, with the exception of the connections between xyloglucans and cellulose microfibrils, covalently connected. Xyloglucans and cellulose microfibrils were shown to be connected by hydrogen bonds. Xyloglucan chains were shown to be covalently connected to the pectic polysaccharides (Figure 1.1) via interactions between the reducing ends of the xyloglucans and the galactan side chains of the rhamnogalacturonan. The pectic polysaccharides were covalently bound to the extensin cell wall proteins by attachment to the serine residues of the extensin through the arabinogalactan chains of the pectic polysaccharides (Keegstra *et al.*, 1973).

Soon after this primary cell wall model was published, it was accepted as the standard model of all dicot primary cell walls. Several years later, however, several groups of cell biologists (Monro *et al.*, 1976; Jarvis *et al.*, 1981; Ring and Selvendran, 1981; O'Neill and Selvendran, 1983) found that the glycosidically interlinked structure was incorrect for certain dicot primary cell walls. Moreover, Carpita (1987) and Monro *et al.* (1976) also proposed that the wall's coherence was primarily due to non-covalent interactions between its polymers.

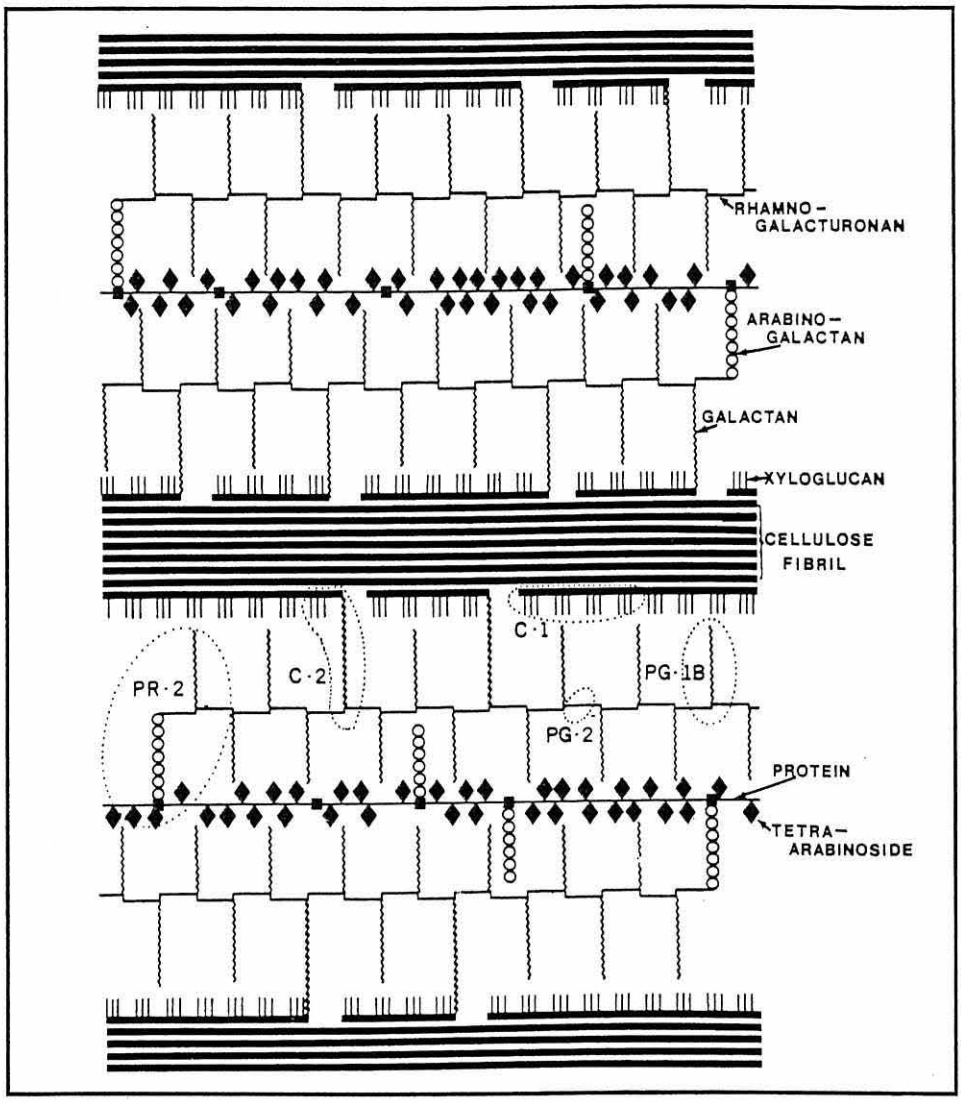


Figure 1.1. Primary cell wall model by Keegstra *et al.* (1973).

In 1992, Talbott and Ray claimed that they had obtained molecular size and separability data indicating that the sycamore wall model did not apply to pea. They now believe that their investigations have provided evidence, which best supports, instead, a model in which the cell wall polymers are non-covalently associated. Their work provided further evidence that the sycamore primary cell wall model was not valid for growing dicot primary walls in general.

Recently, Carpita and Gibeaut (1993) proposed a new model for the primary cell wall of dicot plants (Figure 1.2). They claimed that the model of Keegstra *et al.* (1973) failed to explain the direction of the microfibrils

movement relative to each other during cell expansion or elongation even though the sycamore model had elegantly illustrated possible mechanisms for growth.

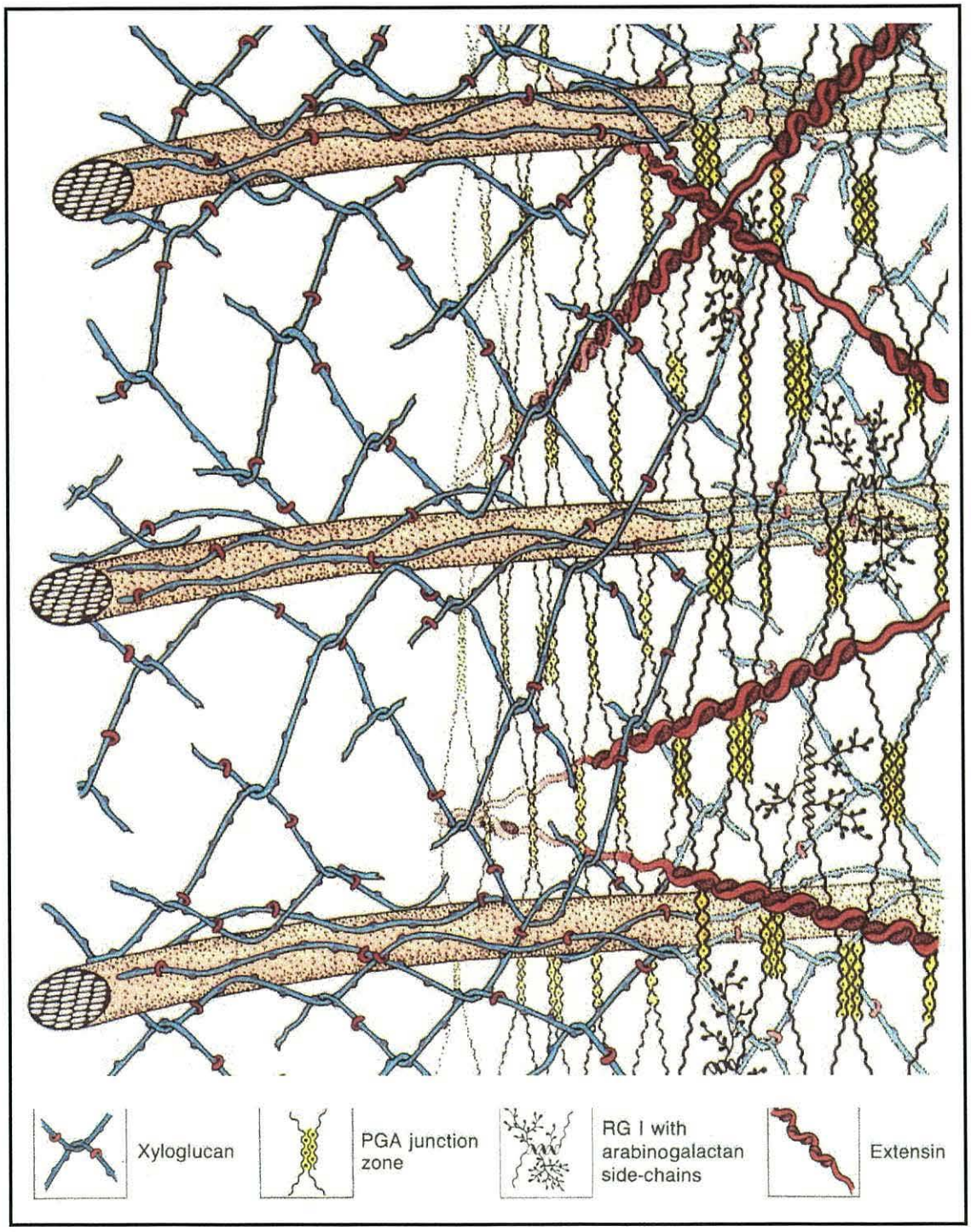


Figure 1.2. The primary cell wall model taken from Carpita and Gibeaut (1993).

In their primary cell wall model (Figure 1.2), Carpita and Gibeaut proposed that the cellulose microfibrils were hydrogen bonded to the xyloglucan chains and the cellulose-xyloglucan framework was embedded in an independent pectin matrix. Interestingly, Carpita and Gibeaut proposed that cross-linking of xyloglucans in the wall (in addition to tightly binding the xyloglucans to the cellulose microfibrils) strengthened the wall. Cleavage or dissociation of xyloglucans by hydrolases (or transglycosylases) loosens and separates the microfibrils resulting in wall expansion, but after displacement, extensin molecules inserted radially interlock the separated microfibrils to cease further stretching.

1.3 Cell wall components

Primary cell walls are composed of approximately 90% polysaccharides and 10% protein (McNeil *et al.*, 1984), and the proteins are themselves glycoproteins. In addition, there are other substituents such as methyl esters and ethers, and acetyl and feruloyl esters. Cellulose is the main polysaccharide component of the cell wall. It constitutes about 20 - 30% of the total polysaccharide component of the primary wall (McNeil *et al.*, 1984). Other polysaccharide components of the primary cell wall are xyloglucans, chitin, β-1,4-mannans, β-1,3-xylans, glucomannans, galacto-glucomannans, β-D-glucans and pectic substances such as polygalacturonic acid and rhamnogalacturonan I (McNeil *et al.*, 1984).

The primary cell wall proteins can be divided into two classes - the structural proteins and the proteins that have enzymatic activities. There are three major classes of structural proteins in primary cell walls. These three classes include the hydroxyproline-rich glycoproteins (HRGPs), the proline-rich glycoproteins (PRPs) and the glycine-rich glycoproteins (GRPs) (José and Puigdomenech, 1993). Amongst these three classes of structural proteins, the best-known and characterised structural proteins are the HRGPs. These three protein classes may be related to each other, most obviously because each of them, with the exception of the GRPs, contain

hydroxyproline, and less obviously in the case of the GRPs because they have amino acid sequence similarity to the HRGPs (Showalter, 1993).

Various enzymes are located in the primary cell walls of higher plants such as peroxidases, a family of hydrolases, xyloglucan endotransglycosylase (XET), pectinesterases and polygalacturonase (McNeil *et al.*, 1984).

1.4 Polysaccharides

Polysaccharides can be divided into two groups - the microfibrillar polysaccharides and the matrix polysaccharides (Brett and Waldron, 1990). The microfibrillar polysaccharides are very long unbranched molecules which, within the cell wall, are aggregated together in bundles called microfibrils. Within most type of microfibrils the polysaccharides are parallel to each other. In addition, the majority of the molecules constituting the core of the structure are so regularly arranged that they form a highly crystalline phase (Goodwin and Mercer, 1988).

The microfibrils are embedded in a non-crystalline polysaccharide matrix. The matrix polysaccharides are the non-cellulosic components, and can be divided into hemicellulosic and pectic compounds. The matrix polysaccharides are more often than not multi-branched molecules containing several different species of monosaccharide residues. The matrix is often described as "amorphous" but this cannot always be the case for it is clear that some relatively linear matrix polysaccharides lie parallel to the microfibrils (Goodwin and Mercer, 1988).

1.4.1 Microfibrillar polysaccharides

Cellulose is the most common microfibrillar polysaccharide found in the plant. However, three other microfibrillar polysaccharides do occur. These are chitin, which is present in the majority of fungi (the remainder have cellulose), the mannans which occur in the green algal families *Codiaceae*, *Dasycladaceae* and *Derbesiaceae*, and the xylans which occur in the green algal families *Bryopsidaceae*, *Caulerpaceae*, *Udotuceae* and *Dichotomosiphonaceae* (Goodwin and Mercer, 1988).

1.4.1.1 Cellulose

Cellulose is the best known of all the plant cell wall polysaccharides, and is particularly abundant in the secondary cell wall (McNeil *et al.*, 1984). It is characterised by long chains of unbranched D-glucose residues linked by β(1→4) glycosidic bonds. They vary considerably in length, and the average number of glucose residues is about 8000 per molecule (Goodwin and Mercer, 1988). In the secondary wall, the number of D-glucose of cellulose may be as high as 15000, but in the primary wall it is considerably less, in the region of 3 - 5000 (Smith, 1993).

A cellulose microfibril consists of a bundle of cellulose molecules arranged with its long axis parallel to that of the other molecules. The cross-section of the bundle is usually oval. The cellulose molecules constituting the core of the microfibril are arranged in a perfect three-dimensional array and thus constitute a crystal lattice (Goodwin and Mercer, 1988). The cellulose chains within the microfibril are stabilised by hydrogen bonds formed between (intermolecular) and within (intramolecular) chains. X-ray diffraction patterns indicate the presence of hydrogen bonds between the pyranose ring oxygen atom (O-5) of one glucose residue and the C-3 hydroxyl of an adjacent residue in the same chain (intramolecular bonding) (Goodwin and Mercer, 1988). Bonds between chains (intermolecular bonding) occur between hydroxyl groups at C-6 and the oxygen of the glycosidic linkage (O-1) in adjacent chains (Northcote, 1972). This arrangement has therefore made the cellulose of secondary cell wall have a rather high degree of crystallinity (McNeil *et al.*, 1984). The situation is however less clear in primary cell walls.

Since the cellulose molecules have a range of lengths (as determined by the degree of polymerisation) and so do the microfibrils, it is evident that relatively few cellulose molecules will run the whole length of the microfibril. Most will not. There will therefore be regions along the length of the microfibril where some cellulose molecules end, others begin and the majority passes straight through. In these regions the crystal lattice is distorted to some extent (Goodwin and Mercer, 1988).

Within the plant kingdom there appear to be two types of cellulose microfibrils which differ in their cross-sectional area. The cross-sectional areas of the microfibrils of all higher plants and most lower plants have external long and short diameters of 8.5 and 4.5 nm respectively. The long and short diameters of the crystalline core of these microfibrils are 5 nm and 3 nm respectively. The cross-section of the crystalline core is estimated to cut across about 50 cellulose molecules. In certain green algae, in particular *Valonia* and members of the Cladophorales, the external cross-sectional diameter of long and short microfibrils are 18.5 nm and 11.5 nm respectively with a crystalline core of long and short diameters of 17 nm and 11 nm respectively. The cross-section of the core is estimated to cut across about 500 cellulose molecules (Goodwin and Mercer. 1988).

1.4.1.2 Chitin

Chitin constitutes the microfibrillar component of the cell walls of most fungi. It consists of unbranched long chains of N-acetyl-D-glucosamine residues linked by β(1→4) glycosidic linkages. The arrangements of chitins in the microfibril are analogous to that of the celluloses. X-ray diffraction studies show that the microfibril is highly crystalline. The chains of chitin within the microfibril are stabilised by intermolecular hydrogen bonding between the C-3 hydroxyl group of one N-acetyl-D-glucosamine residue and the glycosidic oxygen of the next chain. The chitin molecules within the microfibril are anti-parallel (Goodwin and Mercer, 1988).

1.4.1.3 β-1,4-Mannans

These types of microfibrillar molecules are found in the cell walls of the green alga genera *Acetabularia*, *Batophora*, *Codium*, *Cymopolia*, *Dasycladus*, *Derbesia*, *Halicoryne* and *Neomeris*. They are remarkably simple and uniform. The β-1,4-mannan molecules are composed of D-mannose residues linked together by β(1→4) glycosidic bonds. The β-1,4-mannan molecules are aggregated together within the cell walls of these algae as microfibrils that are structurally analogous to cellulose microfibrils (Goodwin and Mercer, 1988).

1.4.1.4 β-1,3-xylans

These types of microfibrils are embedded in a matrix that is rich in glucans. The microfibrils are made up of β-1,3-xylan molecules, which are unbranched chains of D-xylose residues linked together by β(1→3) glycosidic linkages. The ratio of xylan to glucan is about 4:1 except for the genus *Bryopsis* in which the ratio is about 1:1. The crystalline structure of these microfibrils is quite different to that of cellulose or the β-1,4-mannan microfibrils. This arises because the β(1→3) glycosidic linkages of the xylan cause its chain to curve (Goodwin and Mercer, 1988).

1.4.2 Matrix polysaccharides

The matrix polysaccharides of the plant cell wall are divided into two classes - the hemicelluloses and the pectins. This classification is based upon solubility differences between these two classes of matrix polysaccharides. The hemicelluloses are a group of polysaccharides which are extracted by 4 M KOH solution at room temperature, whilst the pectins are a group of polysaccharides which are extracted from the cell wall by prolonged treatment with boiling water (Goodwin and Mercer, 1988)

1.4.2.1 Hemicelluloses

Xyloglucans (XG) are the best studied of the hemicellulose polysaccharides (Bauer *et al.*, 1973). They are the major components of the polysaccharides in the primary cell walls of plants (de Silva *et al.*, 1993). Xyloglucans are also the major cell wall storage polysaccharides in certain seeds including that of nasturtium, *Tropaeolum majus* L. (Edwards, 1985). The basic structure of xyloglucans consists of a backbone of β(1→4)-linked D-glucosyl residues with D-xylosyl side chains α-linked to O-6 of some of the glucosyl residues (Darvill *et al.*, 1980). Additional side chain residues, β-D-galactosyl and α-L-arabinosyl are added to the O-2 of some xylosyl units (Bacic *et al.*, 1988). Addition of an α-L-fucosyl residue to the O-2 of a subtending galactosyl unit to produce a trisaccharide side chain attached to

alternate heptasaccharide units, will produce a nonasaccharide (Figure 1.3) (Carpita and Gibeaut, 1993).

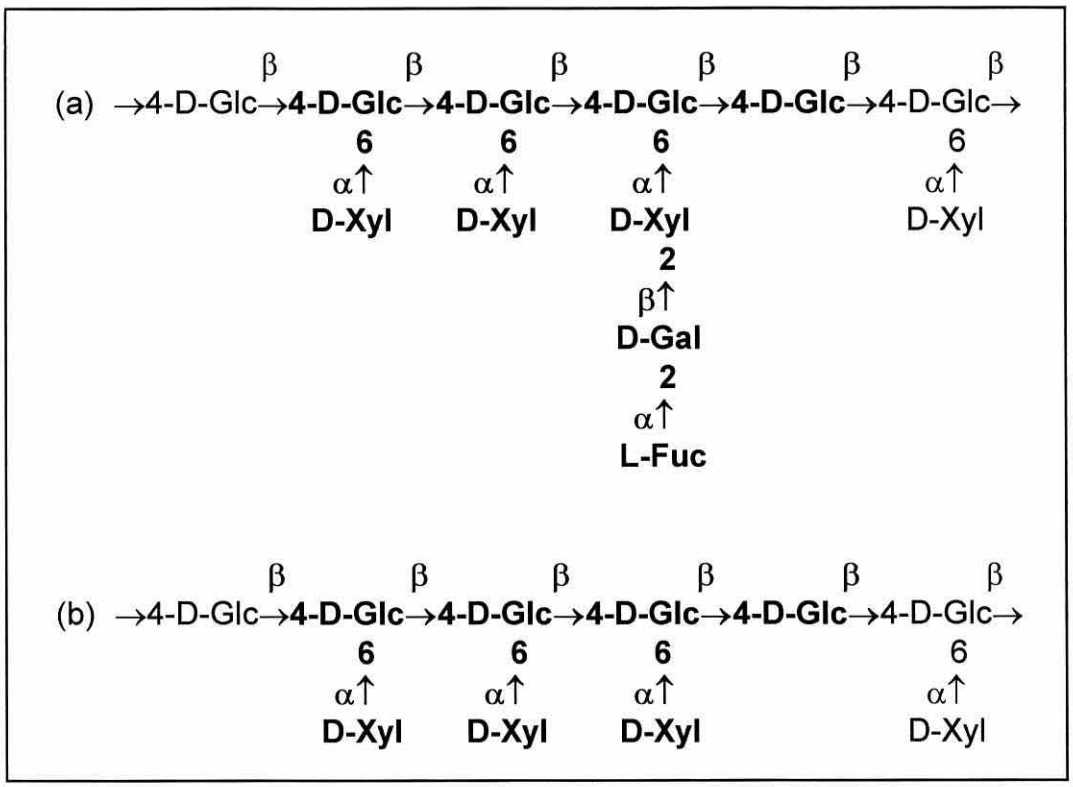


Figure 1.3. A nonasaccharide (a) and heptasaccharide (b) units of xyloglucan.

The presence of the trisaccharide side chain in the XG backbone has two significant functions. First, the trisaccharide blocks the cellulose-binding side of the chain, but in the second, it could straighten the backbone (Carpita and Gibeaut, 1993). Levy *et al.* (1991) proposed that this straightened conformation may facilitate the close packing of XG to cellulose.

The primary cell wall contains about equal amounts of XG and cellulose. Not all of the XG coats the cellulose microfibrils. Much of the XG must span the milieu between the microfibrils (Carpita and Gibeaut, 1993). Electron microscopy analysis shows that the XGs are in the range between 20 nm to over 700 nm long, but most of them are about 200 nm (McCann *et al.*, 1990) and these are long enough to span the distance between two microfibrils and bind to each of them (Carpita and Gibeaut, 1993). Extracted

XGs are soluble in aqueous solution and interaction amongst the XG chains has also been shown (Carpita and Gibeaut, 1993).

X-ray diffraction patterns of *Tamarindus* XG show that it forms crystalline structures when intermolecular water evaporates (Taylor and Atkins, 1985). Millane and Narasaiah (1992) have suggested that several conformations of side chain groups could stabilise the linear structure of XGs. These studies therefore suggest that XGs may occupy two distinct regions *in muro* - one binds tightly to the exposed faces of glucan chains in the cellulose microfibrils, and a second spans the distance to the next microfibril and simply interlocks with other XG molecules to space and lock the microfibrils into place (Figure 1. 2) (Carpita and Gibeaut, 1993).

A regulatory function for XGs has also been proposed. They are believed to be involved in the control of cell wall elongation (Labavitch, 1981). This function was evidenced by the finding that small amounts of XG were released from the cell wall during auxin-promoted cell wall growth (Labavitch and Ray, 1974). Fry *et al.* (1992) found that breaking and re-joining of XGs was catalysed by the enzyme xyloglucan endotransglycosylase (XET) allowing wall expansion.

Other members of the hemicelluloses are the xylans, glucomannans, galactoglucomannans, and β-D-glucans. Xylans are the most abundant non-cellulosic polysaccharides in the majority of angiosperms. They account for 20 - 30% of the dry weight of woody tissues (Aspinal, 1980). They are mainly components of the secondary wall but in monocots they are also found in the primary wall of suspension-cultured cells (Burke *et al.*, 1974). Xylans are polymers of a D-xylose backbone linked with a β(1-4) glycosidic linkage (Aspinal, 1980).

Glucomannans and galactoglucomannans are the main group of mannose-containing polysaccharides from the cell walls of higher plants (Aspinal, 1980). They are the major cell wall components (12 -15%) of gymnosperms, with residues of D-glucose and D-mannose in the approximate ratio of 1:3. These polysaccharides form a family of β(1→4)-linked D-glycans in which α-D-galactose residues, if present, are attached as single unit side

chains by α(1→6) linkages to D-mannose and probably also D-glucose residues in the main chain (Aspinal, 1980).

1.4.2.2 Pectic substances

Pectins are not only important cell wall matrix polysaccharides, but also occur in some plant juices (Goodwin and Mercer,1988). The primary cell walls of dicots contain approximately 35% of pectic polysaccharides (Darvil *et al.*, 1980). The area between primary cell walls of adjoining cells, known as the middle lamella, is thought to be particularly rich in pectins (Hall, 1976). Two fundamental constitutents of pectins are polygalacturonic acid (PGAs) and rhamnogalacturonan I (RGI).

PGAs are helical homopolymers of α(1→4)-D-galacturonic acid (GalA) (McNeil *et al.*, 1984), thus called homogalacturonans. PGAs are difficult to purify because they tend to be insoluble (McNeil *et al.*, 1984). No pure PGAs have been isolated from primary cell walls without treatments that are likely to cleave covalent bonds. The PGAs contain up to about 200 GalA units and are about 100 nm long (Carpita and Gibeaut, 1993). The GalA backbone of PGAs may, however, also contain rhamnosyl residues (Jarvis, 1984). In the presence of Ca^{2+}, the PGA molecules can be cross-linked to form 'junction zones' (Rees, 1977), linking two anti-parallel chains (Jarvis, 1984; Powell *et al.*, 1982). At low Ca^{2+} concentrations, two chains are thought to form a stable junction with maximum strength at about 14 GalA units (Jarvis, 1984). If sufficient Ca^{2+} is present, some interrupting esterified GalA can be tolerated in the stable junction zone. With excess Ca^{2+} available, four-chain or higher-order stacking of PGA chains is possible (Walkinshaw and Arnott, 1981; Jarvis, 1984). This Ca^{2+} cross-linking of PGA chains was suggested to be very important in defining the porosity of the cell wall by the PGAs (Baron-Epel *et al.*, 1988).

The contorted rod-like rhamnogalacturonan I molecules are heteropolymers of α(1→2)-L-rhamnosyl-α(1→4)-D-GalA disaccharide units (Figure 1.4) (Lau *et. al.*, 1985). They have side chains of D-galactosyl, L-

arabinosyl and small amounts of L-fucosyl residues, but the arrangement of these side chains is not really known (McNeil *et al.*, 1984).

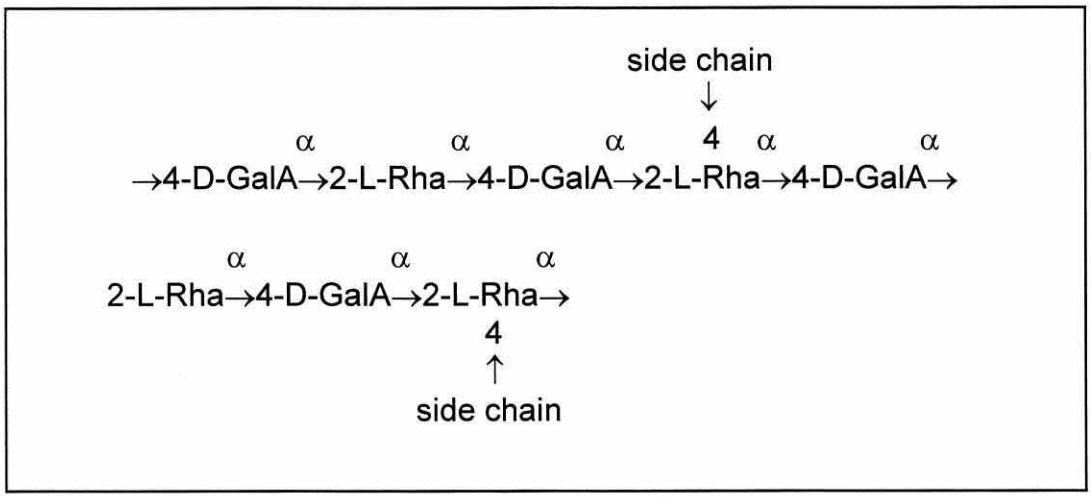


Figure 1.4. The backbone glycosyl-residue of rhamnogalacturonan I.

The length of RGI is unknown but it could contain as many as 300 L-rhamnosyl and 300 D-galactosyluronic acid residues. Approximately half of the L-rhamnosyl residues are branched with glycosyl side chains at D-4. The side chains average about seven glycosyl residues in length (McNeil *et al.*, 1984). The side chains of RGI also constitute arabinans, arabinogalactans I and arabinogalactans II (Carpita and Gibeaut, 1993). Stretches of PGAs are also present at the ends of RGI molecules. These stretches of PGA could link these two types of polymers, but the rhamnosyl units of RGI and their side chains interrupt the Ca^{2+} junctions (Carpita and Gibeaut, 1993).

1.5 Cell wall proteins

1.5.1 Hydroxyproline-rich glycoproteins (HRGPs)

Hydroxyproline-rich glycoproteins are the main structural protein class in the plant cell wall. There are three main classes of HRGP's - the extensins, arabinogalactan proteins (AGPs) and solanaceous lectin (José and Puigdomenech, 1993). In dicot plants, the term 'HRGP' is usually referred to extensins (José and Puigdomenech, 1993). In addition, the HRGPs are the

most important supplier of proline and hydroxyproline to the cell wall (Cassab and Varner, 1988).

1.5.1.1 Extensins

1.5.1.1.1 Structure

Extensins, as a family of HRGP's are found in the cell wall of higher plants. In dicot plants, extensins are characterised by being rich in hydroxyproline and serine and other amino acids such as valine, threonine, lysine, tyrosine, histidine and an unusual tyrosine derivative, isodityrosine, composed of two tyrosyl units cross-linked by a diphenyl ether bridge (Lamport, 1969; Fry, 1982; Showalter, 1993). Extensins usually contain the repetitive pentapeptide motif Ser-Hyp-Hyp-Hyp-Hyp (Ser-Hyp_4). Most of the hydroxyproline residues are glycosylated with one to four arabinosyl residues and some of the serine residues are glycosylated with a single galactose unit (Figure 1.5) (McNeil *et al.*, 1984; Showalter, 1993). In solution, extensins form a polyproline II helical structure. Under the electron microscope they appear as a rod-like structure (Showalter, 1993). The backbone of extensin consists of a highly basic polypeptide of M_r ~ 40000 daltons (Stuart and Varner, 1980) with isoelectric points of ~10 (Showalter, 1993).

Extensin is approximately 50% carbohydrate, about 96% of which consists of arabinosyl residues and 4% galactosyl residues (Stuart and Varner, 1980). Extensin molecules are insoluble in the cell wall. It has been proposed that the insolubility of extensins is due to the formation of intramolecular and intermolecular isodityrosine cross-links between the extensin molecules (Cooper *et al.*, 1987). In a primitive dicot plant (sugar beet), different extensin molecules are present in the cell wall (Li *et al.*, 1990). This sugar beet extensin lacks the repeating pentapeptide motif Ser-$(Hyp)_4$, but it does contain a repeating motif Ser-Hyp-Hyp-[X]-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys, where [X] represents either Val-His-Glu or Lys-Tyr-Pro (Li *et al.*, 1990). In monocots, different types of extensin exist. For example, in the monocot maize, a threonine-hydroxyproline-rich glycoprotein (THRGP) and a

histidine-hyroxyproline-rich glycoprotein (HHRGP) are present. The HHRGP is also rich in alanine (Kieliszewski and Lamport, 1988).

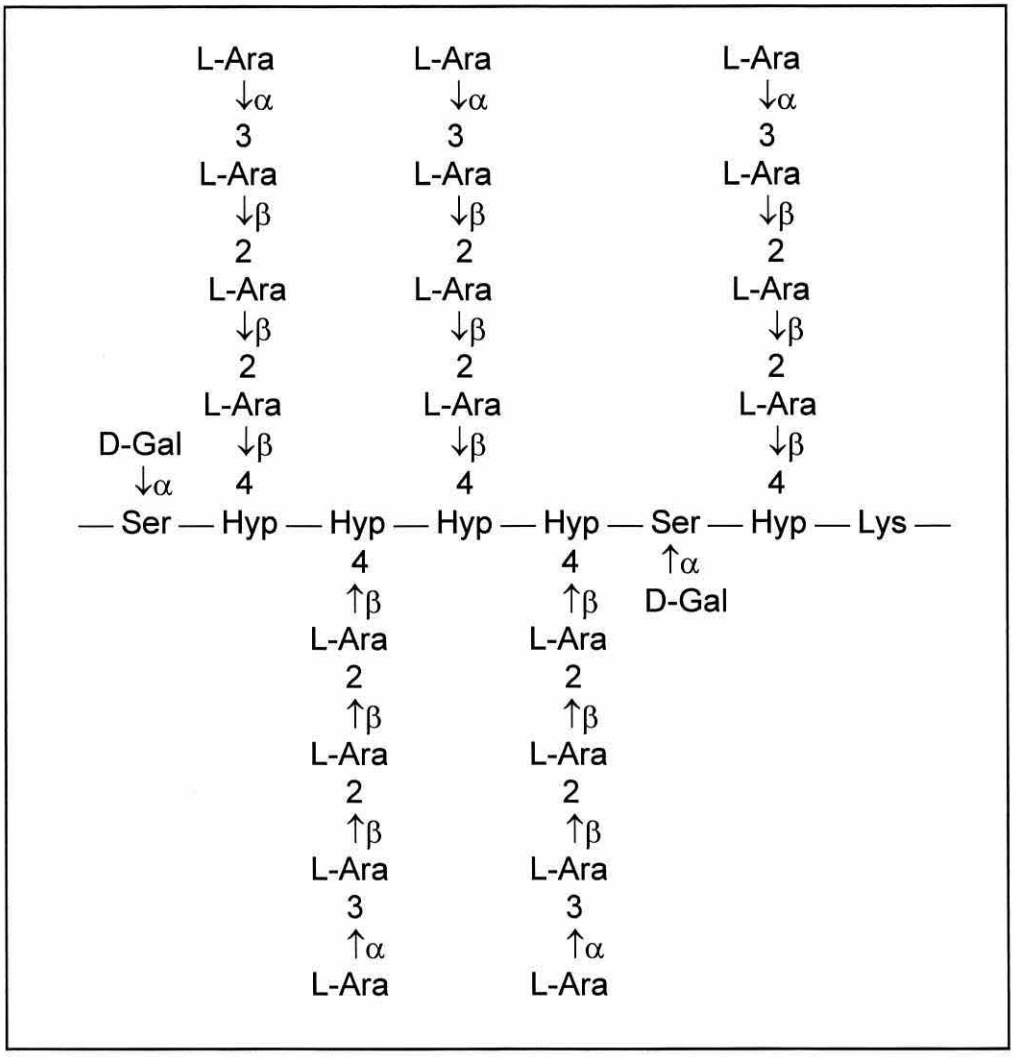


Figure 1.5. Proposed structure of part of extensin of dicot primary cell walls.

Studies show that the THRGP is rich in threonine and proline in addition to hydroxyproline, lysine and serine. It also contains two amino acid repeat motifs, Thr-Pro-Lys-Pro-Thr-$(Hyp)_2$-Thr-Tyr-Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp and Ala-Thr/Ser-Lys-Pro-Pro. Only one repeat motif (Ser-Hyp_4) is present in this THRGP. The serine residues and approximately half of the hydroxyproline residues are not glycosylated, and it is predicted that the THGRP exists in a random coil conformation (Kieliszewski and Lamport, 1987; Kieliszewski *et al.*, 1990). Studies on a number of peptides of the HHRGP

have shown the presence of amino acid sequence repeats of Ala-Hyp-Hyp-Hyp, Ala-Hyp-Hyp-Hyp-Hyp and Ser-Hyp_{1-3}. However, at this moment it is unclear how these repeat motifs are arranged (Kieliszewski *et al.*, 1992).

In gymnosperms, two HRGPs were purified from Douglas fir suspension-cultured cells (Kieliszewski *et al.*, 1992; Fong *et al.*, 1992). One of them has sequences very similar to the PRPs (Kieliszewski *et al.*, 1992) and the other contains both Ser-Hyp_4 and Ala-Hyp repeat motifs (Fong *et al.*, 1992). *Pinus* also contains a cell wall HRGP (Bao *et al.*, 1992). Amino acid analysis on this HRGP shows that the protein contains 24% proline and 11% hydroxyproline. The amino acid sequences of this HRGP remain to be elucidated (Bao. *et al.*, 1992).

Lower plants appear to have other versions of HRGPs. At least two sets of HRGPs have been found in the green alga *Chlamydomonas* (Adair and Snell, 1990). One set is found in the cell walls of vegetative and gametic cells, whereas the other set is found in the cell walls of zygotic cells. Under the electron microscope, both sets appear as rod-like molecules. The HRGPs in the zygotic cells are found to have Pro-Pro-Pro and $(Ser\text{-}Pro)_n$ repeat motifs (Woessner and Goodenough, 1989). In the vegetative cells, the HRGPs are characterised by having Pro-Pro-Pro, Pro-X-Pro, Pro-X-X-Pro and Leu-Pro sequence repeats (Adair and Apt, 1990). A similarity between both sets of these HRGPs is they have the sequence Leu-Leu-Hyp-Hyp (Adair and Snell, 1990). In sugar beet *(Beta vulgaris)*, one HRGP has been isolated from suspension-cultured cells (Li *et al.*, 1990). This HRGP has an insertion sequence inside the pentamer Ser-$(Hyp)_4$ producing Ser-Hyp_2-X-Hyp_2-Tyr-Hyp-Val-Tyr-Lys repeat where X is either Val-His-Glu of Lys-Tyr-Pro (Li *et al.*, 1990).

Extensin was first purified and analysed from carrot discs by Stuart and Varner (1980). Since then several groups of researchers have successfully isolated extensins from various plant tissues including potato tuber (Leach *et al.*, 1982), tobacco callus (Mellon and Helgeson, 1982), tomato cell suspension-cultures (Smith *et al.*, 1984), soybean seed coats (Cassab *et al.*, 1985), cucumber and sycamore-maple suspension-cultured cells (Heckman *et*

al., 1988) mellon callus (Mazau *et al.*, 1988), *Capsicum* cell walls (Biggs and Fry, 1990), tobacco callus (Kawasaki, 1991), cell walls of cotton suspension-cultures (Xi *et al.*, 1995), and tomato (Brownleader *et., al.*, 1995).

Extensin cDNA and genomic clones encoding the gene for extensin have also been isolated from various plants such as carrot (Chen and Varner, 1985a), tomato (Showalter *et al.*, 1985), tobacco (Memelink *et al.*, 1987; Keller and Lamb, 1989; Tire *et al.*, 1994; Hirsinger *et al.*, 1997), oilseed rape (Evans *et al.*, 1990), almond (Garcia-Mas *et al.*, 1992), maize (Stiefel *et al.*, 1990), rice (Caelles *et al.*, 1992), soybean (Hong *et al.*, 1994), tobacco protoplasts (Parmentier, *et al.*, 1995) and southern pea, *Vigna unguiculata* (Arsenijevic-Maksimovic *et al.*, 1997). Table 1.1 shows the cDNA and genomic clones encoding the cell wall protein extensin that have been isolated from numerous plants.

1.5.1.1.2 Biochemical characterisation

Most extensins are highly basic molecules. Generally, extensins contain hydroxyproline and arabinose, are abundant in lysine and are poor in aspartate and glutamate. Most of the hydroxyproline is found in Ser-Hyp_4 peptide sequences (José and Puigdomenech, 1993). Most of the hydroxyproline residues are glycosylated and the degree of hydroxyproline glycosylation varies between plant species. According to Lamport and Miller (1971), in extensins in the dicotyledonous species, hydroxyproline residues are glycosylated with tri- and tetra-arabinosides, and many serine residues are glycosylated with galactose (Figure 1.5) (Lamport *et al.*, 1973). Extensins in dicot plants may have a carbohydrate content higher than 60% (Lamport and Miller, 1971). In monocots, only 30% of the hydroxyproline is glycosylated, mostly as Hyp-Ara (McNeil *et al.*, 1984). The best-characterised salt-extractable extensin so far is the one present in carrot root cell walls isolated by van Holst and Varner (1984). The amino acid composition of this extensin is mostly Hyp, Ser, His, Tyr, Lys and Val. The abundance of Lys and the low content of Asp and Glu contribute to the high isoelectric point observed in this molecule. This extensin consists of 35% protein and 65%

carbohydrate. Three or four arabinosyl residues are glycosylated to Hyp residues and galactose is glycosylated to serine (Lamport *et al.*, 1973).

Table 1.1. cDNA and genomic clones encoding cell wall extensins.

Plant	Name	References
Carrot	DC 5	Chen and Varner (1985a)
	DC 5A1	Chen and Varner (1985b)
Tomato	Tom-17-1	Showalter and Rumeau (1990)
	Tom J-2	Showalter and Rumeau (1990)
	Tom 5	Showalter *et al.* (1985)
	Class I-UG-18	Showalter *et al.* (1991)
	Class I	Showalter *et al.* (1991)
	Class I-W17-1	Showalter *et al.* (1991)
	Class I-WY	Showalter *et al.* (1991)
	Class I-W6	Showalter *et al.* (1991)
	Class I-Tom J-10	Zhou *et al.* (1992)
	Class II-uJ-2	Showalter *et al.* (1991)
	Class II-u1	Showalter *et al.* (1991)
	Class II-u2	Showalter *et al.* (1991)
	Class II-Tom-L4	Zhou *et al.* (1992)
Tobacco	CNT 1	Memelink *et al.* (1987)
	npext	De Loose *et al.* (1991)
	HRGP nt3	Keller and Lamb (1989)
	NaPRP 3	Chen *et al.* (1992)
	NaPRP 3g12	Chen *et al.* (1992)
	NaClass I	De S Goldman *et al.* (1992)
	NaClass II	De S Goldman *et al.* (1992)
	NaClass III	De S. Goldman *et al.* (1992)
	6Pext 1.2	Parmentier *et al.* (1995)
	Ext 1.4	Hirsinger *et al.* (1997)
Vigna unguiculata	ext 3	Arsenijevic-Maksimovic *et al.* (1997)
	ext 127	Arsenijevic-Maksimovic *et al.* (1997)
	ext 26	Arsenijevic-Maksimovic *et al.* (1997)
Bean	Hyp 2.13	Corbin *et al.* (1987)
	Hyp 3.6	Corbin *et al.* (1987)
	Hyp 4.1	Corbin *et al.* (1987)
Sunflower	HaGX 3	Adams *et al.* (1992)
Petunia	CW 6	Showalter and Rumeau (1990)
	CW 7	Showalter and Rumeau (1990)
Antirrhinum	ptl 1	Baldwin *et al.* (1992)
Oilseed rape	*extA*	Evans *et al.* (1990)
	PRR_t566	Evans *et al.* (1990)
	PRR_t999	Evans *et al.* (1990)

	PRR_t592	Evans *et al.* (1990)
	PRR_t1214	Evans *et al.* (1990)
Arabidopsis	aHRGP	Showalter and Varner (1989)
Soybean	*SbHRGP-1*	Hong *et al.* (1994)
	SbHRGP-2	Hong *et al.* (1994)
	SbHRGP-3	Hong *et al.* (1994)
Almond	paHRGP	Garcia-Mas *et al.* (1992)
Chlamydomonas reinhardtii	crHRGP	Woessner and Goodenough (1989)
Volvox carteri	ISG	Ertl *et al.* (1992)
Maize	MC 56	Raz *et al.* (1992)
Teosinte	zdHRGP	Raz *et al.* (1992)
Sorghum	svHRGP	Raz *et al.* (1991)
Rice	*osHRGP*	Raz *et al.* (1992)

Studies on the secondary structure of extensin showed that it is completely folded in the polyproline II conformation (an extended left-handed helix) (van Holst and Varner, 1984). If extensin is deglycosylated, much of the conformation is lost indicating that the carbohydrate moiety of this extensin serves to stabilise this conformation. Electron microscopy studies of extensin confirm these results - it appears as a rod-like structure. Electron micrographs of extensin show thin rod-like molecules with an average length of 80 - 84 nm (van Holst and Varner, 1984).

Studies on extensins from potato tuber (Leach *et al.*, 1982), tobacco callus (Mellon and Helgeson, 1982), tomato cell suspension cultures (Smith *et al.*, 1984) and soybean seed coats (Cassab *et al.*, 1985) showed that Hyp is the major amino acid representing 33 - 42% of the total amino acids. Other abundant amino acids are Ser, His, Lys, Tyr, Val and Pro. Hydroxyproline residues are glycosylated with three or four arabinosyl residues in extensins from tomato cell suspension cultures (Smith *et al.*, 1984), while in soybean seed coat extensin, Hyp is mainly glycosylated with three arabinosyl residues (Cassab *et al.*, 1985).

Studies on amino acid sequences of two different extensin monomers, P1 and P2 from tomato cell suspension cultures, showed that both extensin precursors have highly periodic structures. P1 contains two different peptide blocks: Ser-$(Hyp)_4$-Thr-Hyp-Val-Tyr-Lys and Ser-$(Hyp)_4$-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys, and P2 consists of a single repeating decapeptide, Ser-$(Hyp)_4$-Val-Tyr-Lys-Tyr-Ly (Smith *et al.*, 1986). These results show that two different repeat domains are present in P1 and P2. One of these is the glycosylated Ser-$(Hyp)_4$ sequences repeat domain and the other one is the non-glycosylated repeat domain. According to Smith *et al.* (1986), the glycosylated domain is relatively rigid and the non-glycosylated domain is flexible. They proposed that this is the structure that might allow the weaving of the cellulose microfibrils of the primary cell wall with an extensin network.

The mechanism of rapid insolubilisation of extensins once they are secreted into the wall still remains unanswered. To date, there are several suggestions for the insolubilisation of the extensin in the cell wall. It was first proposed that the insolubilisation was due to the formation of intermolecular isodityrosine cross-links between two tyrosine residues from different extensin molecules (Cooper and Varner, 1984; Lamport, 1986; Fry, 1982), however, no such interaction was found. Instead, such isodityrosine cross-links have only been found intramolecularly (Epstein and Lamport, 1984). Epstein and Lamport (1984) have shown that intramolecular isodityrosine links can form between two unique sequences Tyr-Lys-Tyr-Lys and Tyr-Tyr-Tyr-Lys present in the same extensin molecule. However, other isodityrosine-forming sequences have also been proposed including Pro-Tyr-Pro-Tyr (Fong *et al.*, 1992) and Tyr-Val-Tyr-Lys (Zhou *et al.*, 1992). Fry (1982) proposed that the synthesis of the isodityrosine cross-link was mediated by the release of hydrogen peroxide and catalysed by the cell wall enzyme peroxidase.

With the exception of the extensin gene from *Volvox,* ISG (Ertl *et al.*, 1992), the rest of the extensin genes that have been characterised so far do not have introns in their coding region (José and Puigdomenech, 1993). Some extensin genes from carrot, tobacco and monocotyledenous species have an intron in their 3' non-coding region. According to Raz *et al.* (1992),

the intron sequence of the extensin genes in maize, teosinte and sorghum is very conserved. The presence of introns in the 3' non-coding region is, however, very unusual in both animal and plant systems. The *Chlamydomonas reinhardtii* extensin gene, however, has an intron in the 5' non-coding region. Other introns in 5' non-coding regions have also been reported in the *hsp83* gene in *Drosophila melanogaster* and in the polyubiquitin gene in humans, chickens, sunflower, *Arabidopsis* and maize (José and Puigdomenech, 1993). Until now, it has not been determined whether introns placed in extensin non-coding regions have any regulatory function.

1.5.1.1.3 Extensin classification

In general, extensins can be divided into four subgroups according to the amino acid consensus sequence repeats present in the extensin molecule (José and Puigdomenech, 1993). Group A extensins represent molecules having the amino acid consensus sequence repeat SPPPPSPSPPPYYYK (Table 1.2) and variations of these repeats. Most of the tomato extensins are included in this group, along with the extensins from bean and petunia.

Table 1.2. Amino acid sequence repeats of the group A extensin.

Groups repeat	Number of repeats (Name/Plant)	Reference
SPPPPSPSPPPPYYYK	3 (Tom 17-1, tomato)	Showalter and Rumeau (1990)
	6 (Hyp 2.13, bean	Corbin *et al.* (1987)
	4 (Hyp 3.6, bean)	
	nd (Class I -uG18, tomato)	Showalter *et al.* (1991)
	nd (Class I-w17-1, tomato)	
	nd (Class I-wY, tomato)	
SPPPPSPSPPPPYY/VYK	ND (Class I-w6, tomato)	Showalter *et al.* (1991)
SPPPPSPSPPPPYYY	3 (CW 7, petunia)	Showalter and Rumeau (1990)
SPPPPSPSPPPPTY$_{1-3}$S	nd (Class II-uJ-2, tomato)	Showalter *et al.* (1991)
SSPPPPSPSPPPPTY$_{1-3}$	2 (Class II-Tom L-4, tomato)	Zhou *et al.* (1992)

SPPPPSPSPPPP	2 (Tom J-2, tomato)	Showalter and Rumeau (1990)
SPPPPKHSPPPPYYYH	11 (Hyp 4.1, bean)	Corbin *et al.* (1987)
SPPPPSPKYVYK	19 (Class I-Tom J-10, tomato)	Zhou *et al.* (1992)
SPPPPYYYKSPPPPSP	8 (Class I-Tom J-10, tomato)	Zhou *et al.* (1992)
SPPPPYYYK/S	nd (Class I-uG, tomato)	Showalter *et al.* (1991)
$SP_{4\text{-}5}$TPSYEHP	nd (Class II-u1, tomato) nd (Class II-u2, tomato)	Showalter *et al.* (1991)
$SP_{2\text{-}5}$TPSYEHPKTP	4 (Class II-Tom L-4, tomato)	Zhou *et al.* (1992)

The SPPPPTPVYK repeat motif is the main sequence for repeats belonging to group B (Table 1.3). These repeats sometimes alternate in the same extensin sequence with repeats belonging to group C (Table 1.4). Group B repeats are represent in tomato, *Arabidopsis*, oilseed rape, sugar beet, *Antirrhinum*, carrot, tobacco, petunia and Douglas fir extensins.

Table 1.3. Amino acid sequence repeats of the group B extensin.

Groups repeat	Number of repeats (Name/Plant)	Reference
SPPPPTPVTKTK	7 (DC5A1, carrot)	Chen and Varner (1985b)
SPPPPTPVYK	6 (CNT 1, tobacco) 11 (npext, tobacco) 1 (CW 6, petunia)	Memelink *et al.* (1987) De Loose *et al.* (1991) Showalter and Rumeau (1990)
SPPPPTPITK	1 (CW 6, petunia)	Showalter and Rumeau (1990)
LPP[DTDPAD]PP	1 (ptl 1, *Antirrhinum*)	Baldwin *et al.* (1992)
SPPPPVYK	4 (*extA*, oilseed rape) nd (PRR566, oilseed rape) nd (PRR_t999, oilseed rape) nd (PRR_t1449, oilseed rape)	Evans *et al.* (1990)
VYKSPPPP	12 (HaGX 3, sunflower)	Adams *et al.* (1992)
SPPPPVYH	8 (*extA*, oilseed rape) nd (PRR_t592, oilseed rape) nd (PRR_t566, oilseed rape) nd (PRR_t999, oilseed rape) nd (PRR_t1449, oilseed rape)	Evans *et al.* (1990)

VHKSPPPP	5 (HaGX 3, sunflower)	Adams *et al.* (1992)
SPPPPVH	21 (Tom 5, tomato) nd (aHRGP, *Arabidopsis*)	Showalter *et al.* (1985) Showalter and Varner (1989)
SPPPPVA	8 (Tom 5, tomato) nd (aHRGP, *Arabidopsis*)	Showalter *et al.* (1985) Showalter and Varner (1989)
SPPPPVKHY	nd (PRR_t592, oilseed rape)	Evans *et al.* (1990)

Group C represents extensins with the sequence SPPPPKK followed by a very degenerate tail (Table 1.4). Extensins from tomato, melon, carrot, almond tree, tobacco, petunia and oilseed rape can be found in this group.

Table 1.4. Amino acid sequence repeats of the group C extensin.

Groups repeat	Number of repeats (Name/Plant)	Reference
SPPPPKKPYYPPHTPVYK	8 (CNT 1, tobacco)	Memelink *et al.* (1987)
SPPPPKKPY/HYPPHTPVYK	8 (npext, tobacco)	De Loose *et al.* (1991)
SPPPPK/VKPYHPSPTPTH-PS/APVYK	5 (npext, tobacco)	De Loose *et al.* (1991)
SPPPPKKPYHPSPTPY	1 (CW 6, petunia)	Showalter and Rumeau (1990)
SPPPPKKXYEYK	nd (PRR_t592, oilseed rape)	Evans *et al.* (1990)
SPPPPKKHYEYK	7 (*extA*, oilseed rape) nd (PRR_t566, oilseed rape) nd (PRR_t999, oilseed rape) nd (PRR_t1449, oilseed rape)	Evans *et al.* (1990)
HHYKYK	4 (DC5A1, carrot)	Chen and Varner (1985b)
SPPPPKH	7 (DC5A1, carrot)	Chen and Varner (1985b)
SPSPPKH	4 (paHRGP, almond)	Garcia-Mas *et al.* (1992)

Extensin molecules with amino acid repeats rich in threonine, such as PPTYTP, PPTYKP, ATKPP, TPKPT and QPKPT/NP are included in group D (Table 1.5). Molecules with the repeat SPKPP (without threonine) are,

however, also included in this group. Repeats belonging to this group are also present in maize, teosinte, rice and sorghum.

Table 1.5. Amino acid sequence repeats of the group D extensin.

Groups repeat	Number of repeats (Name/Plant)	Reference
PPTYTP	13 (MC 56, maize W 64A)	Stiefel *et al.* (1988)
	15 (zmHRGP, maize AC 1503)	Stiefel *et al.* (1990)
	13 (zmHRGP, maize W 22)	Raz *et al.* (1992)
	15 (zdHRGP, teosinte)	
	4 (svHRGP, sorghum)	Raz *et al.* (1991)
	3 (*osHRGP*, rice)	Caelles *et al.* (1992)
PPTYKP	11 (*osHRGP*, rice)	Caelles *et al.* (1992)
SPKPP	12 (MC 56, maize W 64A)	Stiefel *et al.* (1988)
	9 (zmHRGP, maize AC 1503)	Stiefel *et al.* (1990)
	8 (zmHRGP, maize W 22)	Raz *et al.* (1992)
	10 (zdHRGP, teosinte)	
	4 (svHRGP, sorghum)	Raz *et al.* (1991)
TPKPT	12 (MC 56, maize W 64A)	Stiefel *et al.* (1988)
	11 (zmHRGP, maize AC 1503)	Stiefel *et al.* (1990)
	9 (zmHRGP, maize W 22)	Raz *et al.* (1992)
	11 (zdHRGP, teosinte)	
	1 (svHRGP, sorghum)	Raz *et al.* (1991)
ATKPP	2 (MC 56, maize W 64A)	Stiefel *et al.* (1988)
	1 (zmHRGP, maize AC 1503)	Stiefel *et al.* (1990)
	2 (zmHRGP, maize W 22)	Raz *et al.* (1992)
	3 (zdHRGP, teosinte)	
	3 (svHRGP, sorghum)	Raz *et al.* (1991)
QPKPT/NP	9 (*osHRGP*, rice)	Caelles *et al.* (1992)

1.5.1.1.4 Cellular localisation

Extensins are known to be associated with cell walls. With the use of techniques such as the western blot, tissue print immunoblot and immunoelectron or immunolight microscopy, the cellular localisation of extensins in the plant has been successfully determined. As summarised in Table 1.6, extensin gene expression and localisation varies between plant to plant and among cell and tissue types. This variation is probably due to the

different functions extensins perform in different cell types and tissues (Showalter, 1993).

Table 1.6. Tissue localisation of extensins.

Plant system	Tissue localisation	Reference
Soybean stems and petioles	Cambium cells, in a few layers of cortex cells surrounding primary phloem, and in some parenchyma cells around the primary xylem; abundant in hypocotyl apical regions	Ye and Varner (1991)
Soybean roots	Two to three layers of cortex cells around vascular bundles and in protoxylem; abundant in root apical regions	Ye and Varner (1991)
Soybean seeds	Sclerenchyma tissues (palisade epidermal and hourglass cells) of seed coats	Cassab and Varner (1987)
Bean stems and petioles	Cambium cells and in a few layers of cortex cells surrounding primary phloem	Ye and Varner (1991)
Tobacco with bean extensin-GUS transgene	Subset of pericycle and endodermal cells involved with lateral root initiation	Keller and Lamb (1989)
Tomato stems and petioles	Outer and inner phloem	Ye *et al.* (1991)
Tomato root	Minor components of cortical and parenchyma cell walls	Benhamou *et al.* (1990a)
Tomato stems	Xylem, phloem, epidermis, cortical parenchyma, pith	Li and Showalter (1996)
Tomato root	Xylem, endodermis, outer cortical cells epidermis	Li and Showalter (1996)
Green tomato fruits	Epidermis; protoxylem metaxylem and to adjacent phloem and parenchyma tissue (in pericarp); epidermis of the ovule, ovule with the exception of the embryo sac, in the tracheary elements leading to the ovule	Li and Showalter (1996)
Petunia stems	Outer phloem	Ye *et al.* (1991)
Tobacco stems	Outer phloem	Ye *et al.* (1991)

Tobacco young stem nodes	Cortical cell surrounding the leaf trace, outer phloem cells in the stem and in the leaf trace, xylem, phloem fibres of the outer phloem	Tire *et al.* (1994)
Tobacco roots	In differentiating secondary xylem elements and in part of the cortex; over the differentiating secondary xylem elements, the vascular cambium; associated with the lignified cell wall of xylem vessel elements	Tire *et al.* (1994)
Carrot roots	Phloem parenchyma cells	Stafstrom and Staehelin (1988)
Carrot and potato leaves	Primary xylem	Li and Showalter (1996)
Carrot stems	Xylem, outer and inner phloem, cortical parenchyma cells in direct contact with the outer phloem	Li and Showalter (1996)
Carrot lateral and tap roots	Xylem, epidermis	Li and Showalter (1996)
Rape with rape extensin-GUS transgene	Phloem of rape roots	Shirsat *et al.* (1991)
Maize	Predominantly to sites of early vascular differentiation in embryos, leaves, and roots; these sites included xylem elements and surrounding sclereenchyma in leaves and metaxylem and proto-xylem in roots	Stiefel *et al.* (1990)
Potato stems	Cortex, metaxylem, protoxylem, phloem, pith	Li and Showalter (1996)
Potato tuber	Epidermal and peridermal, vascular cells	Li and Showalter (1996)

1.5.1.1.5 Expression

Extensin gene expression can be affected by various conditions and treatments such as wounding, fungal and viral infections, elicitors, ethylene as well as developmental events (Stiefel *et al.*, 1988; Ludevid *et al.*, 1990; Fritz *et al.*, 1991; Ruiz-Avila *et al.*, 1991; Showalter, 1993).

Extensin mRNA synthesis in response to wounding was first shown by Chen and Varner (1985b) in their study in carrot storage root tissue. Two transcripts of different sizes, 1.5 kb and 1.8 kb were synthesized 24 h after wounding. It was found that both transcripts originated from the same gene (but from different transcription initiation sites placed at a distance of 300 bp between each other). It was proposed that the initiation of transcription from the two initiation sites was controlled by different regulatory signals. The 1.5 kb transcript began to accumulate 8 h after the tissue was wounded, reaching a maximum after 24 h (Tierney *et al.*, 1988). However, studies by Ecker and Davis (1987) on peeled stored carrot roots showed that the 1.5 kb transcript accumulated 1 h after the tissue was incubated in a stream of moist air. During both treatments, the 1.5 kb transcript showed a dramatic increase over the 1.8 kb transcript. Ecker and Davis (1987) also showed that during the preparation of protoplasts, the 1.5 kb transcript was induced to accumulate after 16 h, reaching a maximum level after 24 h.

In bean, the expression of three extensin genes (Hyp 2.13, Hyp 3.6 and Hyp 4.1) was studied by Corbin *et al.* (1987). Hypocotyls of bean were subjected to excision-wounding. Within 1.5 h, Hyp 3.6 transcripts were strongly induced to a maximum level, while the Hyp 2.13 and Hyp 4.1 transcripts were induced later, reaching a maximum after 12 h. Between 12 h and 24 h, expression of Hyp 3.6 decreased, whereas the level of the Hyp 2.13 and Hyp 4.1 transcripts remained constant.

Studies on the *extA* gene from oilseed rape by Shirsat *et al.* (1996b) showed that it was expressed to a high level in the roots of normal plants. No significant level of *extA* transcripts was detected in leaves, stems and petioles. However, after wounding, significant levels of *extA* transcripts were detected in leaves after 36 h and increased to a maximum level at 48 h. In wounded petioles, *extA* transcripts were seen after 11 h and reached a maximum level at 48 h. In wounded stems, accumulation of transcripts were first detected at 17 h and reached a maximum level between 36 h and 48 h. In roots, however, expression of the *extA* gene decreased after the tissues were wounded and a very low level of *extA* transcript was detected 11 h after application of the

wounding stimulus. By using the GUS histochemical assay, Shirsat *et al.* (1996b) analysed the expression of *extA*-GUS promoter reporter fusions in transgenic plants and showed that high levels of *extA*-driven GUS expression were detected in cortical parenchyma cells at the point where the axillary flowering branch joined the main stem in transgenic tobacco plant. The highest level of expression was detected in the region where the point of probable maximum tensile stress was located.

Studies on Class I and Class II tomato extensin genes (Adams *et al.*, 1992) showed that the expression of both genes decreased after application of a wounding stimulus in roots. In stems, transcripts from both genes increased locally 8 h - 12 h after the tissues were wounded. In tobacco, studies on expression of the *pCNT1* gene showed that the gene was induced one day after the leaves were wounded (Memelink *et al.*, 1993). The *6Pext1.2* gene has also been shown to be induced by wounding in protoplasts and leaf strips (Parmentier *et al.*, 1995). Wounding of leaves and stems also induced the accumulation of the Ext 1.4 transcript to high levels (Hirsinger *et al.*, 1997).

The ability of pathogens to induce the expression of extensin genes have also been reported. By using the tomato extensin genomic probe Tom 5, Showalter *et al.* (1985) have detected extensin transcripts in bean hypocotyls after injection with *Colletotrichum lindemuthianum*. These transcripts were detected both in incompatible and compatible interactions. The same study performed by Corbin *et al.* (1987) using bean extensin probes (Hyp 2.13, Hyp 3.6 and Hyp 4.1) showed that three transcripts corresponding to the probes were induced by both the compatible and the incompatible interactions. Hyp 2.13 was preferentially induced in the compatible reaction, whereas Hyp 3.6 and Hyp 4.1 were strongly induced in both types of interactions.

In cucumber seedlings, a significant level of extensin was detected in the hypocotyls 12 h after they were inoculated with *Cladosporium cucumerinum* (Hammerschmidt *et al.*, 1984). The level of extensin continued to increase until 48 h, and an average of 61.4% increase in extensin content was detected. Later, Stermer and Hammerschmidt (1987) found that extensin

accumulated in the apical zones of hypocotyls after the tissues were heat-shocked at 50^{0}C for 40 s. However, extensin content doubled 72 h after the heat-shocked tissues were inoculated with *C. cucumerinum*. According to Tire *et al.* (1994), extensin transcripts accumulated to a high level in tobacco leaves 48 h after the tissues were infected with the pathogen *Pseudomonas syringae*. Induction of extensin gene expression has also been detected in tobacco roots after they were infected with the root knot nematode *Meloidogyne javanica* (Niebel *et al.*, 1993).

Elicitors by themselves are also able to induce extensin gene expression as shown by pathogen infection. Showalter *et al.* (1985) using the tomato extensin probe Tom 5, showed that extensin transcripts accumulated in bean suspension-cultured cells 4 h after the cells were treated with *Colletotrichum lindemuthianum* elicitors. Expression increased from 6 h to 12 h and remained constant thereafter. However, when the same study was performed using bean Hyp 2.13, Hyp 3.6 and Hyp 4.1 probes, the transcripts corresponding to the respective probes were only detected after 24 h (Corbin *et al.*, 1987). Tierney *et al.* (1988) have also shown that the 1.5 kb and 1.8 kb extensin transcripts are expressed when carrot suspension-cultured cells are grown for 5 days in the presence of 5 μg/ml of a crude endogenous elicitor fraction.

Extensin gene expression is also developmentally regulated. Tobacco *pCNT1* extensin transcripts were shown to be expressed at a high level in roots, were less prevalent in stems and low in leaves (Memelink *et al.*, 1993). In soybean, studies on three extensin genes *SbHRGP-1*, *SbHRGP-2* and *SbHRGP-3* showed that expression of all three genes was detected at the highest level in mature zones of the hypocotyl followed by the elongation zone. The lowest level of transcripts was detected in the apical zone (Hong *et al.*, 1994). Developmentally regulated expression of the *SbHRGP-3* gene has also been recently shown by Ahn *et al.* (1996). *SbHRGP-3* gene expression increased with seedling maturation and was relatively high in the roots of soybean seedlings and in the mature regions of the hypocotyls. In wounded tissues of transgenic tobacco plants, expression of the *SbHRGP-3* gene

required the presence of sucrose, and its expression was specific to phloem tissues and cambium cells of leaves and stems (Ahn *et al.* (1996). In pea, expression of *ext 3*, *ext 26* and *ext 127* extensin genes was only detected in root hairs (Arsenijeric-Maksimovic *et al.*, 1997). In tomato, mRNA transcripts encoded by a genomic clone, Ext 1.4 were only detected in stems, roots, ovaries and germinating seeds in healthy plants (Hirsinger *et al.*, 1997).

Several other conditions have also able to affect the expression of extensin genes. Stems of ethylene treated melon seedlings have shown an increased in the accumulation of extensins as reported by Esquerre-Tugaye *et al.* (1979). In addition, the increase in extensin expression mediated through ethylene is paralleled by an increasing resistance of the plant to *C. lagenarium* injection (Esquerre-Tugaye *et al.*, 1979). According to Tierney *et al.* (1988), expression of the 1.5 kb and 1.8 kb extensin transcripts was only slightly elevated 24 h after the carrot roots were treated with ethylene. But when the roots were stored in cold conditions and treated with ethylene and oxygen for 72 h, the 1.8 kb transcript level increased while the 1.5 kb transcript level decreased. Weiser *et al.* (1990) have shown that after germination of pea seedlings exposed to cold conditions, more than a three-fold increase in total extensin transcripts accumulation was detected in epicotyls. Specific transcripts of sizes 1.5, 1.8, 2.3, 2.6, 3.5, 4.5 and 6.0 kb were identified. The induction of extensin genes in response to ozone has also been reported. A strong increase of extensin transcript level was detected within a few hours after plants were treated. The induction occured in needles and hypocotyls of Scot pine, needles of Norway spruce and leaves of European beech (Schneiderbauer *et al.*, 1995).

1.5.1.1.6 Functions

When extensin was first identified many years ago (Lamport, 1967), the function of this protein was proposed to be in the regulation of cell wall extension, thus the name extensin. However, with the recent discovery of expansins by McQueen-Mason *et al.* (1992) and xyloglucan endo-

transglycosylase (XET) by Smith and Fry (1991), it is unlikely that extensins are the primary determinants of cell wall elongation.

To date, the actual function of extensins in plant systems has not yet understood. It has been proposed that extensins may play a role in the plant defence mechanism against pathogen attack. The observation of the accumulation of extensins and mRNA transcripts encoding for extensins in plants as a result of pathogen attack were shown by Clarke *et al.* (1981), Esquerre-Tugaye and Lamport (1979), Esquerre-Tugaye *et al.* (1979), Showalter *et al.* (1985), Corbin *et al.* (1987), Templeton *et al.* (1990), Mouly *et al.* (1992), Memelink *et al.* (1993), Hammerschmidt *et al.* (1994), Tire *et al.* (1994) and Parmentier *et al.* (1995). Even though the exact role of extensin in plant defense system is unknown, the accumulation of extensins as a result of wounding and elicitor treatments of plants as shown by Chen and Varner, (1985b), Showalter *et al.* (1985), Ecker and Davis (1987), Corbin *et al.* (1987), Tierney *et al.* (1988), Memelink *et al.* (1993), Tagu *et al.* (1992), Ahn *et al.*, (1996) and Shirsat *et al.* (1996b), strongly supports this suggestion. Furthermore, the accumulation of extensin proteins and transcripts in response to ethylene treatment as shown by Esquerre-Tugaye *et al.* (1979), Tierney *et al.* (1988), Showalter *et al.* (1992) and Memelink *et al.* (1993) further supports the suggestion of the involvement of extensin in plant defence mechanisms. This is because one of the earliest detectable events during plant-pathogen interactions is a rapid increase in ethylene biosynthesis (Cassab and Varner, 1988). It has also been proposed that the ethylene produced in response to biological stress is a signal for plants to activate defence mechanisms against invading pathogens (Ecker and Davis, 1987).

Two possible mechanisms have been proposed for the possible involvement of extensin in the plant resistance to pathogens. The first mechanism suggests that extensin agglutinates bacteria and thereby prevents their spread (Leach *et al.*, 1982). This agglutination response probably results from positively charged extensin molecules interacting ionically with the negatively charged surfaces of certain plant pathogens (Leach *et al.*, 1982; Mellon and Helgeson, 1982; van Holst and Varner, 1984). The second

mechanism suggests that extensin, by virtue of its tight cross-linking, renders the wall indigestible by invading pathogens (Hammerschmidt *et al.*, 1984). It has also been proposed that extensin plays a role in restricting cell expansion (Klis, 1976; Fry, 1982; Lamport and Epstein, 1983) due to the tight cross-linking of extensins in the cell wall (Fry, 1982; Lamport and Epstein, 1983). Carpita and Gibeaut (1993) suggest that wall expansion happens after the cross-linked cell wall xyloglucans have been cleaved by hydrolases (or transglycosylases), thus loosening the cellulose microfibrils and allowing separation. After displacement, however, extensin molecules are inserted radially, interlock the separated microfibrils to cease further stretching. To date, how extensins are cross-linked in the wall is not really known.

Recently, studies on transgenic tobacco transformed with an *extA* promoter-β-glucuronidase (GUS) coding sequence fusion have shown that the GUS gene was expressed in cortical parenchyma cells at the point where the axillary flowering branch joined the main stem. In the area where the maximum tensile stress would seem to be exerted by the weight of the axillary flowering branch, more expression of GUS was observed. Furthermore, when a 10 g weight was hung on an axillary stalk, a strong band of GUS expression extending through the parenchyma cells of the pith and cortex was seen in the node (Shirsat *et al.*, 1996a). Results from this study therefore suggest that extensin synthesis contributes to the tensile strength of cells.

1.5.1.2 Arabinogalactan Proteins (AGPs)

1.5.1.2.1 Structure and cellular localisation

Arabinogalactan proteins (AGPs) are found in most higher plants. They are generally very soluble and are heavily glycosylated. The protein moiety of AGPs is usually between 2% - 10%, and is rich in hydroxyproline, serine, alanine, threonine and glycine (Showalter, 1993). The glycosylated protein component is resistant to proteolysis (Jermyn, 1980) presumably because of its substitution with bulky carbohydrate groups (Fincher *et al.*, 1983). AGPs have isoelectric points in the range of pH 2 to 5 (Showalter, 1993). According

to Gleeson *et al.* (1985), the amino acid sequences of three carrot AGPs and one ryegrass AGP contain Ala-Hyp repeats. Gleeson *et al.* (1989) have also found that some of the deglycosylated ryegrass AGP peptides also contain Ala-Hyp repeats.

Carbohydrates account for most of the AGPs mass. The major monosaccharides are D-galactose and L-arabinose. The proportion of D-galactose:L-arabinose varies between 10:90 and 85:15 with most of the AGPs containing more D-galactose than L-arabinose. Other components of the monosaccharides are L-rhamnose (up to 11%), D-mannose (up to 16%), D-xylose (up to 7%), D-glucose (up to 4%), D-glucuronic acid and its 4-O-methyl derivative (up to 28%) and D-galacturonic acid and its 4-O-methyl derivative (up to 26%). In most of the AGPs, however, D-galactose and L-arabinose predominate (Fincher *et al.*, 1983). Structural analysis of ryegrass AGP has shown that one in every two D-galactose residues of the β(1→3)-linked D-galactosyl polysaccharide backbone are branched with β(1→6)-linked D-galactosyl side chains, and every D-galactosyl residue of the β-D-galactosyl side chains is either β(1→3)- or β(1→6)-linked with L-arabinosyl residues (Figure 1.6) (Fincher *et. al.*, 1983).

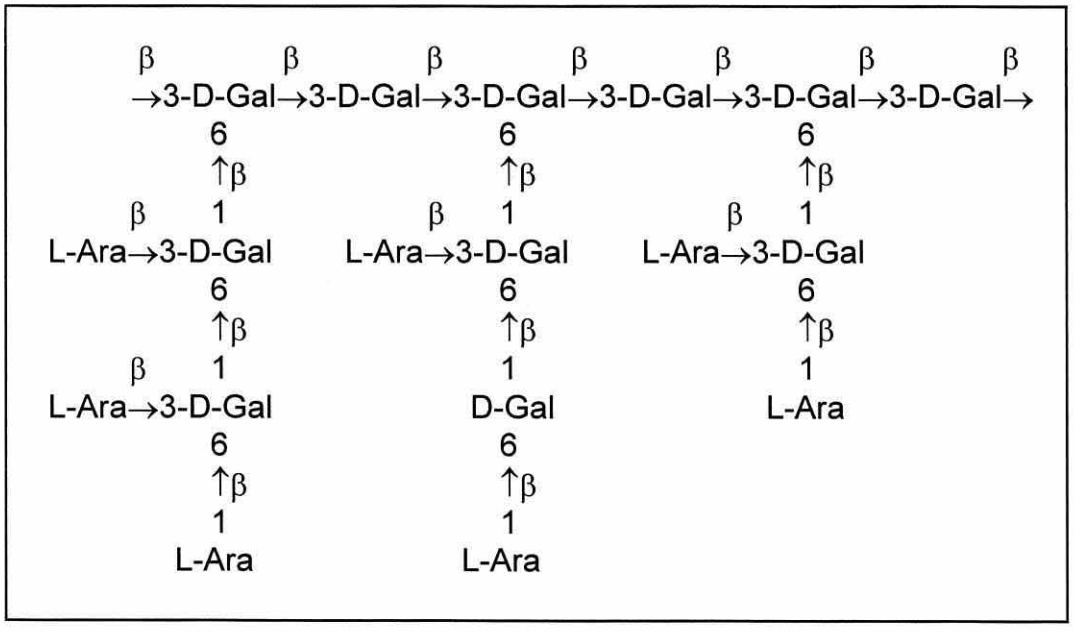


Figure 1.6. Tentative side chain structure of ryegrass (*Lolium multiflorum*) endosperm arabinogalactan protein.

According to Fincher *et al.* (1983) and Bacic *et al.* (1987), attachment of such carbohydrate moieties to the protein core occurs via β-D-galactosyl-hydroxyproline linkages. Circular dichrometry study of AGPs showed that approximately 30% of the protein moiety is in a polyproline II helix (van Holst and Fincher, 1984). Little is known however, about the conformation of the rest of the protein or of the carbohydrate chains.

Cellular localisation of AGPs has proved difficult because of their extreme solubility. Several groups of researchers have, however, been able to localise the AGPs in the tissue of several plant species using cytochemical analysis. Anderson *et al.* (1977) have shown that AGPs were associated with granules in the peripheral cytoplasm of cultured *Lolium multiflorum* endosperm cells. They have also been found to be associated with the aleurone layer of cereal seeds, especially the cytoplasm-wall interface (Anderson *et al.*, 1977), with vesicles in the intercellular spaces of cotyledon parenchyma cells of legume seeds (Clarke *et al.*, 1975), with the cell membrane in leaf parenchyma cells of *Zantedeschia* and *Alocasia* and in secretory ducts of *Hedera helix* leaves (Clarke *et al.*, 1978), and associated with the stigma surface and style canal of *Gladiolus* (Knox *et al.*, 1976). AGP has also been found within the protoplasts of pollen grains (Clarke *et al.*, 1979) and associated with the surfaces of plant protoplasts from both mono- and dicotyledonous callus cells (Larkin, 1977a). In addition, AGPs were shown to be associated with the plasma membrane (Pennel *et al.*, 1989; Norman *et al.*, 1990).

1.5.1.2.2 Expression and functions

Studies on the accumulation of AGPs in soybean root nodules showed that genes coding for the proteins were developmentally regulated (Cassab, 1986). In this study, seeds of soybean were infected with *Bradyrhizobium japonicum* USDA 3I1b 110, and were planted to allow the plants to produce root nodules. As the nodule ages, the AGP level declines from 8.8 μg/mg dry weight in two-week-old nodules to 4.2 μg/mg dry weight in ten-week-old nodules. Analyses on ten-week-old uninfected plant tissues showed that

AGPs were also present in roots, leaves, flowers, pods and seeds (Cassab, 1986). Evidence for the developmental regulation of plasma membrane AGPs in flowers, embryos and root has also been shown (Pennel and Roberts, 1990; Pennel *et al.*, 1991). A study by Herman and Lamb (1992) using immunogold electron microscopy has shown that plasma membrane AGPs were not only localised to tobacco leaf plasma membranes but also to intravacuolar multivesicular bodies.

There is no general function for AGPs that has been established so far. AGPs have been proposed to act as glues and lubricants (Fincher *et al.*, 1983). These suggestions were made based on their predominantly extracellular location and their various biochemical and physical properties (Fincher *et al.*, 1983). Showalter (1993) has also suggested that AGPs are likely to function in cell-cell recognition. The presence of AGPs in the middle lamella of the wall and in the style of angiosperms and in the medulla of root nodules as shown by Cassab (1986) may support this suggestion. Other suggested functions for AGPs are in wound healing, frost hardiness and drought resistance (Clarke *et al.*, 1979; Lamport and Catt, 1981) but there has been no strong evidence presented to support these functions (Fincher *et al.*, 1983).

1.5.1.3 Solanaceous lectins

1.5.1.3.1 Structure

Solanaceous lectins are a member of the lectin group of proteins. They can be distinguished from other lectins by their restricted occurrence in solanaceous plants, their ability to agglutinate oligomers of N-acetylglucosamine, their predominantly extracellular location, and their unusual amino acid and carbohydrate composition. Hydroxyproline and arabinose are the major constituents of solanaceous lectins (Showalter and Varner, 1989).

Potato tuber lectin (PTL) is the most well studied member of the solanaceous lectins. It is a glycoprotein with a molecular weight of 50 kDa. It

consists of 50% carbohydrate and 50% protein by weight (Allen *et al.*, 1978; Matsumoto *et al.*, 1983). Arabinose is the main component of the carbohydrate moiety of solanaceous lectins and is linked to the protein component by hydroxyproline residues. To a lesser extent, galactose residues are linked to serine (Allen *et al.*, 1978; Ashford *et al.*, 1982). The protein moiety of solanaceous lectins is rich in hydroxyproline, serine, glycine and cysteine (Allen *et al.*, 1978). According to Showalter (1993), the protein moiety consists of at least two distinct domains, one being rich in serine and hydroxyproline and containing the carbohydrate moiety, and the other being rich in glycine and cysteine. The ability of PTL to bind N-acetylglucosamine oligomers is known to be associated with the glycine-cysteine-rich domain (Allen *et al.*, 1978; Ashford *et al.*, 1981). It is likely that this domain will be homologous to that found in other chitin binding proteins such as wheat germ agglutinin, barley lectin, rice lectin, stinging nettle lectin, hevein, potato Win 1 and 2, poplar Win 8, bean chitinase, potato chitinase and tobacco chitinase (Showalter, 1993).

The serine-hydroxyproline-rich domain bears a striking biochemical resemblance to the extensins. Solanaceous lectins and extensins are similar in several aspects. Both are abundant in serine, hydroxyproline and arabinose, and they also have a similarity in term of their extracellular location. Also in term of carbohydrate-protein linkages, tri- and tetra-arabinose residues are attached to protein molecules to hydroxyproline residues and galactose residues are attached to serine (Showalter, 1993).

1.5.1.3.2 Expression and functions

Potato tuber lectin (PTL) has been shown to accumulate in potato tuber in response to wounding (Casalongue and Pont-Lezica, 1985) and viral infection (Scheggia *et al.*, 1988). As shown by Sequeira and Graham (1977), PTL agglutinates avirulent strains of *Pseudomonas solanacearum*. Tissue print immunoblot studies by Pont-Lezica *et al.* (1991) showed that PTL is accumulated in a developmental fashion, with initial deposition occuring in the

cortex and pith regions of the tuber and subsequent distribution occuring uniformly in the whole tuber.

The physiological function of solanaceous lectins is unknown. It has been proposed that these glycoproteins involve various forms of cell to cell interaction (Showalter, 1993). They may also be involved in sugar transport, stabilisation of seed storage proteins and the control of cell division (Showalter, 1993). The results described by Casalongue and Pont-Lezica (1985) suggest that these glycoproteins are involved in wound healing. The role of PTL in the plant defence mechanism has also been proposed (Sequeira and Graham, 1977; Scheggia *et al.*, 1988).

1.5.2 Proline-rich glycoproteins (PRPs)

1.5.2.1 Structure

Apart from extensins, another type of cell wall proline-rich protein has also been identified. These proteins are termed the proline-rich glycoproteins (PRPs). There are two characteristics that distinguish PRPs from extensin; PRPs lack the characteristic Ser-$(Hyp)_4$ motif, and their mode of expression is different (José and Puigdomenech, 1993).

Generally, all the PRPs are characterised by the presence of Pro-Pro repeat motifs that are contained within a variety of other larger repeat units. (Showalter, 1993). For example, PRPs in soybean, alfalfa and pea are characterised by the presence of the repeating pentapeptide sequence Pro-Pro-X-Y-Lys, where X and Y can be valine, tyrosine, histidine and glutamic acid. In some cases, an extension of proline residues beyond the other two proline residues occurs (Showalter, 1993). PRP sequences show a large heterogeneity in their respective amino acid composition. The deduced proteins from soybean *SbPRP 1*, *SbPRP 2* and *SbPRP 3* sequences lack His and Ser, have moderate amounts of Glu and are high in Tyr and Lys (Hong *et al.*, 1987, 1990). According to Raines *et al.* (1991), wheat WPRP1 lacks in His, Ser and Tyr. It also has highly basic and acid amino acids such as Lys and Glu. Franssen *et al.* (1987) showed that soybean ENOD2 contains similar

amounts of His, Tyr and Lys and high levels of Glu but lacks Ser. Carrot *p33* has been shown to contain a high level of His and Lys and a moderate amount of Ser, Tyr and Glu (Chen and Varner, 1985a).

In dicots, PRPs lack carbohydrate or are only lightly glycosylated (Datta *et al.*, 1989) and contain approximately equimolar quantities of proline and hydroxyproline (Averyhart-Fullard *et al.*, 1988; Datta *et al.*, 1989; Kleis-San Francisco and Tierney, 1990). In monocots, a PRP gene was first isolated and characterised by Jose-Estanyol *et al.* (1992) in maize. The protein of this gene consists of an N-terminal proline-rich domain with numerous Pro-Pro-Tyr-Val and Pro-Pro-Thr-Pro-Arg-Pro-Ser repeats and a C-terminal proline-poor domain that is hydrophobic and contains several Cys residues (Jose-Estanyol *et al.*, 1992).

Proline-rich glycoproteins can be divided into four classes according to their abundance in amino acid repeats in their sequences. Group A represents PRPs where the most represented repeat is PPVYK and their derivatives (Table 1.7) as V can be replaced with Y, I and H, also Y with K, E, G and H, and K with D, N and T. Most of the group A PRPs are soybean PRPs. Tomato, carrot and maize PRPs are also included in this group.

Table 1.7. Amino acid sequence repeats of group A PRPs.

Repeat	Number of repeats (Name/Plant)	Reference
PPVYK	29 (*SbPRP 1*, soybean)	Hong *et al.* (1987)
	17 (*SbPRP 2*, soybean)	Hong *et al.* (1990)
	6 (*SbPRP 3*, soybean)	
	19 (1A10, soybean)	Averyhart-Fullard *et al.* (1988)
	1 (*p33*, carrot)	Chen and Varner (1985b)
PPYV	16 (zmHyPRP, maize)	Jose-Estanyol *et al.* (1992)
PPVYT	3 (*p33*, carrot)	Chen and Varner (1985b)
	1 (*SbPRP 1*, soybean)	Hong *et al.* (1987)
PPVKK	1 (*SbPRP 1*, soybean)	Hong *et al.* (1987)
PPYKK	2 (*SbPRP 3*, soybean)	Hong *et al.* (1990)
PPVHK	5 (*p33*, carrot)	Chen and Varner (1985b)

PPVEK	3 (1A10, soybean) 6 (*SbPRP 1*, soybean) 16 (*SbPRP 2*, soybean)	Averyhart-Fullard *et al.* (1988) Hong *et al.* (1987) Hong *et al.* (1990)
PPVEN	1 (*SbPRP 2*, soybean)	Hong *et al.* (1990)
PPVED	1 (*SbPRP 3*, soybean)	Hong *et al.* (1990)
PPTEK	1 (*SbPRP 2*, soybean)	Hong *et al.* (1990)
PPHEK	17 (ENOD 2, soybean)	Franssen *et al.* (1987)
PPYGK	1 (*SbPRP 2*, soybean) 1 (*SbPRP 3*, soybean)	Hong *et al.* (1990)
PPIEK	1 (*SbPRP 3*, soybean) 1 (1A10, soybean)	Hong *et al.* (1990) Averyhart-Fullard *et al.* (1988)
PPIHK	3 (*p33*, carrot)	Chen and Varner (1985b)
PPIYK	1 (1A10, soybean) 4 (*SbPRP 1*, soybean) 2 (*SbPRP 2*, soybean)	Averyhart-Fullard *et al.* (1988) Hong *et al.* (1987) Hong *et al.* (1990)
PPI/HVK/S	8 (TPRP-F 1, tomato)	Salts *et al.* (1991)
PPXTPK/T	8 (TPRP-F 1, tomato)	Salts *et al.* (1991)
PPPEYQ	6 (ENOD 2, soybean)	Franssen *et al.* (1987)
PPPEHQ	3 (ENOD 2, soybean)	Franssen *et al.* (1987)
PPEHQ	2 (ENOD 2, soybean)	Franssen *et al.* (1987)

Group B represents PRPs with amino acid repeats of PEPK and their derivatives (Table 1.8). Wheat and carrot PRPs are represented in this group.

Table 1.8. Amino acid sequence repeats of group B PRPs.

Repeat	Number of repeats (Name/Plant)	Reference
PEPK	43 (WPRP 1, wheat)	Raines *et al.* (1991)
MPPEPKPEPKPEP	14 (WPRP 1, wheat)	Raines *et al.* (1991)
PEPMPK	16 (WPRP 1, wheat)	Raines *et al.* (1991)
PMPK	4 (WPRP 1, wheat)	Raines *et al.* (1991)
PX	10 (DC 2.15, carrot)	Aleith and Richter (1990)

Bean PvPRP 1 is a dominant group C PRP which is characterised by having PVHPPVKPPV repeats and their derivatives (Table 1.9), while maize HyPRP is the only member in group D and characterised by having PPTPRPS repeats (José and Puigdomenech, 1993).

Table 1.9. Amino acid sequence repeats of group C PRPs.

Repeat	Number of repeats (Name/Plant)	Reference
PVHPPLNPP	1 (PvPRP 1, bean)	Sheng *et al.* (1991)
PPHPPLKPPV	1 (PvPRP 1, bean)	Sheng *et al.* (1991)
PIHPPLNPPV	1 (PvPRP 1, bean)	Sheng *et al.* (1991)
PVHPPVKPPV	3 (PvPRP 1, bean)	Sheng *et al.* (1991)
PVHPPV	1 (PvPRP 1, bean)	Sheng *et al.* (1991)
PVHP	1 (PvPRP 1, bean)	Sheng *et al.* (1991)
PV/LPPL/IP	nd (SF 19, sunflower)	Herdenberger *et al.* (1990)

1.5.2.2 Expression

Wounding and developmental events are the two main factors that have contributed most to studies on the expression of PRP genes in plant tissues. Carrot *p33* PRP was the first PRP gene shown to respond to wounding. Studies by Tierney *et al.* (1988) showed that the mRNA encoding the *p33* protein increased markedly within 1 h after carrot storage roots were wounded. Expression was maximal after 2 h and was maintained for the next 24 h. Treatment of the carrot roots with ethylene did not induce *p33* expression. However, treatment of wounded roots with ethylene inhibited the accumulation of *p33* mRNA transcripts. Incubation of carrot suspension-cultured cells with a crude endogenous elicitor fraction also failed to induce the accumulation of *p33* mRNA transcripts (Tierney *et al.*, 1988).

The soybean *SbPRP 1* and *SbPRP 2* genes have also been shown to be induced by wounding (Suzuki *et al.*, 1993). *SbPRP 1* transcripts were

detected in the roots of soybean seedlings within 2 h after wounding and were present at a high level in the roots and elongating hypocotyls 20 h after wounding. In contrast, *SbPRP 2* transcripts increased transiently and rapidly through the soybean seedlings after wounding. Wounding has also been shown to induce expression of the *SbPRP 2* gene in leaves, but the pattern of expression was distinct from that seen in seedlings, and reached a maximum 20 h after wounding. Treatments of the mature hypocotyls and roots of seedlings with indoleacetic acid and naphthalene-1-acetic acid have also induced the expression of the *SbPRP 2* gene (Suzuki *et al.*, 1993).

Soybean PRP genes are also developmentally regulated. *SbPRP 1* is mainly expressed in the mature roots and hypocotyls of germinating seedlings (Hong *et al.*, 1989), and is induced by water deficit in elongating cells from hypocotyls (Creelman and Mullet, 1991). *SbPRP 2* is expressed in apical and elongating hypocotyls and in elongating and maturing roots. *SbPRP 3* has limited expression in mature and elongating hypocotyls, but is mainly expressed in three-week-old stems and leaves. All of the genes are expressed in the seed pod, especially *SbPRP 3*, and in the seed coat, although *SbPRP 2* does not appear until days 24 and 28 after anthesis. No transcript corresponding to these genes is detected in the cotyledons, but they are present in soybean cultured cells (Datta *et al.*, 1989; Hong *et al.*, 1989).

In sunflower, the *SF1*, *SF2* and *SF33* PRP genes are developmentally regulated (Evrard *et al.*, 1991). Expression of these genes was first detected shortly before flower opening, and continued during pollination. Expression of the *HW101* and *HW103* PRP genes in English ivy was studied by Woo *et al.* (1994). The *HW101* gene was expressed at a higher level in juvenile laminae, petioles and stems compared to identical tissues in mature plants, whereas the *HW103* gene was expressed to a higher level in mature compared to juvenile *in vitro*-cultured petioles. The *HW103* gene was also expressed in developing seeds.

Studies on the *PTGRP* gene in *Lycopersicon chilense* showed that this gene was significantly down-regulated by water stress (Yu *et al.*, 1997). The *PTGRP* transcript level decreased 5- to 10-fold in leaves and stems after 4 - 8

days of water stress, and re-accumulated when the plants were re-watered. Abscisic acid, mannitol and NaCl treatments of suspension-cultured cells of *L. chilense* also inhibited the expression of the *PTGRP* gene (Yu *et al.*, 1997).

1.5.2.3 Cellular localisation and functions

As well as extensins, PRPs have been shown to be localised in various tissues in plant systems. The cellular localisation of PRPs is summarised in Table 1.10 below. The definite function of PRPs in plant systems is unknown and subject to much speculation. Studies on the expression of the ENOD2 PRP gene in soybean and pea (van de Wiel *et al.*, 1990) suggests that the proteins are involved in nodule morphogenesis.

Table 1.10. Tissue localisation of PRPs .

Plant system	Tissue localisation	Reference
Soybean stems	Xylem vessel elements of young stems and in both phloem fibers of older stems	Ye *et al.* (1991)
Soybean stems (*SbPRP 1*)	Phloem. Xylem and epidermis	Wyatt *et al.* (1992)
Soybean stems (*SbPRP 2*)	Epidermis, cortical cells, phloem and pith parenchyma	Wyatt *et al.* (1992)
Soybean stems (*SbPRP 3*)	Endodermis	Wyatt *et al.* (1992)
Soybean roots	Corner walls of the cortex and in the protoxylem	Ye *et al.* (1991)
Soybean seeds (*SbPRP 1*)	Group of sclerid cells of the seed coat near the hilum	Wyatt *et al.* (1992)
Soybean seeds (*SbPRP 2*)	Primary to the aleurone layer of the seed coat	Wyatt *et al.* (1992)
Soybean nodules (ENOD 2)	Nodule parenchyma (i.e., inner cortex)	van de Wiel *et al.* (1990)
Tomato stems	Xylem vessel elements and fibers; some in outer and inner phloem fibers	Ye *et al.* (1991)
Petunia stems	Xylem vessel elements and fibers; some in outer phloem fibers	Ye *et al.* (1991)

Tobacco stems	Xylem vessel elements and fibers	Ye *et al.* (1991)
Tobacco stylar	Cells of the transmitting tract of the style	Chen *et al.* (1993)
Potato stems	Xylem vessel elements and fibers	Ye *et al.* (1991)
Maize embryo	Scutellum and in nonvascular cells from the embryo axis	Jose-Estanyol *et al.* (1992)
Pea nodules	Nodule parenchyma (i.e., inner cortex)	van de Wiel *et al.* (1990)
Sunflower flower	In the single cell layer of the anther epidermis	Evrard *et al* (1991)
Juvenile and mature petioles of English ivy	Phloem parenchyma, the inner cortex adjacent to the phloem, in cells surrounding ducts	Sanchez *et al.* (1995)

They may function specifically in the erection of a cell wall oxygen barrier for the oxygen-sensitive nodule. The involvement of PRPs in the lignification process has also been suggested by Ye *et al.* (1991) as some PRPs have been shown to be associated with lignified tissues. It has also been proposed that the PRPs are involved in mechanisms resulting in the rapid hardening of the wall as a protection against environmental stress (Bradley *et al.*, 1992). PRPs may also play a role in the maturation of cell walls (Cassab and Varner, 1988), but there is still lack of conclusive evidence for these functions.

1.5.3 Glycine-rich glycoproteins (GRPs)

1.5.3.1 Structure

Glycine-rich glycoproteins (GRPs) represent a relatively newly discovered class of plant proteins. The general characteristic of GRPs are that they contain $(Gly\text{-}X)_n$ repeat motifs, where X is most frequently Gly but can also be Ala or Ser (Showalter, 1993). However, different repetitive motifs have also been identified in isolated clones (Table 1.11). Glycine-rich glycoproteins appear to contain various amounts of Gly depending on the plant species.

Table 1.11. Amino acid sequence repeats in GRPs.

Repeat	Number of repeats (Name/Plant)	Reference
G_n-X	30 (*atGRP-2*, *Arabidopsis*)	de Oliveira *et al.* (1990)
	21(*atGRP- 4*, *Arabidopsis*)	
	30 (*atGRP-5*, *Arabidopsis*)	
	nd (*GRP 1.8*, bean)	Keller *et al.* (1988)
	nd (*GRP 1.0*, bean)	
	33 (hvGRP, barley)	Rohde *et al.* (1990)
$(G_nAG_n)_n$-F/H	19 (*atGRP-1*, *Arabidopsis*)	de Oliveira *et al.* (1990)
G_4N/RYQ	6 (*atGRP-3*, *Arabidopsis*)	de Oliveira *et al.* (1990)
G-X-G-X	nd (*PTGRP-1*, petunia)	Condit and Meagher (1986)
GYGYGYG	3 (*Osgrp-1*, rice)	Lei and Wu (1991)
G_{2-6}-R	6 (class III-wM, tomato)	Showalter *et al.* (1991)
	9 (class III-wN, tomato)	
	6 (class III-uE-7, tomato)	
G_{2-6}-YP	6 (class III-wM, tomato)	Showalter *et al.* (1991)
	6 (class III-wN, tomato)	
$SP_4SPSP_4Y_3K$	nd (class IV-w1-8, tomato)	Showalter *et al.* (1991)
and G_{2-6}-R/Y-P	nd (class IV-w10-1, tomato)	
G_{2-5}-R	11 (class V-uA-3, tomato)	Showalter *et al.* (1991)
	6 (class V-uK-4, tomato)	

Soybean seed coat GRP contains the least amount of Gly - 11% (Rackis *et al.*, 1961), while the glycine-richest protein containing 73% glycine is found in *Brassica napus* (Bergeron *et al.*, 1993). Condit and Meagher (1986) were the first to isolate a gene, *PTGRP*1 from petunia which codes for a protein containing 67% Gly. Since then, several other groups have successfully isolated and characterised GRP cDNAs or genes from tomato (Godoy *et al.*, 1990; Showalter *et al.*, 1991), *Arabidopsis* (de Oliveira *et al.*, 1990; Quigley *et al.*, 1991), petunia (Linthorst *et al.*, 1990), tobacco (van Kan *et al.*, 1988; Obokata *et al.*, 1991), carrot (Sturm, 1992), oilseed rape (Bergeron *et al.*, 1993) and barley (Molina *et al.*, 1997). The protein coded by the *PTGRP*1 gene had a signal peptide indicating that it could be transported

out of the cytoplasm. A model for the mature protein was proposed as a β-pleated sheet with 8 anti-parallel strands (Condit and Meagher, 1986).

Generally, GRPs can be divided into two groups depending on their location in the cell. The first group contains the cell wall associated GRPs; examples of this group are the petunia GRPs (Condit and Meagher, 1986; Linthorst *et al.*, 1990), pumpkin GRP (Varner and Cassab, 1986), tomato GRPs (Godoy *et al.*, 1990; Showalter *et al.*, 1991), *Arabidopsis* GRPs (de Oliveira *et al.*, 1990; Quigley *et al.*, 1991), tobacco GRPs (van Kan *et al.*, 1988; Obokata *et al.*, 1991; Yasuda *et al.*, 1997), carrot GRP (Sturm, 1992) and oilseed rape GRP (Bergeron *et al.*, 1993). The second group of the GRPs are located in the cytoplasm. Members of this latter group are maize GRP (Gomez *et al.*, 1988), rice GRP (Mundy and Chua, 1988) and barley GRP (Molina *et al.*, 1997).

1.5.3.2 Expression

The genes coding for GRPs, like those coding for HRGPs and PRPs are expressed in response to a variety of developmental and exogenous stimuli. However, most of them were shown to be developmentally regulated. Studies on the petunia *PTGRP*1 gene showed that the gene is expressed mainly in stems, leaves and to a lesser extent in flower and no expression is detected in roots (Condit and Meagher, 1986, 1987). In stems and leaves the level of *PTGRP*1 transcripts decreases with developmental age of the tissue (Condit, 1993).

In bean, the *GRP 1.8* gene is expressed in young hypocotyls, developing ovaries and roots. In young hypocotyls, significant levels of *GRP 1.8* transcripts are detected 7 days after germination and reach a maximum at 9 days. No expression is detected after the tissues are 16 days old (Keller *et al.*, 1988). In response to wounding, expression of *GRP 1.8* is inhibited 12 h after the young hypocotyls are wounded (Keller *et al.*, 1988). *Arabidopsis* GRP genes are also differentially regulated. *atGRP-1* and *atGRP-2* are expressed in roots, stems, leaves, seed pod and flowers. *atGRP-3* is expressed in stems and leaves. *atGRP-4* is expressed at a low level in stems,

leaves and flowers. *atGRP-5* is expressed in stems and seed pods and at a low level in leaves (de Oliveira *et al.*, 1990).

Two transcripts encoded by the rice *Osgrp-1* gene were shown to be differentially regulated. The 0.6 kb transcript was detected in whole rice seedlings in all developmental stages, while the 0.9 kb transcript was mainly expressed in 30-day-old seedlings, the stage where new tillers were vigorously growing and the vascular system was actively differentiating (Lei and Wu, 1991). Studies on two oilseed rape GRP genes, *BnGRP10* and *BnGRP22* showed that expression of both genes is only detected in roots and stems of seedlings (Bergeron *et al.*, 1993, 1994). Expression of *BnGRP22* decreases 30 days after germination (Bergeron *et al.*, 1994). The tobacco *NT16* GRP gene was shown to be highly expressed in unorganised callus as well as in shoot-forming calli. In normal plants, the level of *NT16* transcripts was high in roots, and low in stems, whereas no transcripts were found in flowers or leaves. Mechanical wounding, however, induced the accumulation of *NT16* transcripts in leaves (Yasuda *et al.*, 1997).

In tomato, the class III GRP transcripts are highly expressed in stems 24 h after wounding. Expression was detected locally and at a distance from the wounded tissue. A low level of expression of the gene is also detected in wounded leaves. Expression of the class V GRP gene is high in unwounded roots and lower in unwounded stems. Wounding decreased expression of the class V GRP in both roots and stems. Unwounded and wounded leaves contained little of the class V gene transcript. Abscisic acid treatment and drought stress also induced the accumulation of class III GRP transcripts in stems (Showalter *et al.*, 1992).

Arabidopsis GRP genes also show different responses to external stimuli. Salicylic acid first increases and then reduces the accumulation of *atGRP-1* transcripts, *atGRP-2* and *atGRP-5* responses are retarded, while *atGRP-3* transcripts increase continuously with time. After dessication, *atGRP-1* and *atGRP-2* transcripts disappeared, *atGRP-3* transcripts first increased but then reduced and *atGRP-5* was stable. Ethylene and abscisic acid only stimulated *atGRP-3* moderately (de Oliveira *et al.*, 1990).

Both barley *HvGRP2* and *HvGRP3* genes were induced by cold treatment and *Erysiphe graminis* infection. Ethylene treatment only induced the accumulation of *HvGRP2* transcripts, whereas methyl jasmonate switched off the expression of both genes. The two genes, however, did not respond to bacterial pathogen infections (Molina *et al.*, 1997).

1.5.3.3 Cellular localisation and functions

As shown in Table 1.12, GRPs are located mostly in vascular bundles in stems, particularly the xylem elements. However, GRPs are also detected in soybean petioles and roots (Ye and Varner, 1991), bean seed (Keller *et al.*, 1989b) and in maize embryos (Gomez *et al.*, 1988).

Table 1.12. Tissue localisation of GRPs.

Plant system	Tissue localisation	Reference
Soybean stems and petioles	Primary xylem, primary phloem, and in newly differentiated secondary xylem	Ye and Varner (1991)
Soybean roots	Primary xylem	Ye and Varner (1991)
Bean stems	Protoxylem tracheary elements of the vascular system	Keller *et al.* (1989b)
Bean stems	Protoxylem, primary xylem and phloem, and newly differentiated secondary xylem	Ye and Varner (1991)
Bean stems	Protoxylem, cell corners of protoxylem and metaxylem elements, and phloem	Ryser and Keller (1992)
Bean seeds	Tracheary elements of the vascular tissue of seed coats	Keller *et al.* (1989b)
Tomato stems	Xylem vessel elements and fibers; some in outer and inner phloem fibers	Ye *et al.* (1991)
Petunia stems	Vascular tissue (phloem and cambium) and to a layer of cells at the epidermis	Condit *et al.* (1990)
Petunia stems	Xylem vessel elements and fibers; some in outer phloem fibers	Ye *et al.* (1991)
Petunia stems	Primary phloem, pith parenchyma and cortex collenchyma	Condit (1993)

Petunia stems	Primary phloem, outer collenchyma cells and palisade cells	Condit (1993)
Tobacco stems	Xylem vessel elements and fibers	Ye *et al.* (1991)
Tobacco with bean *GRP 1.8*-GUS transgene	Protoxylem tracheary elements of the vascular system	Keller *et al.* (1989a); Keller and Baumgartner (1991)
Maize embryo	Scutellar epidermal cells surrounding embryo axis; epidermal cells of leaves	Gomez *et al.* (1988)

Studies on the GRPs are not as well advanced as studies on extensins and PRPs, and there is therefore even less of an idea about their actual function. It has been proposed that the function of GRPs is dependent on the type of GRPs; whether they are cell wall associated or cytoplasmic GRPs (Showalter, 1993). Cell wall GRPs have been proposed to be involved in providing elasticity as well as tensile strength during vascular development (Showalter, 1993). It has also been proposed that cell wall GRPs may be involved in the wound healing process (Showalter, 1993; Yasuda *et al.*, 1997). The induction of GRP genes following wounding treatment has been shown by Condit and Meagher (1987), Showalter *et al.* (1991, 1992) and Yasuda *et al.* (1997) and may therefore support this hypothesis. Due to the presence of GRP transcripts at a high level in tobacco roots and shoot-forming calli as shown by Yasuda *et al.* (1997), it was suggested that GRPs may be involved in root and shoot formation. The cytosolic GRPs have been proposed to play roles in drought tolerance as well as in the wound healing process (Showalter, 1993). However, to date, no firm evidence is available to support these hypotheses.

1.5.4 Cell wall enzymes

Several enzymes have been detected in the cell walls of higher plants, and the possibility has been considered that they may play a role in cell wall metabolism. Most of these enzymes are usually glycoproteins.

Peroxidases are widespread in plant tissues, and are particularly abundant in cell walls (Fry, 1979). Peroxidases are present in the form of

isoenzymes whose pattern of expression is tissue specific, developmentally regulated, and controlled by environmental stimuli (Lagrimini and Rothstein, 1987). A possible function of peroxidases is the oxidative coupling of wall polymer-bound phenols to yield diferulate and isodityrosyl residues (Fry, 1979, 1980, 1982, 1983). It has also been shown that peroxidases can inactive the hormone indoleacetic acid (IAA) by oxidation (Palmieri *et al.*, 1978). The importance of peroxidases in the plant defense response to pathogen attack has also been suggested (Gaspar *et al.*, 1982; Sequeira, 1983).

Hydrolases are another class of plant proteins that have been postulated to have a potential role in plant defense (Birecka and Miller, 1974). They are usually localised to the lytic compartment of cells, which includes the vacuoles and the cell wall space (Matile, 1975), even though they are frequently found in the cytoplasm (Dey and Campillo, 1984). The hydrolases that have been found in the wall compartment are β-1,3-glucanase, cellulase, arabinosidases, β-fructofuranosidases, α-galactosidases, β-galactosidases, α- and β-mannosidases, trehalases, β-glucosidase, β-glucuronidase, β-xylosidases and acid phosphatases.

Pectinesterases and polygalacturonase are abundant in ripening tomato fruit (Cassab and Varner, 1988). Pectinesterases are involved in the conversion of protopectin to soluble pectin and pectate, in plant maturation processes and in plant defence mechanisms (Cassab and Varner, 1988), while polygalacturonase is involved in cell wall degradation (Rushing and Huber, 1984). Both enzymes are believed to be involved in fruit ripening (Cassab and Varner, 1988). It has been reported that the red-ripe tomato fruit contains at least 2000 times the level of polygalacturonase mRNA compared to the immature-green fruit (Della Penna *et al.*, 1986).

The most recent cell wall enzyme discovered is xyloglucan endotransglycosylase (XET) (Fry *et al.*, 1992). The activity of this enzyme was found in growing portions of a wide range of species including dicotyledons, graminaceous monocotyledons, a moss and liverwort (both sporophyte and gametophyte). Fry *et al.* (1992) proposed that the enzymic

activity of XET was to cut and re-join xyloglucan molecules, thus loosening the cell wall and allowing the wall to expand.

1.6 Aim of this study

Plant cells are constrained by a tough, yet flexible, polymeric wall that determines cell shape, permits high turgor pressures to develop, and confers important mechanical advantages. However, these walls present a special problem to growing cells, which must expand and at the same time keep the wall strong enough to withstand the large mechanical stresses that arise from turgor pressure. These stresses may exceed 10^8 N m^{-2} (Cosgrove, 1993) because the expansive forces generated by turgor are focused on the thin cell wall. Because of this mechanical situation, plant cells cannot simply deposit more material in the wall to make it extend, but the polymeric network that confines the cell must slip to create a new surface area, while still maintaining sufficient structural integrity to resist large tensile forces.

The cell wall is also an essential element of plant protection against the attack of microbes and resistance to stress. Plant pathogens must penetrate the barrier of the wall in order to attack the plant. During infection, pathogens secrete a complex mixture of cell wall-degrading enzymes. However, plants have within their walls, proteins that can specifically inhibit the wall-degrading enzymes secreted by microbes. The walls of plant cells also contain enzymes that can degrade the walls of microbes (Cline and Albersheim, 1981) and in some cases release molecules from the walls of the microbes that can activate a defense response in the plant (Ayers *et al.*, 1976). Thus, when plant cell walls are exposed to attack by micro-organisms, it is apparent that there is a considerable interaction between molecules of the plant cell wall and molecules of microbial origin. These molecular interactions determine whether a plant can be successfully invaded by micro-organisms.

Changes that take place when plants experience pathogen attacks and stress conditions are perceived at the cell wall. Because cell wall proteins are one of the major components of the cell wall, they may play a very important role in plant adaptations against these stress conditions. One of the best

studied of the cell wall proteins is extensin. Extensins have been shown in many plant systems to be induced by conditions such as mechanical injury, tensile stress and pathogen infections.

In oilseed rape plants, two genes, *extA* (Evans *et al.*, 1990) and *extB* (Gatehouse *et al.*, 1990) representing two different gene families encoding for extensins have been isolated. Studies by Evans and colleagues showed that *extA* was specifically expressed in the root. However, studies by our group showed that the gene was also expressed in vascular system (Shirsat *et al.*, 1991), in leaf, petiole and stem on wounding (Shirsat *et al.*, 1996a) and in regions of the plant which experience tensile stresses (Shirsat *et al.*, 1996b). Evans *et al.* (1990) have shown that *extA* is a member of a complex multigene family containing at least 6 members. Because *extA* is the only member of the family isolated so far, the aim of this study was to isolate other member(s) of the gene family. This aim was achieved by the construction of a mini genomic library and the library was then screened against the coding sequence of the *extA* gene. After several batches of screening, one clone was successfully isolated.

In order to characterise the isolated gene, it was restriction mapped and expression studies were performed. Studies were done in order to see if the isolated gene was induced by mechanical wounding in leaf, petiole and stem as already shown for the *extA*. In order to support the hypothesis that extensin proteins are functioned in plant defense, studies on the expression of the gene were performed in leaf. Detached leaves were treated with several compounds (abscisic acid [ABA], sodium salicylate and methyl jasmonate [MeJ]) which have been shown to be able to induce expression of several genes involved in plant defense systems. The effect of these compounds on the expression pattern of the *extA* gene in leaf was also tested.

However, during the first year, the study concentrated on root growth in rice, *Oryza sativa* (Appendix I). Two varieties of upland rice were used, *Oryza sativa* variety Azucena (long rooted plants) and *Oryza sativa* variety Bala (short rooted plants). Both plants are drought resistant but use different adaptation mechanisms for drought resistance. In Azucena, a long root

system is the main drought avoidance mechanism, while increasing the rate of stomatal closing when plants experience water stress is the main drought avoidance mechanism in Bala. Because the long root system was the main drought avoidance mechanism in the upland rice (Yoshida and Hasegawa, 1982), the aim of the study was to isolate a gene(s) which was involved in controlling root growth in Azucena, with the hope that the gene could be used in the development of a new transgenic drought resistant variety of rice. To achieve this, the elongation zone of Azucena roots was used to construct a root cDNA library. The library was then screened against cDNA probes prepared from the elogation zones of Azucena root subtracted against cDNA from the elongation zones of Bala roots. Unfortunately, the project was stopped after more than one year due to the failure to isolate a positive clone hybridising to the probe.

Chapter 2

Materials and methods

Chapter 2

Materials and methods

2.1 Chemicals and reagents

Most of the general laboratory chemicals and reagents were from BDH Merck Ltd., Lutterworth and from Fisher Scientific UK, Loughborough, Leicestershire.

Ethylenediaminetetraacetic acid (EDTA), hexadecyltrimethyl-ammonium bromide (CTAB), polyvinylpyrrolidone (pvp), sucrose, 2-mercaptoethanol, bovine serum albumin (BSA) fraction V and salmon sperm DNA were from Sigma Chemical, Poole, Dorset.

Agarose (ultrapure), sodium dodecyl sulfate (SDS) and 123bp DNA ladder were from Gibco BRL, Life Technologies Ltd., Paisley.

Caesium chloride and isopropyl-b-D-thiogalactoside (IPTG), were from Melford Laboratories Ltd., Ipswich.

Ficoll 400 and Sephadex G-50 DNA grade F were from Pharmacia Biotech Ltd., Davy Avenue, Milton Keynes.

3-(N-morpholio)propanesulphonic acid (MOPS) was from Promega Ltd., Delta House, Southamptom.

Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), RNA molecular weight marker and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-gal) were from Boehringer Mannheim UK, Bell Lane, Sussex.

α-^{32}P dCTP, yeast extract, tryptone and agar were from Amersham International Plc., Amersham Place, Buckinghamshire, UK.

2'-deoxythymidine-5'-triphosphate (dTTP), 2'-adenosine-5'-triphosphate (dATP), 2'-deoxyguanosine-5'-triphosphate (dGTP), 2'-deoxycytidine-5'-triphosphate (dCTP), ribonuclease A and ampicillin were from NBL Gene Sciences Limited, Northumberland, UK.

Blue-sensitive X-ray film was from Genetic Research Instruments Ltd., Dunmow, Essex, England.

Kodak X-omatic cassette and Kodak X-omatic fine intensifying screen

were from Kodak Scientific Imaging System Ltd., Cambridge.

Biotrans (+) Nylon membrane was from ICN Biomedicals Inc., Eagle House, Bucks.

Most of the restriction enzymes and DNA modifying enzymes were from Promega Ltd., Delta House, Southampton and NBL Gene Sciences Limited, Northumberland.

Bgl II and *Bst*E II restriction enzymes were from Pharmacia Biotech Ltd., Davy Avenue, Milton Keynes.

Other commercially supplied consumable and equipment are acknowledged at the first reference to use.

The water used for this work was distilled water. All water used for DNA and RNA manipulations was autoclaved. The water used for RNA manipulation was DEPC-treated overnight at 37^0C before it was autoclaved.

2.2 Cloning vector

The plasmid used for cloning was the pBluescript SK(+) phagemid from Stratagene.

2.3 Bacterial strain

The *Escherichia coli* bacterial strain used in this work was DH5α (Clontech). The genotype for the strain was *deoR, endA1, gyrA96, hsd R17* ($r_k^- m_k^+$), *recA1, relA1, supE44, thi-1,* Δ*(lacZYA-argFV169),* ϕ*80*δ*lacZ*ΔM15, *F*$^-$, λ^-.

2.4 Bacterial media and growth conditions

Bacterial strains were routinely grown in luria broth (LB) or LB agar [1.5% (w/v]) at 37^0C. For transformation purposes, 2XL broth was used (Table 2.1). When antibiotic selection was required, stock solutions were prepared and an appropriate amount was added to the cooled autoclaved media as detailed in detailed in Table 2.2.

Table 2.1. Preparation of bacterial growth media (g per litre).

	LB	2XL*
Bacto tryptone	10	20
Bacto yeast extract	5	10
Nacl	10	1
Agar**	15	

* 10 ml of 20% (w/v) filter sterilised glucose were added when the media cooled after if was autoclaved.

** If required.

Table 2.2. Antibiotics used in the bacterial media and growth conditions.

Antibiotic	Stock solution	Solvent	Working concentration
Ampicillin	50 mg/ml	70% ethanol	50 μg/ml
Tetracycline	5 mg/ml	ethanol	12.5 μg/ml

2.5 Colour selection by IPTG/X-gal

When the colour selection of the transformants by α-complementation of β-galactosidase was required, IPTG and X-gal were added to the cooled autoclaved media at a final concentration of 0.5 mM and 40 μg/ml respectively. IPTG and X-gal were added to the media separately to prevent precipitation. The stock solutions were as in Table 2.3.

Table 2.3. Preparation of the IPTG and X-gal stock solutions.

Substance	Stock solution	Solvent
IPTG*	100 mM	dH_2O
X-gal	50 mg/ml	DMF

* Solution was filter sterilised before it was used.

2.6 Plant materials

Seeds of oilseed rape plants, *Brassica napus* Cv. Bienvenue were bleach-sterilised and germinated on vermiculite (Vermipel) in a growth room. After 2 weeks, the germinated seeds were hydroponically grown in Hoagland solutions (Table 2.4) (Hoagland and Arnon, 1950) in the growth room at 25^0C with aeration on a 16 h day/8 h dark cycle for another 6 weeks before they were ready for harvesting. Tissues were quickly frozen in liquid nitrogen and kept at -70^0C until they were needed.

Table 2.4. Preparation of hydroponics Hoagland solution for growing oilseed rape.

	Stock solution	For 25 L (ml)
KH_2PO_4	1 M	25
KNO_3	1 M	125
$Ca(NO)_2$	1 M	125
$MgSO_4$	1 M	50
Fe/EDTA	37.33 g/L	25
Micronutrients*	see below	2.5

Micronutrients*

	Per litre (g)
H_3BO_3	28.6
$MnCl_2.4H_2O$	18.1
$ZnSO_4.7H_2O$	2.2
$CaSO_4.5H_2O$	0.8
(or $CaSO_4.2H_2O$)	0.6

2.7 General stock solutions

2.7.1 Denhardt's reagent

Denhardt's reagent was made as a 50X concentration stock solution. 500 ml of 50X concentration stock solution contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone and 5 g of bovine serum albumin (Fraction V, Sigma). It was distributed into aliquots and stored at -20^{0}C.

2.7.2 Denatured, fragmented salmon sperm DNA

Salmon sperm DNA (Sigma type III sodium salt) was dissolved in water at a concentration of 10 mg/ml. The DNA was sheared by passing the solution 12 times rapidly through a 17-gauge hypodermic needle. It was then boiled for 10 minutes and stored at -20^{0}C in small aliquots. Just before use, the solution was heated for 5 minutes in a boiling-water bath and then chilled quickly on ice.

2.7.3 20X SSC

The solution was prepared by dissolving 175.3 g NaCl and 88.2 g sodium citrate in 800 ml dH_2O. pH was adjusted to 7.0 with a few drops of a 10 N solution of NaOH. The volume was adjusted to 1 litre with dH_2O. The solution was kept at room temperature.

2.7.4 TAE

TAE (Tris-acetate) buffer was prepared as a 10X concentration stock solution. 24.2 g Tris base was dissolved in 800 ml dH_2O. 24.2 ml of glacial acetic acid and 20 ml of 0.5 M EDTA, pH 8.0 were then added and the volume of the solution was adjusted to 1 litre with dH_2O. The solution was diluted to 1X concentration for running and preparation of big agarose gels. The buffer was kept at room temperature.

2.7.5 TBE

TBE (Tris borate) was prepared as a 10X concentration stock solution. 1 litre of the solution contains 108 g Tris base, 55 g boric acid and 40 ml of 0.5 M EDTA, pH 8.0. It was used at a 1X concentration for running and preparation of small agarose gels. The buffer was kept at room temperature.

2.7.6 DNA gel loading buffer

The DNA gel loading buffer used was the buffer Type III. 10X concentration of the buffer contains 0.42% (w/v) bromophenol blue, 0.42% (w/v) xylene cyanol FF and 50% glycerol in dH_2O. The solution was autoclaved and kept at 4^0C in a light-tight bottle. It was added to the sample at a 1X final concentration prior to loading the sample on the gel.

2.7.7 1 M Tris.HCl

121.1 g of Tris base was dissolved in 800 ml of dH_2O. The pH of the solution was adjusted to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool to room temperature before final adjustment to the pH was made. The volume of the solution was adjusted to 1 litre with dH_2O and dispensed into aliquots before it was autoclaved. It was then kept

at room temperature.

2.7.8 DNAse-free RNAse

10 mg of Ribonuclease A was dissolved in 1 ml of solution containing 15 mM NaCl and 10 mM Tris.HCl, pH 7.5 in an eppendorf tube. The tube was placed in a boiling-water bath for 15 minutes and then allowed to cool to room temperature slowly. It was then distributed into aliquots and stored at -20^{0}C. 2 μl of this solution was used in the restriction digests of plasmid miniprep samples.

2.7.9 TE buffer

TE buffer was prepared by adding 1 M Tris.HCl of an appropriate pH and 0.5 M EDTA pH 8.0 to a final concentration of 10 mM and 1 mM respectively to dH_2O. It was autoclaved and kept at room temperature.

2.8 General molecular biology methods

2.8.1 Preparation of dialysis tubing

Dialysis tubing (Medicell International Ltd.) was cut into pieces of convenient length (10 - 20 cm). The pieces were then boiled in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA, pH 8.0 for 10 minutes. They were then rinsed thoroughly with distilled water and boiled again in 1 mM EDTA, pH 8.0 for another 10 minutes. The tubing was allowed to cool to room temperature, transferred into 70% (v/v) ethanol and kept at room temperature. Before use, the tubing was rinsed inside and outside with distilled water. Handling of the tubing was not permitted without wearing gloves.

2.8.2 Preparation of phenol

2.8.2.1 Equilibration of phenol

Liquid phenol was removed from the -20^{0}C freezer and allowed to warm to room temperature before it was melted at 68^{0}C. Hydroxyquinoline was

added to the melted phenol to a final concentration of 0.1%. This compound is an antioxidant, a partial inhibitor of RNAse and a weak chelator of metal ions (Sambrook *et al.*, 1989). An equal amount of TE buffer, pH 8.0 was then added and stirred on a magnetic stirrer for 15 minutes. The mixture was left until the two phases separated. The upper aqueous phase was aspirated using a pipette tip attached to a vacuum line equipped with traps. This step was repeated until the pH of the phenolic phase was >7.8 (as measured with pH paper). The equilibrated phenol was then distributed into aliquots in light-tight bottles and stored at -20^{0}C.

2.8.2.2 Phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v)

Before the equilibrated phenol was used it was mixed with chloroform and isoamyl alcohol in the ratio of 25:24:1 (v/v/v) of phenol, chloroform and isoamyl alcohol respectively. The solvents were then mixed thoroughly and kept at -20^{0}C in a light-tight bottle.

2.8.3 Phenol:chloroform:isoamyl alcohol extraction

Proteins from nucleic acid solution were removed by extraction of the solution with phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (24:1; v/v). An equal volume of phenol:chloroform:isoamyl alcohol was added to the nucleic acid solution in phenol resistant tubes or eppendorfs and mixed until an emulsion formed. The two phases were then separated by centrifuging the tubes at 10000 rpm for 5 minutes in either a Beckman J2-21 centrifuge (Beckman) using an appropriate rotor or a Biofuge 15 microcentrifuge (for eppendorfs) (Heraeus Sepatech). The aqueous phase was transferred to a fresh tube. The process was repeated until no protein was visible at the interface of the organic and aqueous phase. Extraction with chloroform:isoamyl alcohol was then followed before the final aqueous phase was collected for further processing.

2.8.4 Precipitation of DNA with ethanol

The volume of the DNA in a tube was first estimated. 1/10 volume of 3.0 M sodium acetate, pH 5.6 was added to the solution and mixed thoroughly.

Two volumes of 100% cold ethanol were added and gently mixed by inverting the tube before storing on ice or at -20^{0}C for 15 - 30 minutes. The time needed to precipitate the DNA varies depending on the size and amounts of the DNA present in the solution. If the size of the DNA was small (<100 nucleotides) or when it was present in small amounts (<0.1 μg/ml), the period of storage was extended to at least 1 hour. The DNA were then recovered by centrifuging at 10000 rpm for 10 minutes in a Beckman J2-21 centrifuge or at 15000 rpm for 10 minutes in a Biofuge 15. However, as discussed above when low concentration of the DNA (in this case <20 ng/ml) or when very small fragments were present, more extensive centrifugation was required. Supernatant was carefully removed from the tube and the tube was then half filled with 70% (v/v) cold ethanol. The tube was re-centrifuged for 5 minutes at essentially the same speed. Supernatant was discarded and the DNA pellet air-dried before it was resuspended in an appropriate amount of distilled water or TE buffer, pH 8.0.

2.8.5 Precipitation of DNA with isopropanol

Instead of using ethanol, DNA were also precipitated with isopropanol. Details of the methods were as for ethanol precipitation but without the addition of 1/10 volume of 3 M sodium acetate, pH 5.6. One volume of isopropanol was used in place of two volumes of ethanol. The advantage of precipitation with isopropanol was that the volume of liquid to be centrifuged was smaller. This was very useful especially when the DNA were present in a large volume of solution. However, isopropanol was less volatile than ethanol and was therefore more difficult to remove. Moreover solutes such as sucrose or sodium chloride were more easily co-precipitated with the DNA. In general, precipitation with ethanol was preferable unless it was necessary to keep the volume of fluid to a minimum.

2.8.6 Restriction endonuclease and DNA modifying enzyme analysis

Restriction digests and DNA modifying enzyme analyses were performed according to the recommendation given by the manufacturers and by Sambrook *et al.* (1989).

2.8.7 Quantification of DNA and RNA

The amount of DNA and RNA present in the solution was quantified by taking an absorbance of an appropriate dilution of the DNA and RNA at 260_{nm} using a UVIKON 860 (Kontron Instruments) spectrophotometer. An OD reading of 1 corresponds to approximately 50 μg/ml for double-stranded DNA, 40 μg/ml for single stranded DNA and RNA, and ~20 μg/ml for single-stranded oligonucleotides (Sambrook *et al.*, 1989).

2.8.8 Autoradiography

The following procedure was applied in order to detect the radioactive signal from the filters that were hybridised against radiolabelled probes in either screening for the mini library, Southern blots, northern blots or RNA dot blots. The filter to be autoradiographed was mounted on a piece of 3MM Whatman paper (Whatman) with a masking tape after the post hybridisation washes were completed. Radioactive marker ink was spotted on the paper in an asymmetrical order to serve as a marker for the filter orientation. The whole filter was then wrapped with a piece of Saran Wrap and exposed to Blue-sensitive X-ray film in a Kodak X-omatic cassette fitted with Kodak X-omatic fine intensifying screen. The cassette was then left at -50^{0}C for the required amount of time. The cassette was allowed to defrost before the film was removed and developed by immersing in Photosol CD18 X-ray developing solution (Photosol Ltd.) for 5 minutes. It was washed with distilled water for one minute and then fixed by immersing in Photosol CF40 fixer solution for 4 minutes. The film was then rinsed with tap water and allowed to air-dry at room temperature. Developing was done in a dark room.

2.9 Competent cells preparation

Competent cells were prepared according to Alexander *et al.* (1984). *Escherichia coli* DH5α strain was grown overnight in 5 ml of 2XL media. 1 ml of an overnight culture was sub-cultured into 100 ml 2XL pre-warmed to 37^{0}C in a 500 ml flask until an OD_{600} of approximately 0.2 was obtained. 2 M sterile $MgCl_2$ was then added to a final concentration of 20 mM. The cells continued to grow until an OD_{600} of approximately 0.5 (0.45 - 0.55) was obtained, then

the cells were quickly cooled on ice. After 2 hours, the cells were centrifuged at 3000 rpm for 5 minutes at 4^0C in a JA-20 rotor in a Beckman J2-21 centrifuge. Supernatant was aspirated, and the bacterial pellet resuspended gently in 50 ml of freshly-prepared ice-cold filter-sterilised Ca^{++}/Mn^{++}, pH 5.5 (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM sodium acetate) and left on ice for one hour. The cells were recovered by centrifuging at 3000 rpm for 5 minutes at 4^0C and then resuspended gently in 5 ml of the ice-cold Ca^{++}/Mn^{++} medium containing 15% (v/v) glycerol. They were then dispersed into 200 μl aliquots and snap frozen in liquid nitrogen. Cells were stored at -70^0C until they were needed.

2.10 DNA procedures

2.10.1 Isolation of plasmid DNA

2.10.1.1 QIAGEN plasmid midi protocol

Isolation of plasmid DNA by this method was done by using material and solutions supplied with the kit (QIAGEN) and performed according the instruction manual supplied. A single colony of bacteria was grown in 100 ml of LB media containing the appropriate antibiotic selection, overnight at 37^0C. The bacterial cells were recovered by centrifuging the growth media at 4000 rpm at 4^0C for 15 minutes in a JA-14 rotor (Beckman) in a Beckman J2-21 centrifuge (Beckman). Supernatant was discarded and the bacterial pellet was resuspended in 4 ml of buffer P1. 4 ml of buffer P2 were then added, mixed and the mixture was incubated at room temperature for 5 minutes. 4 ml of chilled buffer P3 were added to the cell suspension, mixed and incubated on ice for 15 minutes. The mixture was then centrifuged at 10000 rpm at 4^0C for 30 minutes in the JA-14 rotor in the Beckman J2-21 centrifuge to remove cell debris. Supernatant was collected and applied onto a QIAGEN-Tip 100 column equilibrated with 4 ml of QBT buffer. The column was washed two-times with 10 ml of buffer QC and the DNA was eluted by applying 4ml of buffer QF onto the column. The DNA was then precipitated by adding 0.7 volumes of cold isopropanol and the DNA was recovered by centrifuging the

DNA solution at 10000 rpm for 30 minutes at 4^{0}C in a JA-20 rotor in the Beckman J2-21 centrifuge. The DNA pellet was washed with 5 ml of cold 70% ethanol, air-dried and resuspended in a suitable volume of TE buffer.

2.10.1.2 Plasmid mini-prep - alkaline lysis method

This protocol was performed according to Sambrook *et al.* (1989). A single colony of bacteria was grown in 5 ml LB media containing the appropriate antibiotic selection overnight at 37^{0}C. 250 µl of the culture was saved and the remaining culture solution was centrifuged at 2500 rpm for 10 minutes in a Mistral 3000i bench centrifuge (Phillip Harris). The supernatant was discarded and the bacterial pellet resuspended in 200 µl of GET solution (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl, pH 8.0) containing lysozyme at a concentration of 2 mg/ml. The cell suspension was then transferred into an eppendorf tube. Following incubation on ice for 30 minutes, 300 µl of solution 2 (0.2 mM NaOH, 1% (w/v) SDS) was added, mixed by inversion and incubated on ice for 5 minutes. 225 µl of neutralisation solution (3 M potassium acetate, 5 M glacial acetic acid) was then added, mixed by inversion and left on ice for another 5 minutes. The tube was then centrifuged at 15000 rpm for 5 minutes at room temperature in a Biofuge 15 to remove bacterial debris. The supernatant was transferred into a fresh tube and extracted with an equal volume of TE-saturated phenol:chloroform:isaoamyl alcohol and chloroform:isoamyl alcohol as explained in Section 2.8.3. The final aqueous phase was collected and the DNA was ethanol precipitated (Section 2.8.4). The DNA pellet was then re-dissolved in a suitable volume of TE buffer, pH 8.0.

2.10.1.3 Plasmid maxi-prep

Large-scale preparation of plasmid DNA was done by scaling-up the alkaline lysis miniprep method as described in Section 2.10.1.2 above. Bacterial cells were grown in 100 ml LB medium instead of in 5 ml for the miniprep.

2.10.1.4 Quick mini-prep

This quicker method was a modification of the alkaline lysis method by Sambrook *et al.* (1989) as explained in Section 2.10.1.2. Quick mini-preps were performed in small volumes of culture solutions and were very useful when large numbers of clones needed to be screened for example, in selecting nested deletion clones. Bacteria were plated onto LB agar plate containing the appropriate antibiotic selection. Individual colonies were then transferred to 200 μl of LB media containing an appropriate antibiotic selection in a 96 well microtiter plate. The plate was then left at 37^{0}C gently shaking. 50 μl of the growth media was kept and 30 μl of GET solution containing 2 mg/ml of lysozyme was added to the remaining 150 μl of the growth solution. The solution was mixed by pipetting. The plate was left on ice for 15 minutes before 50 μl of lysis solution 2 was added, mixed by pipetting and left on ice for 5 minutes. 30 μl of neutralising solution was then added, mixed and the plate was left on ice for another 5 minutes. The plate was then centrifuged at 2500 rpm for 15 minutes on a microtiter plate rotor (Phillip Harris Scientific) in a Mistral 3000i bench centrifuge. The supernatants were transferred to a fresh microtiter plate containing 1 μl of 10 mg/ml DNAse-free RNAse (Section 2.7.8) and incubated for 30 minutes at room temperature. 100 μl of the solution were transferred to eppendorf tubes containing 10 μl of the gel loading buffer (Section 2.7.6). As much sample as possible was loaded onto big agarose gels and electrophoresis was performed at 80 V for 4 hours as detailed in Section 2.10.3.

2.10.2 Plant genomic DNA extraction

Plant genomic DNA was prepared according to Doyle and Doyle (1987). 5.0 g of tissue was ground in a mortar and pestle with the presence of liquid nitrogen. 25 ml of prewarmed (60^{0}C) 2X CTAB buffer (100 mM Tris.HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) hexadecyl-trimethylammonium bromide (CTAB), 0.2% (v/v) 2-mercaptoethanol) were added and mixed to form a slurry. The mixture was poured into a 50 ml falcon tube and the mortar and pestle was rinsed with 5 ml 2X CTAB buffer, and added to the tube. The tube was incubated for 30 minutes at 60^{0}C with occasional swirling. The

slurry was gently extracted with an equal amount of chloroform:isoamyl alcohol (24:1; v/v) for 10 minutes on a rotating wheel. The tube was then centrifuged at 3000 rpm for 15 minutes at 4^0C in a Mistral 3000i centrifuge to concentrate the layer. The aqueous phase was collected in a Corex tube and the DNA was precipitated with 2/3 volumes of cold isopropanol. The DNA was spooled out with a sterile glass hook (made from a pasture pipette) and transferred to 20 ml of wash buffer (76% (v/v) ethanol, 10 mM ammonium acetate) for 20 minutes. The solution was then discarded. The DNA was air-dried and resuspended in 1 ml resuspension buffer (1 mM ammonium acetate, 0.25 mM EDTA), and further purified by CsCl density gradient centrifugation as detailed in Section 2.10.5.

2.10.3 DNA agarose gel electrophoresis containing ethidium bromide

Agarose gel electrophoresis was carried out in either large gels (200 mm x 170 mm) or small gels (80 mm x 95 mm) according to Sambrook *et al.* (1989). The concentration of the agarose used varies depending on the size of DNA fragment to be separated. The suitable amount of agarose to be used in the gel in order to separate DNA of appropriate sizes is shown in Table 2.5 (Sambrook *et al.*, 1989).

Table 2.5. Range of separation of DNA in gels containing different amounts of agarose.

Amount of agarose in the gel (% [w/v])	Efficient range of separation of linear DNA molecules (kb)
0.3	5 - 60
0.6	1 - 20
0.7	0.8 - 10
0.9	0.5 - 7
1.2	0.4 - 6
1.5	0.2 - 3
2.0	0.1 - 2

A big agarose gel was prepared with 1X TAE buffer (Section 2.7.4) and run in essentially in the same buffer. For a mini gel, 1X TBE (Section 2.7.5) was used instead. Ethidium bromide was included in the gel at a concentration of 0.5 mg/ml. 1/10 volume of 10X gel loading buffer (Section 2.7.6) was added to the DNA solution before it was loaded onto the gel. Electrophoresis was normally continued until the bromophenol blue marker dye had migrated 2/3 of the way along the gel. DNA within the gel was visualised on a transilluminator (UVP Incorporation) and photographed with a Mitsubishi Video Copy Processor (Mitsubishi).

2.10.4 Recovery and purification of DNA fractionated on agarose gels

An agarose gel containing the DNA band of interest was cut out using a sharp scalpel under UV light. The gel slice was placed in a piece of one end-sealed (using a dialysis-tubing clip) of dialysis tubing and 500 μl of TE buffer was added. Another end of the tubing was then sealed just above the gel slice and the tubing was immersed in a shallow layer of electrophoresis buffer in an electrophoresis tank. Electrophoresis was continued at 4 - 5 V/cm for an appropriate time. During this time the DNA electroeluted out of the gel and onto the inner wall of the tube. Before the gel slice was taken out, the bag was placed under the UV light to make sure that the entire DNA was electroeluted out of the gel. The bag was then gently massaged on the side where the DNA accumulated to remove the DNA from the wall. The buffer was then transferred into an eppendorf tube, phenol:chloroform:isoamyl alcohol extracted (Section 2.8.3) before the DNA was ethanol precipitated (Section 2.8.4). The DNA was then resuspended in an appropriate amount of TE buffer, pH 8.0.

2.10.5 Purification of DNA by equilibrium centrifugation in caesium chloride (CsCl)-ethidium bromide gradients

2.10.5.1 CsCl-ethidium bromide gradient centrifugation

Purification of plasmid DNA and plant genomic DNA by centrifugation in CsCl-ethidium bromide gradients was performed according to the method

adopted from Sambrook *et al.* (1989). To the DNA solution, 0.97 g of CsCl was added for every 1 g of solution in sterile distilled water. The mixture was mixed by inverting the tube gently until the CsCl has dissolved. Ethidium bromide was added to the solution to a final concentration of approximately 0.74 mg/ml. The solution was then transferred to a Beckman Ultra-clear Quick Seal Tubes (Beckman) using a 2.5 ml (or equivalent) disposable syringe fitted with a large-gauge needle. The tube was then filled with sterile distilled water solution containing CsCl prepared in the same way. The tubes were then sealed using a Beckman Tube Topper Sealer (Beckman). The tubes were centrifuged at 50000 rpm overnight at 15^0C in a Beckman Vti80 rotor (Beckman) in a Beckman L8-80M ultracentrifuge (Beckman). The DNA was then collected and processed as detailed in Section 2.10.5.2.

2.10.5.2 Removal of ethidium bromide from the DNA solution

To the DNA solution in an eppendorf tube, an equal volume of TE-saturated isoamyl alcohol was added. The two phases were mixed by gently inverting in a rotating wheel for 5 minutes. The top organic phase was discarded and the lower aqueous phase transferred to a new tube. Another equal volume of TE-saturated isoamyl alcohol was added to the tube and the process was repeated until the entire pink ethidium bromide colour disappeared from both the aqueous and the organic phase. Caesium chloride was removed from the DNA solution by dialysis against several changes of TE buffer (pH 8.0) for at least 4 hours in a cold room. When the dialysis was complete, the DNA solution was transferred to a fresh eppendorf tube, ethanol precipitated (Section 2.8.4), and resuspended in an appropriate amount of TE buffer, pH 8.0.

2.10.6 Removal of 5' phosphate groups on cloning DNA vectors with calf intestinal alkaline phosphatase (CIAP)

Removal of 5' phosphate groups from the digested DNA vector was essential as this prevented re-circularisation of the vector during ligation. The method followed was as described in Protocol and Application Guide (Promega, 1991). 5 μg of pSK^+ was digested with the appropriate restriction

enzyme for 2 - 3 hours in a final volume of 50 μl in an eppendorf tube. 3 μl of the restriction digest mixture was then taken and electrophoresed on a 1% small agarose gel in order to check that the reaction had gone to completion. When digestion was seen to be complete, the DNA was treated with CIAP by adding 10 μl of CIAP 10X buffer and 0.01 U CIAP/pmol of ends of plasmid directly into the tube. The final volume of the reaction was made up to 100 μl with sterile distilled water. The tube was then incubated for 60 minutes at 37^0C before 2 μl of 0.5 M EDTA was added to stop the reaction. Calf intestinal alkaline phosphatase was removed by extraction with phenol:chloroform:isoamyl alcohol (Section 2.8.3). The DNA was then ethanol precipitated (Section 2.8.4) and resuspended in a small volume of TE buffer, pH 8.0.

2.10.7 Random primer radiolabelling

Probes were radiolabelled to a high specific activity by a random priming procedure (Feinberg and Vogelstein [1983]). Two eppendorf tubes were prepared containing appropriate mixtures as below;

Tube A	Tube B
10 μl OLB*	100 ng DNA probe
5 μl Klenow polymerase	dH_2O to a final volume of 30.5 μl
2.5 μl 20% (w/v) stock BSA (NBL)	
2.5 μl α^{32}P dCTP	

* The OLB solution contains a mixture of solution A, solution B and solution C with the ratio 10:25:15 respectively.

Solution A	1 ml of solution O plus 18 μl 2-mercaptoethanol, 5 μl 0.1 M dATP, 5 μl 0.1 M dCTP, 5 μl 0.1 M dTTP.
Solution O	1.25 M Tris.HCl, pH 8.0, 0.125 M $MgCl_2$
Solution B	2 M HEPES, pH 6.6
Solution C	random hexamers at OD_{260} = 90 per ml

The DNA probe was denatured by placing tube B in a boiling-water bath for 5 minutes and cooled on ice immediately. It was then transferred to the tube A and incubated at 37^{0}C for at least 4 hours. The radiolabelled probe was separated from non-incorporated ^{32}P dCTP by running the sample through a Sephadex G-50 column (Section 2.10.8) (Sambrook *et al.*, 1989). The radiolabelled probe was denatured in a boiling-water bath for 7 minutes and cooled on ice immediately before it was added to the hybridisation solution.

2.10.8 Sephadex G-50 chromatography

The Sephadex G-50 slurry was prepared by adding 10 g of Sephadex G-50 beads (Pharmacia) to 200 ml of sterile elution buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris.HCl, pH 7.5, 0.1% (w/v) SDS). 10 g of dry Sephadex G-50 beads yield 160 ml of slurry (Sambrook *et al.*, 1989). It was then hydrated for one hour in a boiling-water bath and kept at room temperature. A column was prepared by adding the slurry to a 1 ml disposable syringe fitted with siliconised glass wool at the bottom of a syringe. The slurry was allowed to set and the column was continuously filled with the slurry until it was packed to a level 1 cm below the top of the column. The gel was washed with several volumes of elution buffer before the radiolabelled mixture was applied to the top of the column. Immediately after the radiolabelling mixture entered the column, elution buffer was continuously added from time to time as required. The passage of the radiolabelled DNA probe through the column was monitored by a Mini-monitor G-M tube series 900 (Mini-Instruments Ltd.) and fractions collected in eppendorf tubes.

2.10.9 Capillary transfer of DNA to Biotrans (+) nylon membrane filter

The technique followed was essentially that described by Sambrook *et al.* (1989). After electrophoresis, the agarose gel was transferred to a glass baking dish and any unused areas of the gel trimmed away with a scalpel. One corner of the gel was cut off to serve as a gel orientation marker during the succeeding operations. The gel was denatured in several volumes of denaturing buffer (1.5 M NaCl, 0.5 N NaOH) for one hour with constant gentle

agitation. The gel was then rinsed with distiled water and neutralised by constant gentle shaking in several volumes of neutralisation solution (1 M Tris.HCl, pH 7.4, 1.5 M NaCl). After one hour, the neutralisation buffer was changed and shaking was continued for another one hour. A piece of Biotrans(+) nylon membrane was cut about 1 cm larger than the gel in both dimensions. Gloves were worn when handling the filter. The filter was then floated on the surface of dish of distilled water until completely wet and then immersed in transfer buffer (20X SSC) for at least 5 minutes. The gel was removed from the neutralisation solution and placed on a blotting apparatus. Arrangement of the filter and the gel on the apparatus as illustrated in Figure 2.1. The gel was surrounded with Saran Wrap to protect the transfer buffer from flowing directly from the reservoir to the nappies by passing the gel. Special care was taken to make sure that there were no air bubbles trapped between the filter and the gel and also between the filter and the Whatman 3MM paper as these would block the elution process. The transfer was allowed to proceed overnight (approximately 16 hours) at room temperature. As the process was completed the filter was peeled from the gel and sandwiched between two sheets of dry Whatman paper. The DNA was fixed by baking the filter at 80^{0}C for 1 - 2 hours. Southern hybridisation was then performed as detailed below.

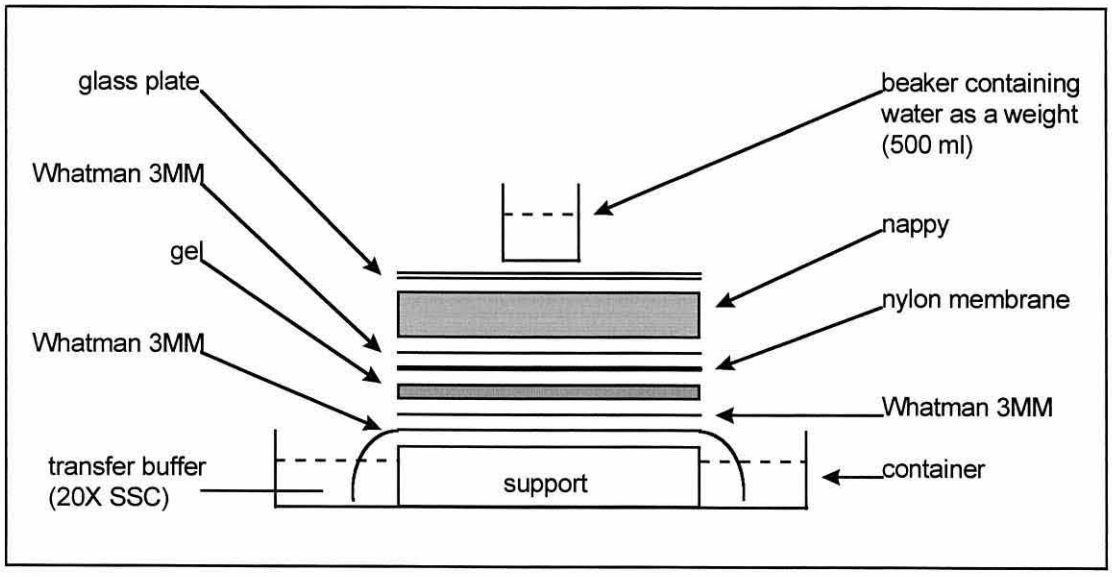


Figure 2.1. Capillary transfer of DNA from agarose gels.

2.10.10 Southern blot hybridisation of DNA samples on the filter

Southern hybridisation of DNA samples transferred onto Biotrans (+) nylon membrane was performed according to Sambrook *et al.* (1989). Hybridisation was done in Techne Hybridisation tubes using a Techne Hybridiser HB-1 oven. Pre-hybridisation was done in a 50 ml pre-hybridisation solution containing 5X Denhardt's (0.1% (w/v) Ficoll Type 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA Fraction V Sigma), 5X SSC, 100 μg of denatured salmon sperm DNA, and 0.5% (w/v) SDS) for 1 - 2 hours at 65^{0}C. Before pre-hybridisation was complete, the radiolabelled probe in an eppendorf tube was denatured by placing the tube in a boiling-water bath for 7 minutes and quickly cooled on ice. Pre-hybridisation solution was then discarded and hybridisation solution (5X Denhardt's, 5X SSC, 100 μg of denatured salmon sperm DNA, and 0.1% (w/v) SDS) was added to the tube. The denatured radiolabelled probe was then added to the tube and hybridisation was left to proceed overnight at 65^{0}C. Post-hybridisation washes were performed at 65^{0}C in 3x SSC, 0.1% SDS for one hour followed by washing in 2X SSC, 0.1% SDS for one hour, then 1X SSC, 0.1% SDS for one hour. High stringency washes at up to 0.1X SSC, 0.1% SDS were performed depending on the level of homology of the radiolabelled probe to the DNA samples on the filter. All washing solutions contained 0.1% SDS. The filter was then autoradiographed as detailed in Section 2.8.8.

2.10.11 Nested deletion of double-stranded DNA

The nested deletion of double-stranded DNA was carried out using the Double-stranded Nested Deletion Kit (Pharmacia P-L Biochemicals) as recommended by the supplier. It was designed to carry out unidirectional deletions with exonuclease III (Exo III). Recombinant plasmids were double digested in an eppendorf tube with two restriction enzymes; one enzyme created a 5'-overhanging or blunt end (nuclease-susceptible end) adjacent to the target sequence and another enzyme produced a 3'-three-base or longer 3'-overhanging end or recessed 3'-end (nuclease-resistant end). Restriction digests were monitored by taking 0.5 μg of the digestion mix and electrophoresed on a small gel (Section 2.10.3). The tube was then heated

for 10 minutes at 70^{0}C to inactivate the enzymes. Extraction with phenol:chloroform:isoamyl alcohol was not necessary. The linearised plasmid was then incubated with Exo III at 30^{0}C according to the manufacturers recommendation. 'Nested' deletions were generated by removing aliquots of samples at time interval during the Exo III digestion as recommended. The samples were then incubated with S1 nuclease to remove single-stranded regions. Half of the nested deletions were analysed by agarose gel electrophoresis, and the other half were re-circularised using T_4 DNA ligase and transformed into *E. coli* DH5α competent cells as detailed in Section 2.12.3. Selection of appropriate sizes of nested deletions was performed by the quick alkaline lysis method (Section 2.10.1.4).

2.10.12 Sequencing

Nested deletion clones were sequenced by using the ABI System DNA sequencer and also manually sequenced using the T7 Sequencing Kit (Pharmacia) according to the instruction supplied by the manufacturer.

2.11 RNA procedures

All equipment (glassware, bottles etc), electrophoresis equipment, tips (yellow and blue tips), solutions and water to be used for RNA work had to be diethylpyrocarbonate (DEPC)-treated and autoclaved before they were used. The purpose of this treatment was to inactivate any RNAse activity.

2.11.1 Diethylpyrocarbonate (DEPC) treatment

Glassware, electrophoresis equipment and yellow and blue tips were DEPC-treated by immersing them in 0.1% (v/v) DEPC solution at 37^{0}C overnight before they were autoclaved. Water and solutions were treated by adding the DEPC to the solution to a final concentration of 0.1% (v/v) and incubated at 37^{0}C overnight before they were autoclaved. Solutions containing Tris could not be treated directly with DEPC as the two compounds react to form a stable complex. They were instead prepared with autoclaved DEPC-treated distilled water and re-autoclaved.

2.11.2 Plant total RNA extraction

Total RNA was extracted from plant tissues using the guanidium hydrochloride method modified from the method of Logemann *et al.* (1987). 5 g of tissue was ground to a fine powder using a mortar and pestle under liquid nitrogen. When the powder was just thawing, 5 ml of RNA extraction buffer (8 M guanidium hydrochloride, 20 mM MES, pH 7.0, 20 mM EDTA, 50 mM 2-mercaptoethanol) was added and mixed to a slurry. 5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was then added and mixed and the mixture was transferred to a Nalgene Oak Ridge tube (BDH). The tube was then centrifuged at 10000 rpm for 10 minutes at 4^0C in a Beckman JA-20 rotor in a Beckman J2-21 centrifuge (Beckman). The aqueous phase was collected and the phenol:chloroform:isoamyl alcohol extraction was repeated until there was no precipitate (protein) seen at the boundary between the two phases. After the final extraction, the aqueous phase was collected in a Corex tube and RNA was precipitated by adding 0.2 volumes of 1 M glacial acetic acid and 0.7 volumes of 100% cold ethanol. The tube was then left in a -20^0C freezer overnight before the RNA was recovered by centrifuging the tube at 10000 rpm for 10 minutes at 4^0C in a Beckman JA-20 rotor in a Beckman J2-21 centrifuge. The RNA pellet was washed with 70% ethanol (Section 2.8.4) and the pellet was left to air-dry at room temperature. The pellet was then resuspended in 200 μl of DEPC-treated water and the RNA was quantified by taking an OD_{260} reading. The RNA was stored under liquid nitrogen in Nalgene cryogenic vials (Nalgene Company) until needed.

2.11.3 RNA agarose gels containing formaldehyde

Electrophoresis of RNA samples were performed on big agarose gels containing formamide/formaldehyde as described by Fourney *et al.* A 1.3% agarose gel was prepared by adding 1.3 g agarose, 10 ml 10X MOPS/EDTA, pH 7.0 and 87 ml of DEPC-treated distilled water to a RNAse-free flask. Agarose was dissolved by microwaving and cooled down to approximately 50^0C. In a fume hood, 5.1 ml of 37% formaldehyde was introduced into the agarose solution, gently mixed and then poured into a RNA-free gel tray to set. It was allowed to sit for one hour before it was used. Prior to loading onto

the gel, the RNA samples in 5 μl in eppendorf tubes were denatured by adding 25 μl of sample buffer (below) and heated to 65^{0}C for 15 minutes. They were then quickly cooled on ice. To the tubes, 1 μl of 1.0 mg/ml ethidium bromide was added and mixed thoroughly. The samples were then loaded on the gel and electrophoresis was performed at 80 V for 4 hours at room temperature. RNA within the gel was visualised on a transilluminator (UVP Inc.) and photographed with a Mitsubishi Video Copy Processor (Mitsubishi). If necessary, the gel was northern blotted as Section 2.11.4 below.

Preparation of the RNA sample buffer.

0.75 ml deionised formamide
0.15 ml 10X MOPS/EDTA
0.24 ml 37% formaldehyde
0.1 ml DEPC-treated dH_2O
0.1 ml glycerol
0.08 ml 10% (w/v) bromophenol blue

2.11.4 Northern blotting

Equal amounts of RNA of approximately 10 μg as determined by spectrophotometric and visual observation of the intensity of ethidium bromide fluorescence were electrophoresed on RNA agarose gels as described in Section 2.11.3. After electrophoresis, the gel was soaked in 0.5 M NaOH (made up in 1X SSC) for 10 minutes at room temperature with gentle shaking. The gel was then transferred to 10X SSC for two-20 minutes periods. At completion, the gel was transferred to the blotting apparatus. The blot was set up as described in Section 2.10.9 using 10X SSC as the transfer buffer. The blot was then left at 4^{0}C overnight. The filter was then removed, sandwiched between two sheets of Whatman 3 MM papers and baked for 2 hours at 80^{0}C to fix the RNA. Northern hybridisation against radiolabelled probes was then performed as detailed in Section 2.11.6.

2.11.5 Dot blotting

Equal amounts of RNA samples in 11 μl in eppendorf tubes were denatured by adding 29 μl of dot blot denaturing buffer (29 μl of denaturing buffer contains 20 μl formamide, 7 μl 37% formaldehyde, 2 μl 20X SSC), heated to 65^{0}C for 15 minutes as for northern blot. A piece of Biotrans (+) nylon membrane was wetted with 10X SSC and placed in a dot blotting vacuum manifold (Anachem). RNA samples were applied to the wells under vacuum and washed with two-100 μl aliquots of 10X SSC. The filter was then air-dried and RNA fixed by baking the filter at 80^{0}C for 1 - 2 hours between two sheets of Whatman 3MM paper. Pre-hybridisation and hybridisation of the filter was performed as for northern hybridisation (Section 2.11.6).

2.11.6 Hybridisation of RNA samples

Hybridisation of dot blots and northern blots of RNA samples was performed according to Shirsat *et al.* (1996). Pre-hybridisation was carried out in 5X Denhardt's, 50% formamide, 5X SSC, 1% SDS, and 100 mg/ml of denatured salmon sperm DNA at 42^{0}C overnight in a Techne Hybridisation tube in a Techne Hybridiser HB-1 oven. Radiolabelled probe was denatured in a boiling-water bath for 7 minutes, cooled on ice and added to the hybridisation solution. Hybridisation was carried out in essentially the same solution as pre-hybridisation solution but containing 2X Denhardt's for 36 - 48 hours at 42^{0}C. Post-hybridisation washes were done with 3X SSC, followed by 2X SSC, then 1X SSC at room temperature, then 0.1X SSC at 42^{0}C and finally 0.1X SSC at 65^{0}C. All washing solutions contained 0.1% SDS and each wash was done twice at 10 minutes each. The filters were then autoradiographed as detailed in Section 2.8.8.

2.12. Construction of *Brassica napus* DNA mini library

2.12.1 Fractionation of *Hind* III-digested rape genomic DNA by sucrose gradient

Hind III-digested rape genomic DNA was fractionated by centrifugation through a 10 - 40% (w/v) sucrose gradient according to Sambrook *et at.*

(1989). A 16 ml of 10 - 40% sucrose gradient was formed in a 17 ml polyallomer tube (Kontron Instruments) using a Gradient Former Model 385 (Biorad). 100 μg of rape genomic DNA was digested with *Hind* III restriction enzyme for 6 hours at 37^{0}C. 10 μl of the reaction mixture was kept while the remainder of the reaction was heated to 60^{0}C for 5 minutes and rapidly cooled on ice. The digested DNA was then diluted to 500 μl with sterile distilled water and carefully loaded on top of the gradient in the tube. The tube was then centrifuged at 26000 rpm for 17 hours at 15^{0}C in a Beckman SW28 rotor (Beckman) in a Beckman L8-80M ultracentrifuge (Beckman). The gradients were fractionated using a Density Gradient Fractionator Model 640 (Fisons Scientific Equipment). In order to fractionate the gradient, the fractionator was first pre-run with 250 ml of sterile washing solution (1 M NaCl, 20 mM Tris.HCl, pH 8.0, 5 mM EDTA). The machine was run at 0.5 ml/minute and 0.3 ml fractions were collected in eppendorf tubes. 25 μl of each fraction was then electrophoresed on a big 0.6% agarose gel at 80 V for 6 hours together with 10 μl of undiluted restriction digest and an appropriate DNA marker. The gel was then Southern blotted (Section 2.10.9) and hybridised against the radiolabelled *extA* coding sequence probe (Section 2.10.7). The remaining fractions containing the DNA fragments of interest were then dialysed overnight against several changes of TE buffer, pH 8.0. The DNA was then recovered by ethanol precipitation (Section 2.8.4) and resuspended in 20 μl of TE buffer, pH 8.0.

2.12.2 Ligation of DNA

Ligation between the DNA vector and fragments of insert was carried out in a 10 μl reaction mixture containing 1 μl 10X T_4 DNA ligase buffer (10X of the buffer contains 300 mM Tris.HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP), 1 μl (3U) T_4 DNA ligase, 100 ng of the plasmid vector and DNA fragment with the picomoles of ends ratio of 1:2 (plasmid:insert) overnight at 16^{0}C. The ligation mixture was then used to transform competent *E. coli* cells.

2.12.3 Transformation of competent *E. coli* cells

An aliquot of frozen competent *E. coli* cells was thawed on ice and used immediately upon thawing. The required amount of DNA or ligation mix was diluted to 100 μl with TE buffer and added to 200 μl of the thawed cells in an eppendorf tube. The mixture was incubated on ice for 30 minutes before it was heat shocked at 37^{0}C for 5 minutes. The mixture was then diluted to 4 ml with 2XL broth pre-warmed at 37^{0}C and incubated at 37^{0}C for 100 minutes with gently shaking. Appropriate amounts of the transformation mixture were then plated on LB agar plates containing selective antibiotic marker. LB plates containing IPTG and X-gal (Section 2.5) were used for the blue and white colony determination. Controls of uncut and unligated cut vector were also included.

2.12.4 Screening of the mini library

2.12.4.1 Plating the transformants

The transformation mixture was plated on sections of large Biotrans (+) nylon membranes laid on big 22 cm x 22 cm LB plates containing an appropriate antibiotic. The transformed cells were evenly dispersed on the filters using a sterile glass spreader leaving an area approximately 0.5 cm wide at the edge of the filter free of bacteria. The plates were left for few minutes at room temperature to allow the fluid to be adsorbed before they were incubated at 37^{0}C overnight or until colonies (0.1 cm - 0.2 cm in diameter) appeared. The filters were peeled from the plate using sterile blunt-ended forceps and laid colony side up on Whatman 3 MM paper for five minutes. Replica filters were then prepared by laying second filters of the same size on the first filters. Care was taken during the preparation of the second filter so that no air bubbles were trapped between the filters. The filters were then firmly pressed together by placing a glass plate of the same size on the top of the duplicated filters. The orientation of the filters was marked by cutting the edges of the filters while they were stacked together. After 5 minutes, the filters were peeled apart and laid on new LB plates containing the same antibiotic selection. The plates were then incubated at

37^{0}C until colonies appeared. The second master plate filter was then kept at 4^{0}C and colonies on the first filter were further processed prior to hybridisation.

2.12.4.2 Lysis of the colonies

Four pieces of Whatman 3 MM paper were prepared. The size of the papers was bigger than the filters. Each of the papers was then saturated with the following solutions;

Paper 1	5% (w/v) SDS
Paper 2	Denaturing solution (0.5 N NaOH, 1.5 M NaCl)
Paper 3	Neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.4)
Paper 4	2X SSC

The Whatman 3 MM papers were then placed on 22 cm x 22 cm plates separately. The first filter containing bacterial colonies was placed on paper 1 with the colony side up for 5 minutes. It was then transferred to the paper 2 for another 5 minutes, then to the paper 3 for 5 minutes and finally to the paper 4 for 5 minutes. After the first filter was moved from paper 1 to paper 2 the second bacterial filter was placed on paper 1. This process was continued until all the filters were treated. During the transfer the edges of the plates were used to remove as much fluid as possible from the underside of the filters. Care was also taken not to get fluid on the side of the filters carrying bacterial colonies. The filters were then laid colony side up on a sheet of Whatman 3 MM paper and allowed to dry at room temperature for 15 minutes. The DNA on the filters was then fixed by baking the filters at 80^{0}C for 1 - 2 hours.

2.12.4.3 Hybridisation of the filters

The library was screened against the coding sequence of the *extA* gene as a probe radiolabelled with α^{32}P-dCTP (Section 2.10.7) following standard protocols as detailed in Section 2.10.10.

2.13 Treatment of oilseed rape plants

2.13.1 Wounding experiments

The protocols followed were detailed by Shirsat *at. al.* (1996). Wounding of stems, petioles and roots were performed by slicing the tissues into segments of ca. 3 mm using sharp scalpel blades. Leaf tissues were wounded by punching out leaf discs using a No 1 cork borer. The wounded tissues were then transferred to petri dishes containing a piece of Whatman 3MM paper wetted with 20 mM sodium phosphate buffer with the presence of 50 μg/ml chloramphenicol, and sealed with parafilm.

Wounding of leaves with dialysis clamps were performed according to Hildman *et al.* (1992). Fully expanded leaves from the six-week old plants were first detached from the plants by cutting the petiole-containing leaves with a sharp blade close to the stem. They were then re-cut in distilled water and subsequently placed in a sterile container containing sterile distilled water. Whole leaves were then wounded by applying dialysis clamps, perpendicular to the main vein. The tissues were incubated in a growth room on a 16 h day/8 h dark cycle for 12 h, 24 h, 36 h and 48 h and then quick frozen in liquid nitrogen and stored at -70^{0}C prior to RNA extraction. Unwounded leaves were incubated in sterile distilled water as a control.

2.13.2 Abscisic acid, sodium salicylate and methyl jasmonate treatments of oilseed rape leaf

Fully expanded leaves from 6 week-old plants were cut off from the plants close to the stem and the petioles were then re-cut in sterile distilled water. Detached leaves were then incubated in a 100 μM abscisic acid (ABA) solution, 1 mM sodium salicylate solution and 50 μM methyl jasmonate solution for 12 h, 24 h, 36 h and 48 h in a growth room on a 16 h day/8 h dark cycle before they were harvested for RNA extraction. For the control, leaves were incubated in sterile distilled water.

Chapter 3

Results

Chapter 3
Results

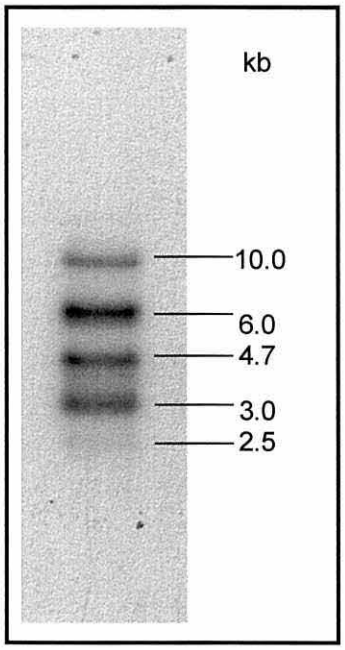
3.1 Analysis of the *Hind* III-digested rape genomic DNA

Preliminary studies showed that a Southern blot of *Hind* III-digested rape genomic DNA (Figure 3.1) contained five bands hybridising to the radiolabelled *extA* coding sequence probe (Shirsat *et al.*, 1991). The sizes of the bands were estimated as 2.5 kb, 3.0 kb, 4.7 kb, 6.0 kb and 10.0 kb respectively. The strongest radioactive signal was localised at the 6.0 kb band, while the signal from the 2.5 kb band was the weakest. The signals from the 3.0 kb, 4.7 kb and 10.0 kb bands were of an equal intensity.

3.2 Fractionation of *Hind* III-digested rape genomic DNA through a 10 - 40% sucrose gradient

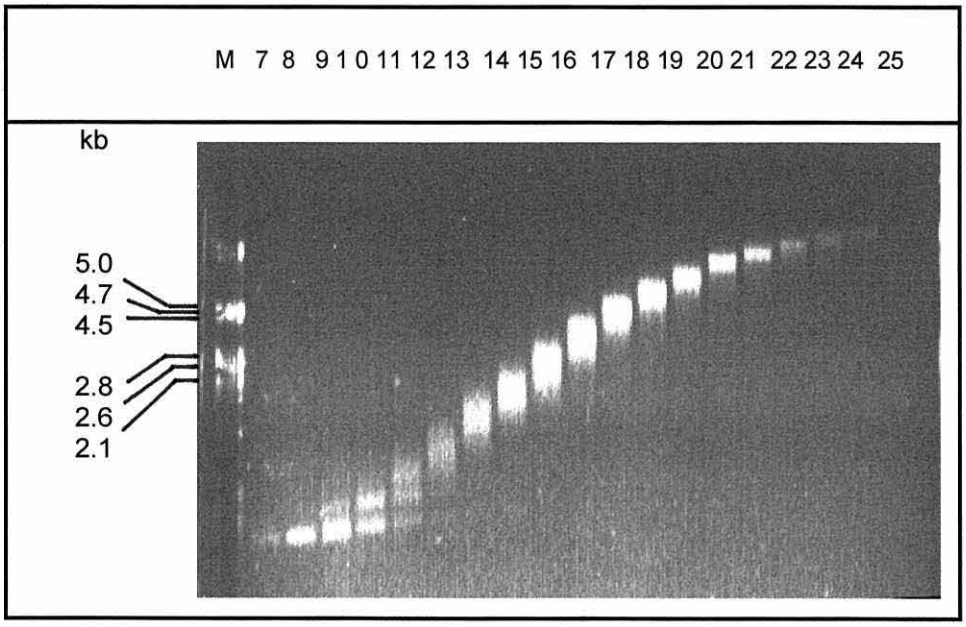
In order to isolate the *extA*-hybridising fragments of the *Hind* III-digested genomic DNA for the purposes of cloning, the DNA was subjected to *Hind* III-digestion and the DNA fragments were fractionated through a 10 - 40% sucrose gradient. An agarose gel analysis of the fractions collected from the gradients (Figure 3.2) showed that DNA fragments of different sizes were well separated. The gel was Southern blotted and hybridised against the radiolabelled *extA* probe (Figure 3.3). Fraction numbers 16 (F16), 17 (F17), 18 (F18), 19 (F19), 20 (F20), 21 (F21) and 22 (F22) contained DNA fragments of different sizes hybridising to the *extA* coding sequence probe. Almost all the fractions contained two different sizes of DNA fragment hybridising to the probe due to cross contamination. The 2.5 kb and 3.0 kb bands were present at almost the same amount in F16. In F17, the majority of the signal was contributed by the 3.0 kb band, while the 2.5 kb band could hardly be seen. The bands of 3.0 kb and 4.7 kb were seen in F18 and F19 respectively, while in F20 only the 6.0 kb fragment was detected. F21 represents two bands of 6.0 kb and 10.0 kb with most of the signal being contributed by the 6.0 kb band. In F22, only

Figure 3.1. Southern blot of the *Hind* III-digested rape genomic DNA hybridised against the *extA* coding sequence.



10 μg of genomic DNA were digested with *Hind* III for 4 hours at 37^{0}C and analysed on a 0.8% agarose gel. The gel was blotted and the nylon membrane was hybridised against the radiolabelled *extA* coding sequence probe. A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5X SSC/0.1% SDS for 1 hour at 65^{0}C.

Figure 3.2. An agarose gel analysis of the *Hind* III fragments of rape genomic DNA fractionated through a 10-40% sucrose gradient.

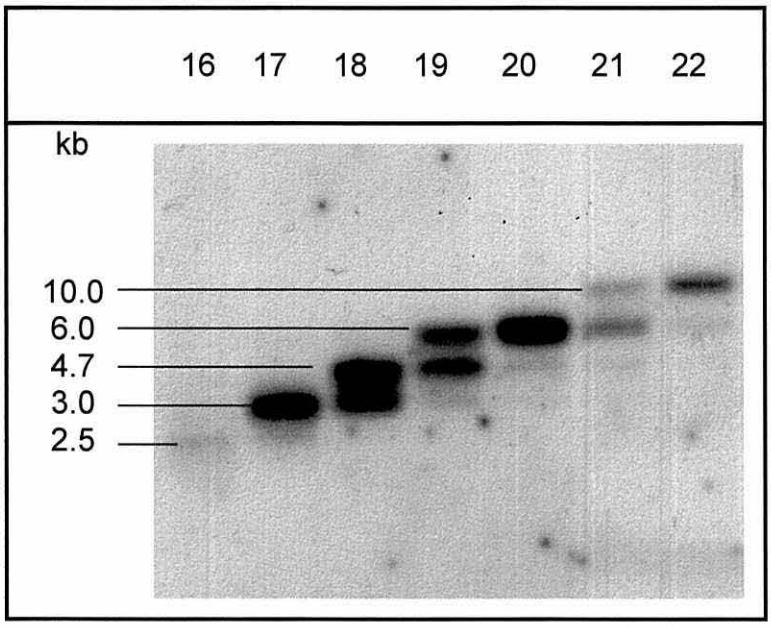


100 μg of rape genomic DNA were subjected to *Hind* III restriction digestion before loading on top of the gradient. The gradient was centrifuged as detailed in Section 2.12.1. After fractions were collected, 25 μl of each fraction was used to run on the gel.

The lanes contained:

M, λ-*Pst* I DNA marker; **7-25**, fraction number.

Figure 3.3. Southern blot of the gel in Figure 3.2 hybridised against the radiolabelled *extA* coding sequence probe.



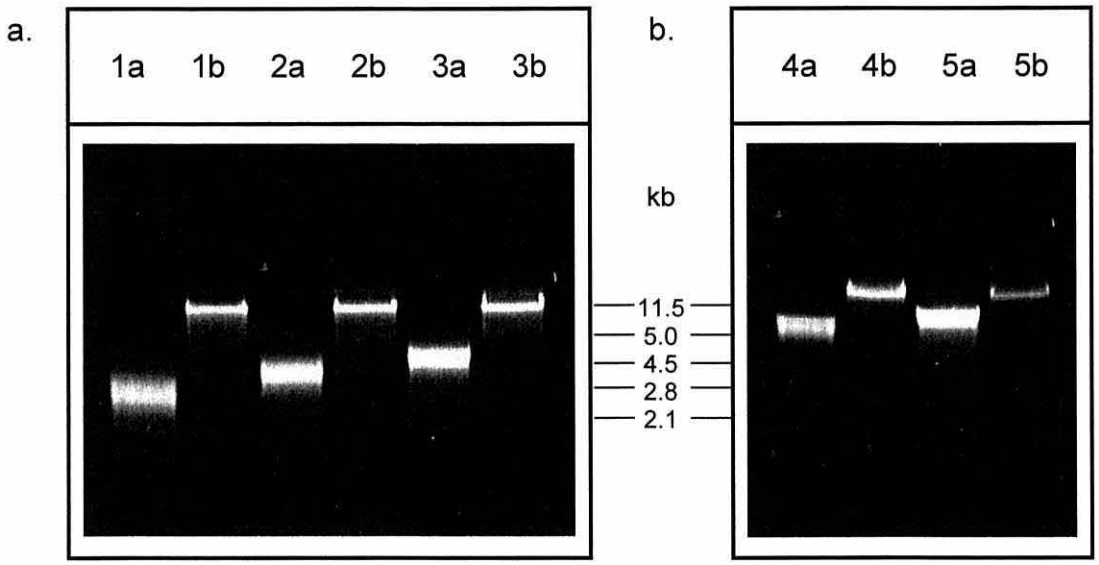
A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5x SSC/0.1% SDS for 1 hour at 65^{0}C. **16-22**, fraction number.

the signal from the 10.0 kb band was detected. Fractions containing the bands of interest were collected and further processed (Section 2.12.1) to purify the DNA. F16, F17 and F18 were collected alone, while F19 and F20 (F19/20), and F21 and F22 (F21/22) were combined.

Cross contamination of the *extA* hybridising fragments in the sucrose gradient fractions (Figure 3.3) occurred because a large volume (0.3 ml) of gradient was collected in each fraction. This could have been avoided by collecting smaller fraction volumes from the gradient. This cross contamination which is clearly seen in F18, F19 and F21 (Figure 3.3) would have reduced the ligation efficiency of the bigger fragment to the pSK^+ vector if the calculation for the picomoles of ends ratio between the inserts and pSK^+ vector had been done based on the smaller fragments present in the same fraction. Because ligations between the pSK^+ vector and insert DNA were performed with a ratio of 1:3 (pSK^+:insert), the calculation for the picomoles of ends ratio in this way caused the ratio for the larger fragment used in the ligation reaction to be less than 1:3 (pSK^+:insert) and would have therefore reduced the ligation efficiency of the fragment. Therefore, in order to avoid this problem, the calculation of the ratio between the pSK^+ vector and insert was based on the larger fragment. This caused the amount of the smaller fragment used in the ligation with the pSK^+ vector to increase to more than 1:3 (pSK^+:insert). In theory, the use of slightly larger amounts of the fragment would have increased the probability of the fragment to self religate and to form tandem oligomers. However, ligation and transformation tests have shown that the efficiency of ligation was not reduced by the presence of a slightly higher amount of the smaller DNA fragments (results not presented).

Before the DNA fragments were ligated into the pSK^+ vector, the ability of the cohesive ends of the fragments to ligate was tested. Agarose gel analyses (Figure 3.4a and b) of the ligation test reactions of the fragments showed evidence of ligation, as judged by the formation of molecules larger than the unligated molecules for all the DNA fractions. It is very important for the cohesive ends on the DNA fragment to maintain their ability to religate with the cohesive complementary ends created with the same restriction cleavage enzyme on the plasmid because if the DNA fragments fail to ligate with the

Figure 3.4. Agarose gel analysis of the ligation test of DNA fragments. 2 μl of each fraction were re-ligated for 2 hours at room temperature and run on a gel.



The lanes contained:

1a, F16 unligated; **1b**, F16 ligated; **2a**, F17 unligated; **2b**, F17 ligated; **3a**, F18 unligated; **3b**, F18 ligated; **4a**, F19/20 unligated; **4b**, F19/20 ligated; **5a**, F21/22 unligated; **5b**, F21/22 ligated.

pSK^+, the ligation efficiency will certainly be reduced and will therefore reduce the number of transformants when the ligation mixture is transformed into competent *E. coli* cells.

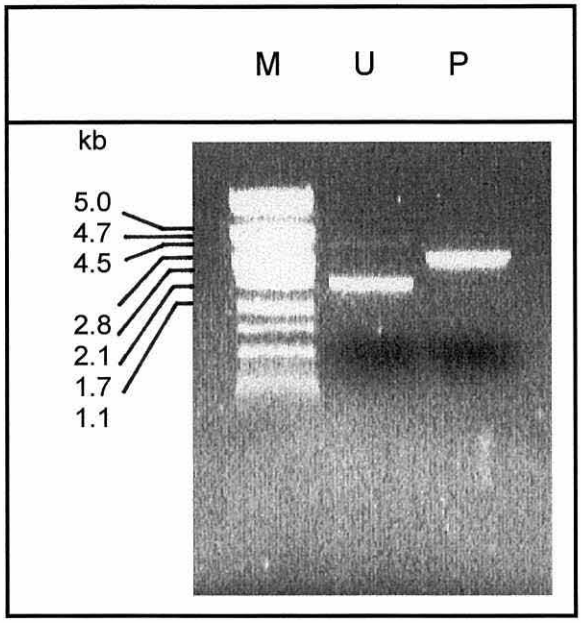
3.3 Preparation of the pSK^+ vector for ligation

Before the pSK^+ plasmid was used for the ligation, it was subjected to *Hind* III digestion in order to produce compatible cohesive ends between the pSK^+ vector and the DNA fragments from the *Hind* III digest of genomic rape DNA. The progress of the restriction digest was monitored by analysing the reaction mixture on an agarose gel (Figure 3.5). The presence of a single band at 2.9 kb showed that the restriction reaction was complete. After the linearised pSK^+ was purified (Section 2.10.4), it was incubated with different amounts of calf intestinal alkaline phosphatase (CIAP) to remove the 5'-end phosphate groups in order to prevent the pSK^+ from self religation (recircularisation). Recircularisation of the pSK^+ during the ligation between pSK^+ and DNA fragments would have reduced the ligation efficiency between the insert and the vector. An agarose analysis of the ligation test performed on the CIAP treated pSK^+ showed that 0.5 U CIAP-treated pSK^+ was unable to re-ligate as only one band of 2.9 kb was detected on the gel (Figure 3.6). DNA bands of higher molecular weight detected in lane 1b (Figure 3.6) showing that incubation of pSK^+ with 0.25 U of CIAP was not sufficient to prevent the pSK^+ from re-ligation. The ability of untreated linearised pSK^+ to ligate was also evident (lane 2b, Figure 3.6).

3.4 Optimisation of ligation

In order to find the best ratio for ligation between the pSK^+ plasmid and the DNA fragments, DNA from fraction 15 (F15) was ligated with the 0.5 U CIAP-treated pSK^+ with the ratio of picomoles of ends being 1:1, 1:2 and 1:3 (pSK^+:F15). Analysis of the ligation tests by transforming the ligation mixture into competent *E. coli* showed that the ratio of 1:3 (pSK^+:insert) gave a total number of transformants of 13160 per 100 ng of pSK^+ (Table 3.1). Ligations with the ratio of 1:1 and 1:2 produced 5240 and 11280 transformants per 100 ng of pSK^+ respectively.

Figure 3.5. The *Hind* III restriction digest of pSK$^+$ analysed on an agarose gel.

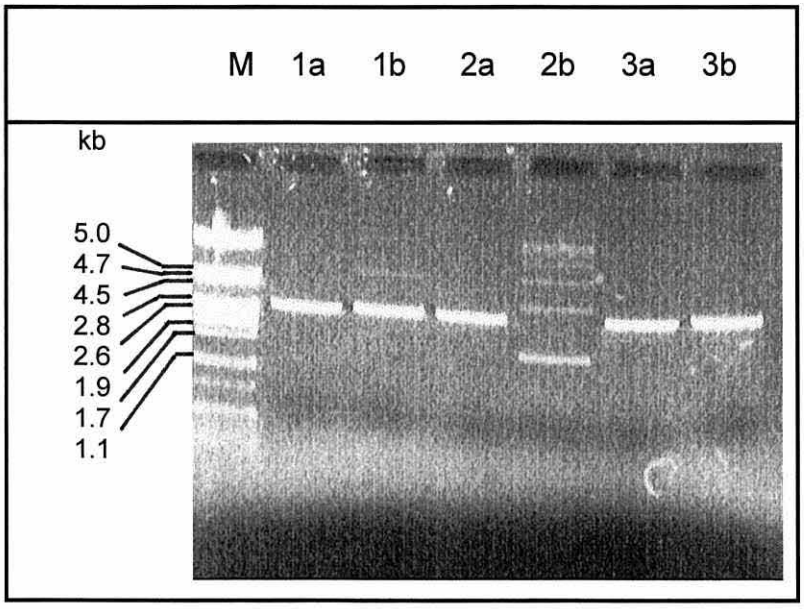


10 μg of pSK$^+$ were subjected to *Hind* III restriction for 2 hours at 37^{0}C. 200 ng of the restriction digest mix was taken and used to run on a gel. 200 ng of uncut pSK$^+$ were included as a control.

The lanes contained:

M, λ-*Pst* I DNA marker; **U**, uncut pSK$^+$; **P**, cut pSK$^+$.

Figure 3.6. An agarose gel analysis of the ligation test of pSK$^+$ treated with calf intestinal alkaline phosphatase (CIAP).



8 μg of *Hin*d III-digested pSK$^+$ were treated with 0.25 U and 0.5 U CIAP respectively for 1 hour at room temperature. 100 ng from each CIAP-treated pSK$^+$ plasmid was ligated for 2 hours at room temperature and used to run on a gel. 100 ng of untreated *Hin*d III-digested pSK$^+$ was included to serve as a control.

The lanes contained:

M, λ-*Pst* I DNA marker; **1a**, 0.25 U CIAP-treated pSK$^+$ unligated; **1b**, 0.25 U CIAP-treated pSK$^+$ ligated; **2a**, untreated pSK$^+$ unligated; **2b**, untreated pSK$^+$ ligated; **3a**, 0.5 U CIAP-treated pSK$^+$ unligated; **3b**, 0.5 U CIAP-treated pSK$^+$ ligated.

After the transformants were plated on LB plates containing IPTG/X-gal, the ligation with the ratio of 1:2 was seen to give the maximum percentage of recombinants (71.9%). Ligations with the ratio of 1:1 and 1:3 produced 61.8% and 69.3% recombinants respectively. Even though the percentage of recombinant colonies from the ligation with the ratio of 1:3 was lower compared to the ligation with the ratio of 1:2 (Table 3.1), the total number of recombinant colonies produced by the ligation with the vector:insert ratio of 1:3 was larger than the one produced by the ligation with the ratio of 1:2. Ligations between the pSK^+ and the DNA fragments were therefore performed with the vector:insert ratio of 1:3. Self re-ligation of the untreated pSK^+ used as a control produced a total number of transformants of 98 133 per 100ng of pSK^+ with all of them being non-recombinants (Table 3.1).

Table 3.1. Efficiency of ligation between pSK^+ and DNA inserts tested with different pSK^+:insert ratios.

Picomole of ends ratio (pSK^+:insert)	Total number of white colonies	Total number of blue colonies	Total number of transformants/ 100 ng pSK^+	% white colonies
1:1	3 240	2 000	5 240	61.8
1:2	8 120	3 160	11 280	71.9
1:3	9 120	4 040	13 160	69.3
pSK^+	-	98 133	98 133	-

3.5 Construction of a rape genomic mini library

DNA fragments from the F16, F17, F18, F19/20 and F21/22 fractions from the sucrose gradient were ligated to the 0.5 U CIAP-treated pSK^+ vector at a ratio of 1:3 (pSK^+:insert), and produced a total number of transformants of 1.589×10^5 per 5.0 μg of the *Hin*d III fragments of rape genomic DNA used. Restriction digest analyses of the plasmids obtained from 22 randomly picked white colonies (Figure 3.7a and b) from the ligated F16 transformation showed that 18 colonies (77.3%) were recombinants. The smallest size of insert was from clone 1, which contained 0.3 kb while the largest insert was approximately 4.5 kb (from clone 16).

3.6 Isolation of the *extA* coding sequence probe

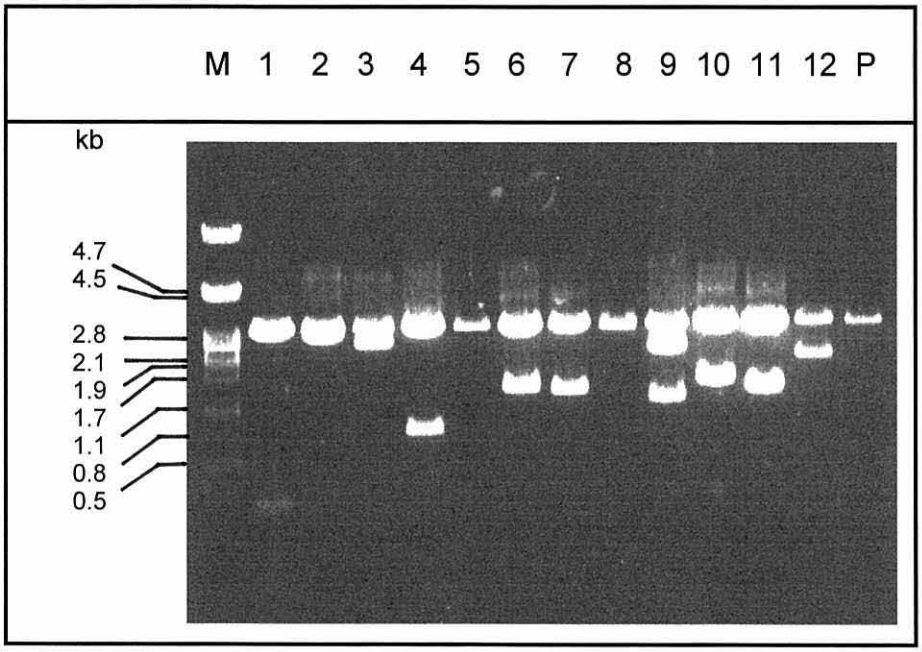
A genomic clone, lambda B31 containing the 1.0 kb of coding sequence of the *extA* gene was originally cloned and isolated by Evans *et al.* (1990). The coding sequence was then sub-cloned into the pUC18 vector into the *Eco*R I and *Hin*d III sites and designated as PRλS5 (Evans *et al.*, 1990). In order to isolate the *extA* coding sequence DNA to use as a probe, the PRλS5 plasmid was double digested with *Eco*R I and *Hin*d III and the probe separated on an agarose gel as shown in Figure 3.8. Two bands (lanes 1, 2 and 3) were clearly separated with the bottom band of 1.0 kb being the *extA* coding sequence. The upper band was the pUC18 vector. An agarose gel slice containing the probe was excised and processed (Section 2.10.4) in order to isolate the probe. An agarose analysis of the isolated *extA* coding sequence probe (Figure 3.9) showed that the DNA was pure as judged by the presence of only one band of 1.0 kb on the gel.

3.7 Screening of the rape mini library

For screening purposes, the library was plated onto 22 x 22 cm agar plates. It is recommended that cells be plated at a density of 2×10^2 colonies per 138 mm plate (Sambrook *et al.*, 1989). This means that the cells are plated at a density of 133 colonies per cm^2. At this density the colonies grow to up to 1 mm in diameter without touching. The 22 x 22 cm plates will therefore accommodate approximately 6.4×10^4 colonies per plate.

Figure 3.7. Restriction analysis of plasmids obtained from 22 white colonies. Cells were grown overnight in 5 ml LB broth containing the selective antibiotic ampicillin for plasmid isolation (Section 2.10.1.2). The final plasmid DNA preparations were resuspended in 50 μl TE buffer. 2 μl of each preparation were used in the *Hin*d III restriction digests. 100 ng of *Hin*d III-digested pSK$^+$ were included as a control.

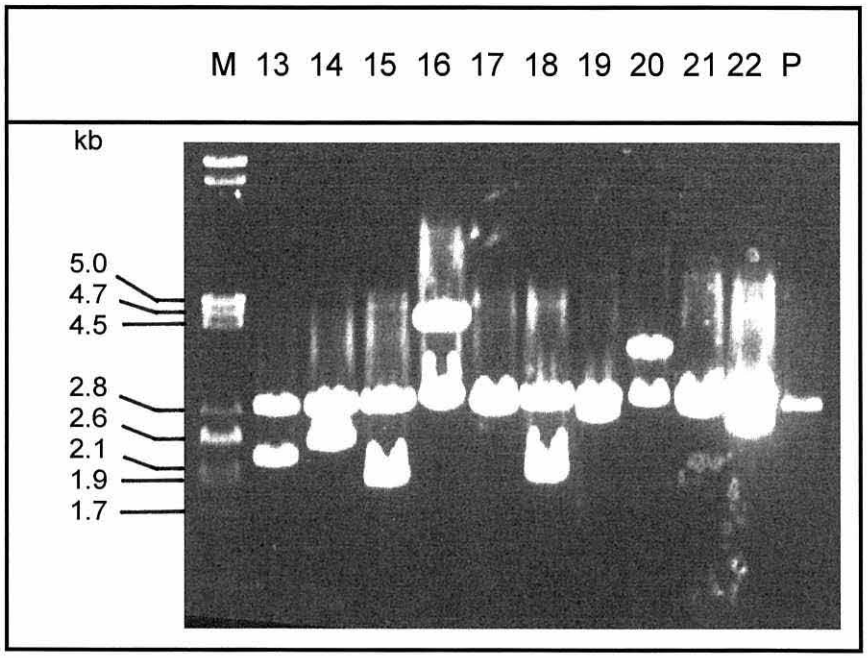
a.



The lanes contained:

M, λ-*Pst* I DNA marker; **1-12**, clone number; **P**, pSK$^+$.

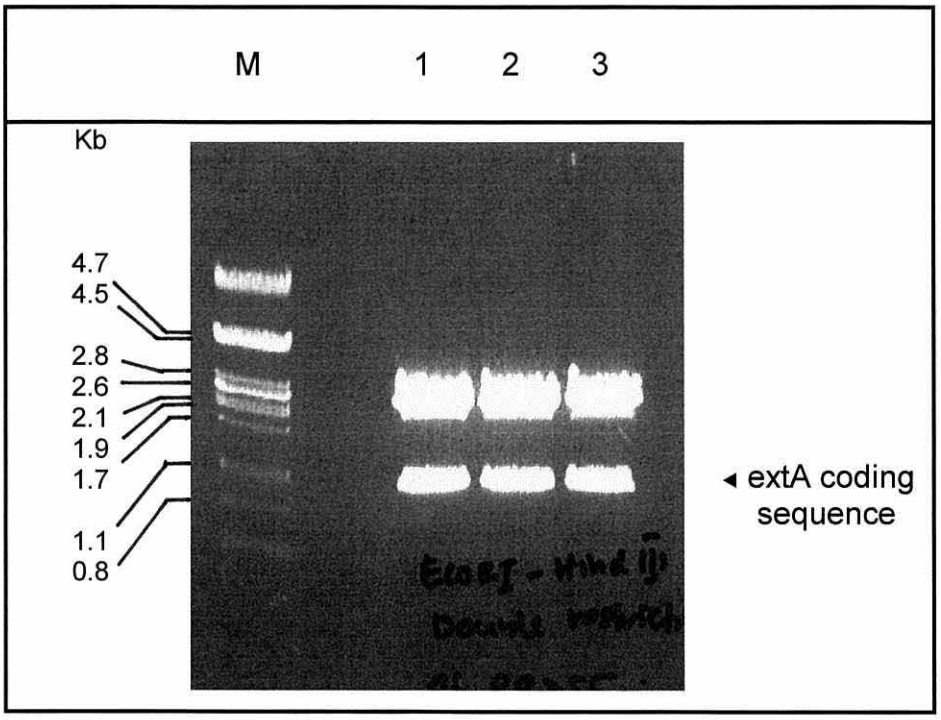
Figure 3.7b. Restriction analysis of plasmids obtained from 22 white colonies.



The lanes contained:

M, λ-*Pst* I DNA marker; **13-22**, clone number; **P**, pSK^+.

Figure 3.8. An agarose gel analysis of the *Hind* III and *Eco*R I double digest of PRλS5 to isolate the *extA* coding sequence DNA fragment.

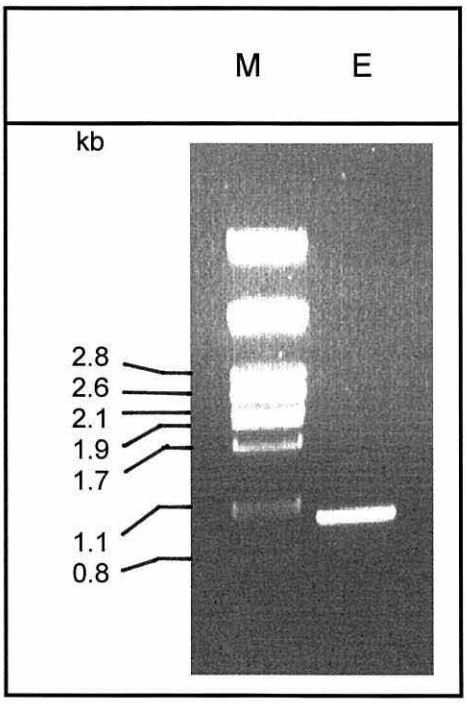


10 μg of PRλS5 were double digested with *Hind* III and *Eco*R I and equally loaded onto each well of a gel. ◂ - *extA* coding sequence probe.

The lanes contained:

M, λ-*Pst* I DNA marker; **1-3**, PRλS5.

Figure 3.9. An agarose gel analysis of the *extA* coding sequence probe after it was electroeluted (Section 2.10.4) from the gel.



200 ng of the probe were loaded onto the gel.

The lanes contained:

M, λ-*Pst* I DNA marker; **E**, *extA* probe.

Because the number of clones obtained in the library was approximately 1.6 x 10^5, 2.48 plates were needed to accommodate all the colonies. Therefore, three 22 x 22 cm plates were used. Each plate therefore had approximately 5.3 x 10^4 colonies or 109 colonies per cm^2. At this density, colonies were allowed to grow bigger than 1 mm in diameter therefore providing more target DNA for hybridisation to the probe. A higher intensity of radioactive signal should therefore be seen on screening.

The rape genomic plasmid library was screened against the radiolabelled *extA* coding sequence probe (Section 2.12.4). Of the 1.589 x 10^5 transformants screened, only one strong radioactive signal was detected (Figure 3.10). Colonies within an area of 1 cm^2 of the signal were picked, re-plated and re-hybridised against the probe for the secondary and tertiary screening. During the secondary screening, enrichment in the number of colonies hybridising to the probe was obtained (Figure 3.11). Tertiary screening of the colonies obtained from a single strong signal colony from the secondary screening showed that 100% of the colonies hybridised to the probe (Figure 3.12).

3.8 Partial characterisation of the isolated clone

The insert was initially characterised by analysing the *Hind* III-digestion of the isolated plasmid from the positive clone on an agarose gel. Two distinct bands of sizes 2.9 kb and 2.5 kb were clearly seen (lane C - Figure 3.13). The upper band was the pSK^+ while the bottom band was an insert. The clone was then designated as pNS1 (plasmid Nik Sidik 1).

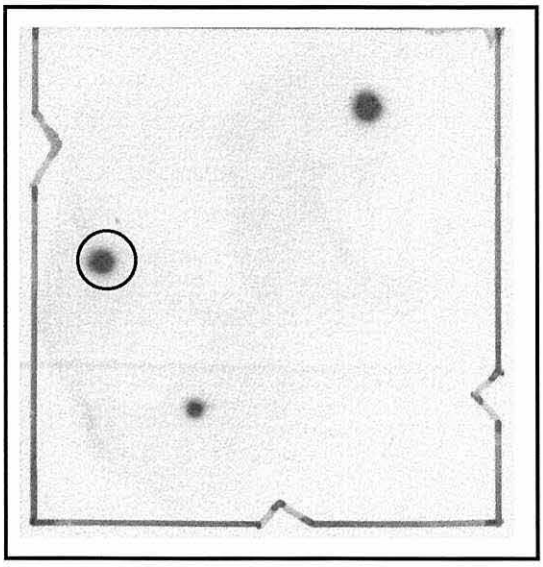
3.9 Restriction enzyme mapping of pNS1

pNS1 was subjected to restriction digestion with several restriction enzymes to locate the restriction enzyme cleavage sites in the pNS1 insert. Two of the gels showing the fragments produced by the restriction digests are shown in Figure 3.14a and b. pNS1 cut by *Sal* I and *Bam*H I produced only one band of 5.4 kb (Figure 3.14a) showing that both enzymes did not cut the insert, because both enzymes have only one restriction enzyme site in the pSK^+ vector polylinker.

Figure 3.10. First screening of the mini library against the radiolabelled *extA* coding sequence probe.

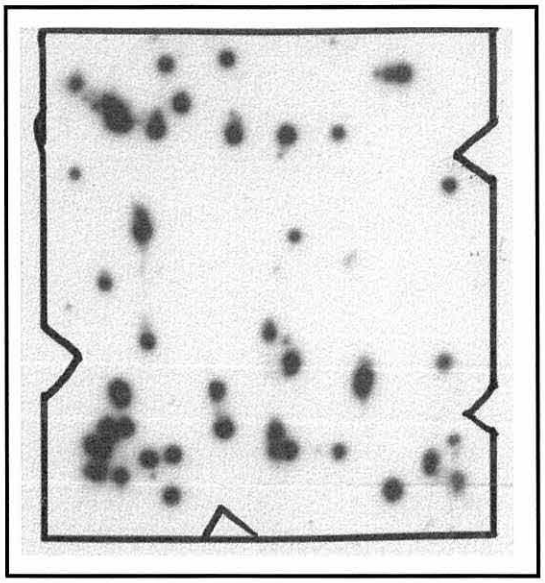
The library was plated on 22 x 22 cm nylon membranes laid on 24 x 24 cm square LB plates at a concentration of approximately 100 colonies/cm^2. Cells were grown until the size of the colonies reached approximately 1 mm. Master plates were prepared and membranes were processed as detailed in Section 2.12.4.1. A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5x SSC/ 0.1% SDS for 1 hour at 65^{0}C. The circle shows the area chosen for secondary screening.

Figure 3.11. Secondary screening of the library against the radiolabelled *extA* coding sequence probe.



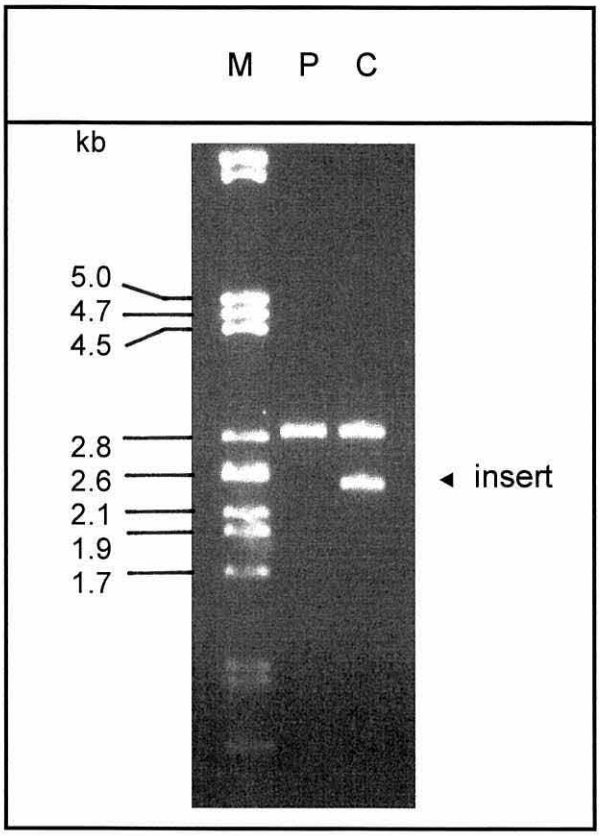
A nylon membrane representing the marked area in Figure 3.10 was cut out and placed in an eppendorf tube containing 0.5 ml LB broth to resuspend the cells. Cells were replated on a fresh nylon membrane at a concentration of 30 colonies/cm^2 so that they were well separated, and grown as detailed in the legend to Figure 3.10. A master plate was prepared and the membrane was processed as detailed in Section 2.12.4.1. A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5x SSC/ 0.1% SDS for 1 hour at 65^{0}C. The circle shows the area chosen for tertiary screening.

Figure 3.12. Tertiary screening of the library against the radiolabelled *extA* coding sequence probe.



A single isolated positive colony obtained from the secondary screening was picked and resuspended in 0.5 ml LB broth in an eppendorf tube. Cells were plated on a nylon membrane at an appropriate concentration so that they were well separated, and grown as detailed in the legend to Figure 3.10. A master plate was prepared and the membrane was processed as detailed in Section 2.12.4.1. A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5x SSC/0.1% SDS for 1 hour at 65^0C.

Figure 3.13. An agarose gel analysis of the insert from the positive clone.



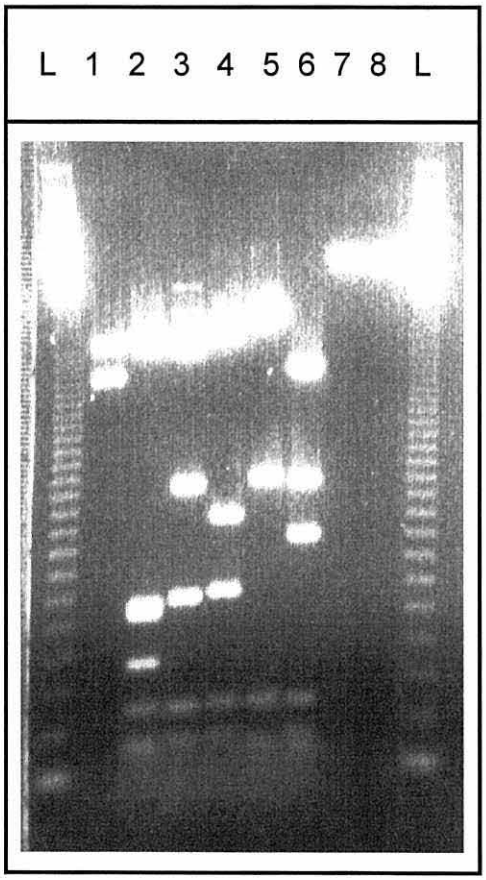
200 ng of plasmid obtained from the positive clone was digested with *Hind* III and on run a gel. 100 ng of *Hind* III-digested pSK$^+$ were included as a control.
◂ - shows the insert.

The lanes contained:

M, λ-*Pst* I DNA marker; **C**, pNS1; **P**, pSK$^+$.

Figure 3.14. Restriction enzyme analysis showing the mapping of pNS1. pNS1 was digested with several combinations of restriction enzymes as detailed below.

a.

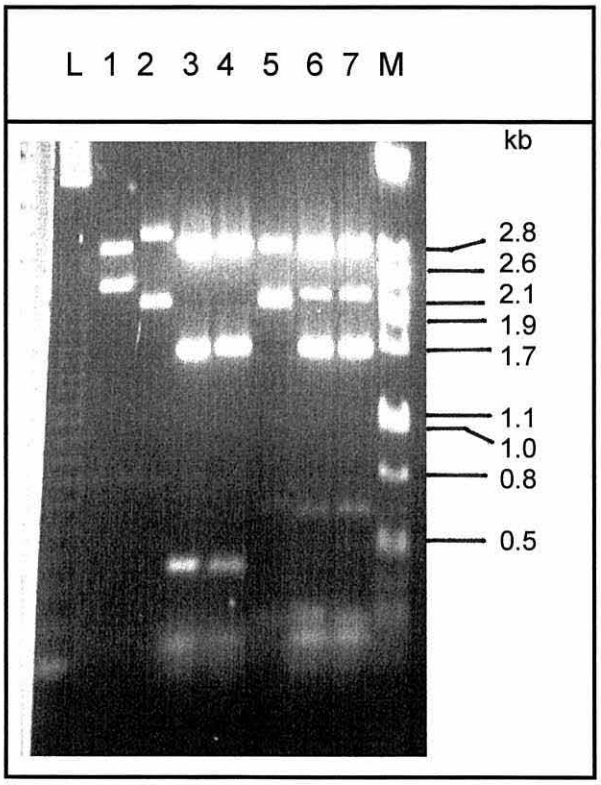


Lanes 2-6 were products of double digestion, while lanes 7-8 were products of a single digestion. 200 ng of *Hin*d III-digested pNS1 were included as a control. **L**, 123 bp DNA ladder; **1**, *Hin*d III-restricted pNS1.

Enzymes used in each lanes were as follow:

2, *Bgl* II/*Acc* I; **3**,*Bgl* II/*Hin*d III; **4**, *Bgl* II/*Eco*R I; **5**, *Bgl* II/*Xho* I; **6**, *Bgl* II/*Pvu* II; **7**, *Sal* I; **8**, *Bam*H I.

b.



Lanes 2-3 were products of a single digestion, lanes 4-6 were products of double digestion, and lane 7 was a product of triple digestion. 200 ng of *Hind* III-digested pNS1 were included to serve as a control. **L**, 123 bp DNA ladder; **M**, λ-*Pst* I DNA marker; **1**, *Hind* III-digested pNS1.

Enzymes used in each lane were as follow:

2, *Eco*R I; **3**, *Acc* I; **4**, *Hind* III/*Acc* I; **5**, *Hind* III/*Eco*R I; **6**, *Eco*R I/*Acc* I; **7**, *Hind* III/*Eco*R I/*Acc* I.

Other enzymes which did not cut the insert were *Pst* I, *Kpn* I, *Sac* I, *Eco*R V, and *Xba* I (results not published). Two fragments produced by the digestion of pNS1 with *Pvu* II, *Xho* I, *Bst*E II, and *Eco*R I showed that they had only one restriction enzyme cleavage site in the insert, whereas the four fragments produced by digestion of pNS1 with *Acc* I and *Bgl* II showed that there were three restriction enzyme sites for both enzymes in the insert. Figure 3.15 illustrates the restriction enzyme map of pNS1 showing the sizes of the various fragments in detail.

In order to locate the *extA*-hybridising region of pNS1, the clone was digested with several combinations of restriction enzymes. This was analysed on an agarose gel (Figure 3.16), Southern blotted and hybridised against the radiolabelled *extA* coding sequence probe. Figure 3.17 shows that the 1.84 kb *Hin*d III-*Bst*E II fragment, the 1.32 kb *Bst*E II-*Xho* I fragment, the 1.14 kb *Xho* I-*Pvu* II fragment, the 0.57 kb *Pvu* II-*Acc* I fragment, the 0.9 kb *Pvu* II-*Eco*R I fragment and the 2.26 kb *Eco*R I-*Hin*d III fragment all hybridised to the probe. Strong radioactive signals were shown by the 1.84 kb, 1.32 kb and 2.26 kb fragments. Signals of medium strength were seen from the 1.14 kb and 0.9 kb fragments, and the weakest signal was obtained from the 0.57 kb fragment.

3.10 Southern blot hybridisation of oilseed rape genomic DNA against the pNS1 probe

In order to determine the number of members of the gene family homologous to pNS1 in the oilseed rape genome, rape genomic DNA was digested with *Hin*d III and run on an agarose gel (Figure 3.18). The gel was then blotted and the nylon membrane was Southern hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1. As shown in Figure 3.19, six bands appear to hybridise with the probe. The sizes of the bands were estimated as 2.5 kb, 3.1 kb, 3.4 kb, 3.8 kb, 7.7 kb and 10.7 kb. Strong radioactive signals were contributed by the 2.5 kb and 7.7 kb bands, moderate signals were given by the 3.1 kb, 3.4 kb and 3.8 kb bands, while the weakest signal was obtained from the 10.7 kb band.

Figure 3.15. Restriction enzyme map of the 2.5 kb pNS1 insert showing restriction enzyme sites.

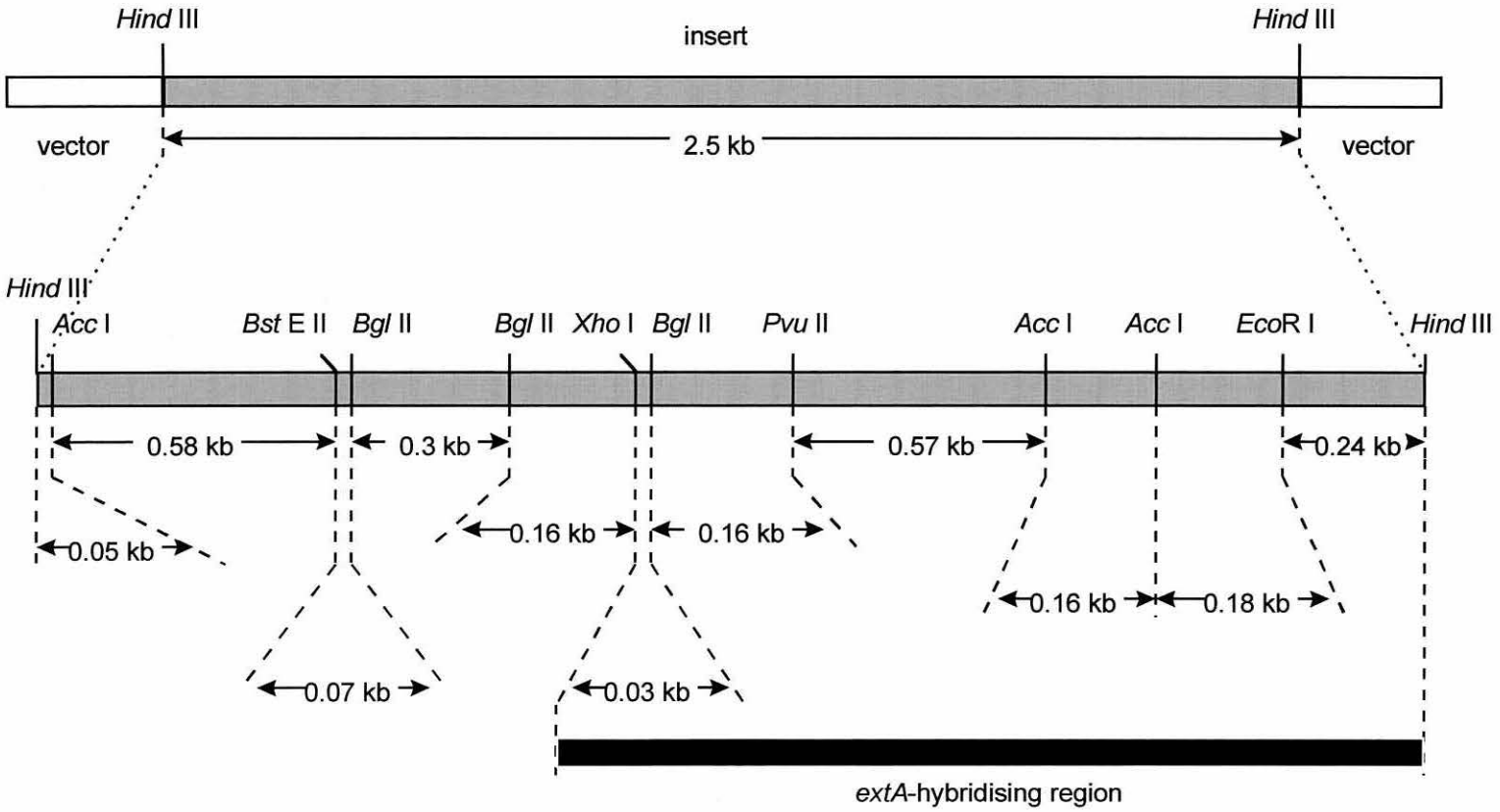
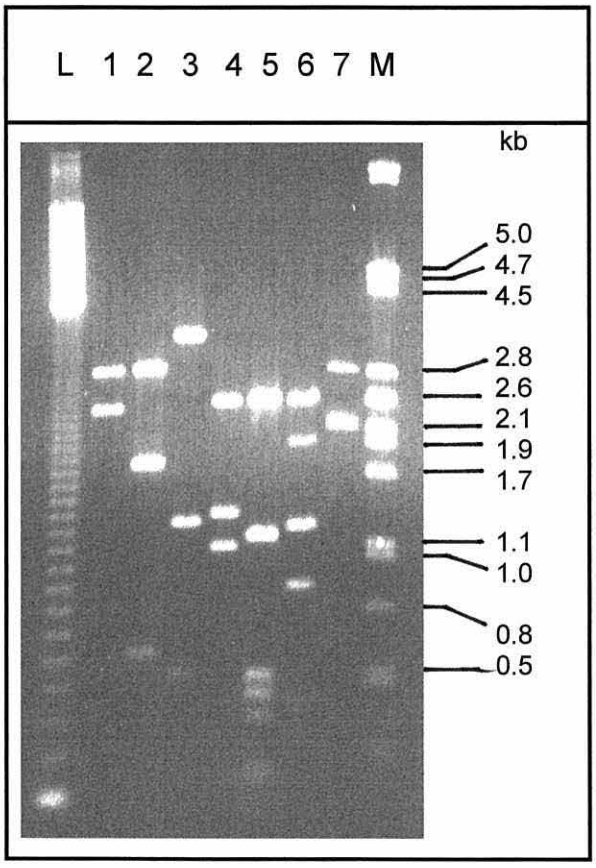


Figure 3.16. An agarose gel analysis of pNS1 cleaved with several restriction enzymes to determine the position of the *extA*-hybridising region of the insert.

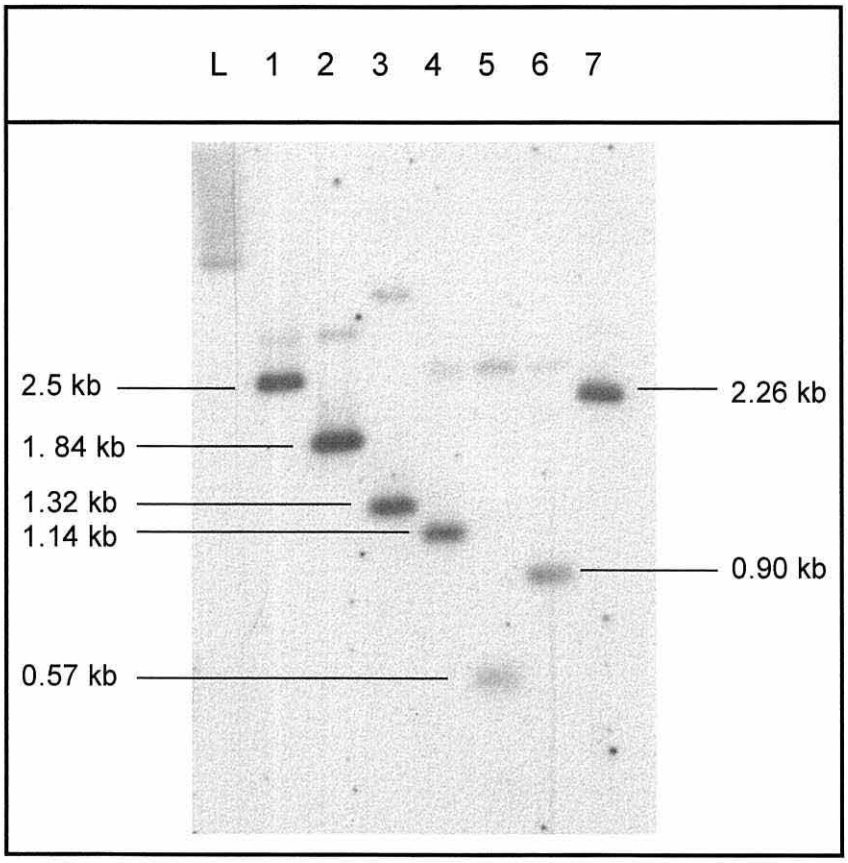


Enzymes used in each lane were as follow:

1, *Hin*d III; **2**, *Hin*d III/*Bst*E II; **3**, *Bst*E II/*Xho* I; **4**, *Xho* I/*Pvu* II; **5**, *Pvu* II/*Acc* I; **6**, *Pvu* II/*Eco*R I; **7**, *Eco*R I/*Hin*d III.

L, 123 bp DNA ladder; **M**, λ-*Pst* I DNA marker.

Figure 3.17. Southern blot of the nylon membrane prepared from the gel from Figure 3.16 hybridised against the radiolabelled *extA* coding sequence probe.

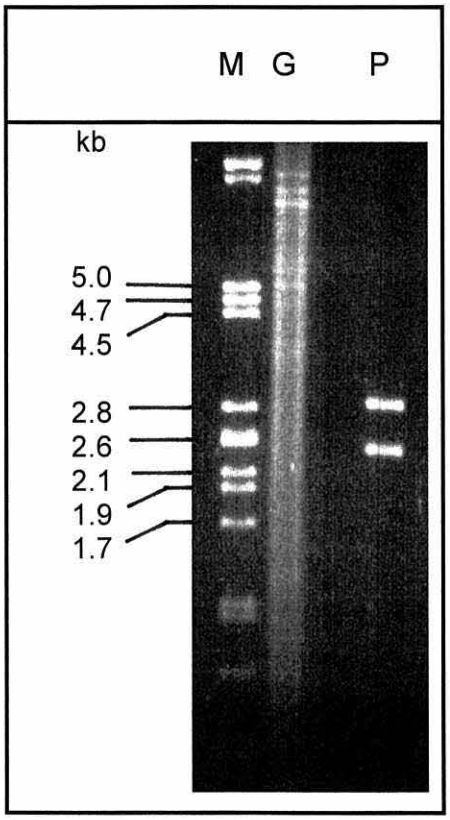


A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5X SSC/0.1% SDS for 1 hour at 65^0C.

Enzymes used in each lane were as follow:

1, *Hin*d III; **2**, *Hin*d III/*Bst*E II; **3**, *Bst*E II/*Xho* I; **4**, *Xho* I/*Pvu* II; **5**, *Pvu* II/*Acc* I; **6**, *Pvu* II/*Eco*R I; **7**, *Eco*R I/*Hin*d III.

Figure 3.18. An agarose gel analysis of *Hind* III-digested rape genomic DNA to determine the members of the extensin gene family homologous to pNS1.

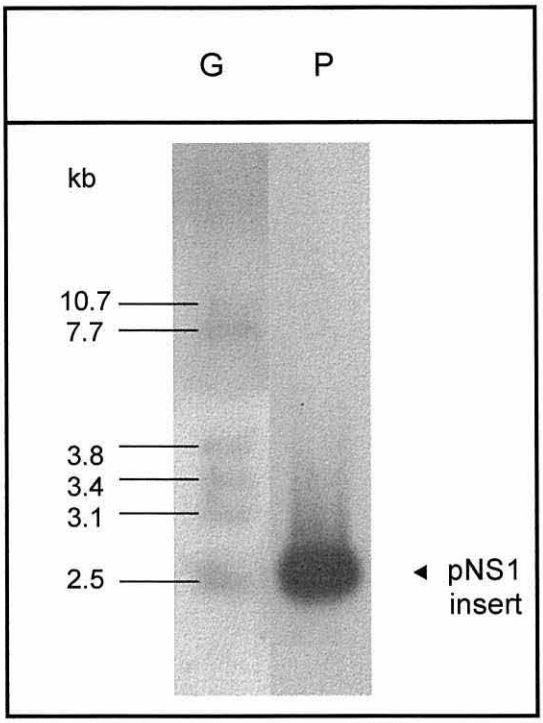


10 μg of rape genomic DNA were digested with *Hind* III and used to run on a gel. 100 ng of *Hind* III-digested pNS1 were also included to serve as a positive control for Southern hybridisation.

The lanes contained:

M, λ-*Pst* I DNA marker; **G**, rape genomic DNA; **P**, pNS1.

Figure 3.19. An autoradiograph of the membrane prepared from the gel in Figure 3.18 hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1.



A standard procedure for Southern hybridisation (Section 2.10.10) was followed. The final post-hybridisation washing stringency was 0.5x SSC/0.1% SDS for 1 hour at 65^{0}C.

The lanes contained:

G, genomic DNA; **P**, pNS1.

3.11 Double-stranded nested deletions of pNS1

Double-stranded nested deletions (Section 2.10.11) were performed on pNS1 in order to produce clones of different sizes to allow sequencing. Two sets of deletions were obtained, i.e., forward deletions and reverse deletions. Forward and reverse directions of deletion were based on the direction in which the clones were going to be sequenced by the forward and reverse M13 primers. For the forward deletions, the nuclease-susceptible end was produced by cutting pNS1 with *Sal* I, while *Kpn* I was used to produce the nuclease-resistant end. For the reverse deletions, *Xba* I was used to produce the nuclease-susceptible end, while *Sac* I was used to produce the nuclease-resistant end. A graphical representation of the strategies used in the deletion experiments is shown in Figure 3.20. Figure 3.21 shows an analysis of 14 fractions produced by the exonuclease III forward deletion of pNS1. The biggest fragment produced was approximately 5.1 kb while the smallest was approximately 2.6 kb. Figure 3.22a and b show two of the gels used to analyse the deleted clones produced after they were re-ligated and transformed into *E. coli*. Clones of different length were successfully produced by the exonuclease III deletion of pNS1. The thick DNA bands seen on the top of each lanes on the gels (◂ - in Figure 3.22a and b) were of bacterial DNA from the cells. Seven clones were finally selected from the forward deletion experiment (Figure 3.23). The clones were designated as pNS10F, pNS11F, pNS12F, pNS13F, pNS14F, pNS15F and pNS16F respectively. From the reverse deletion experiment, eight clones were finally selected (Figure 3.24). The reverse clones were designated as pNS2R, pNS3R, pNS4R, pNS5R, pNS6R, pNS7R, pNS8R and pNS9R respectively. A graphical representation of the clones produced by the deletion experiments is shown in Figure 3.25. The sizes of the forward and reverse clones isolated are shown in Table 3.2 and Table 3.3 respectively.

Figure 3.20. Strategies for the deletion experiments.

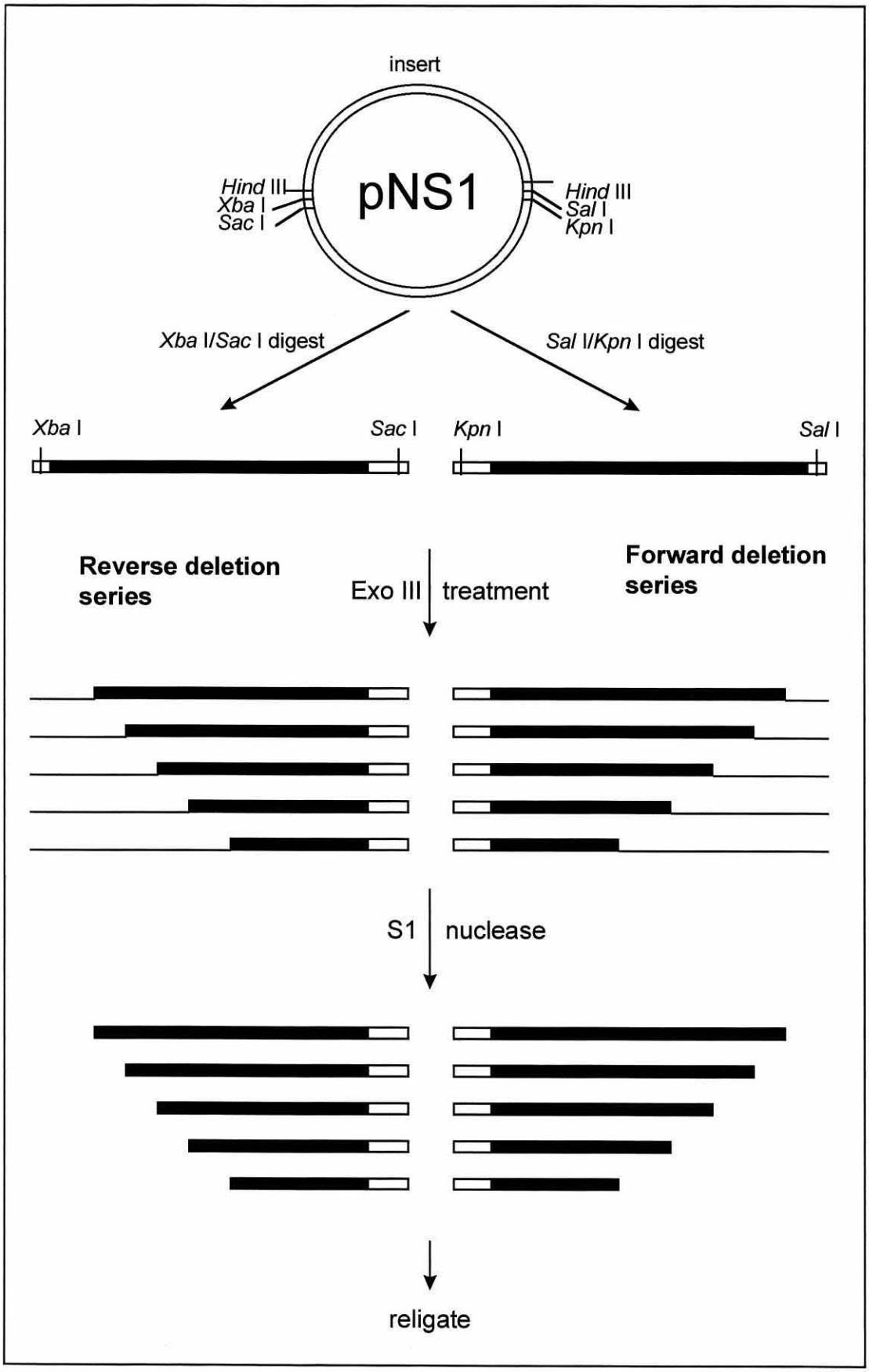
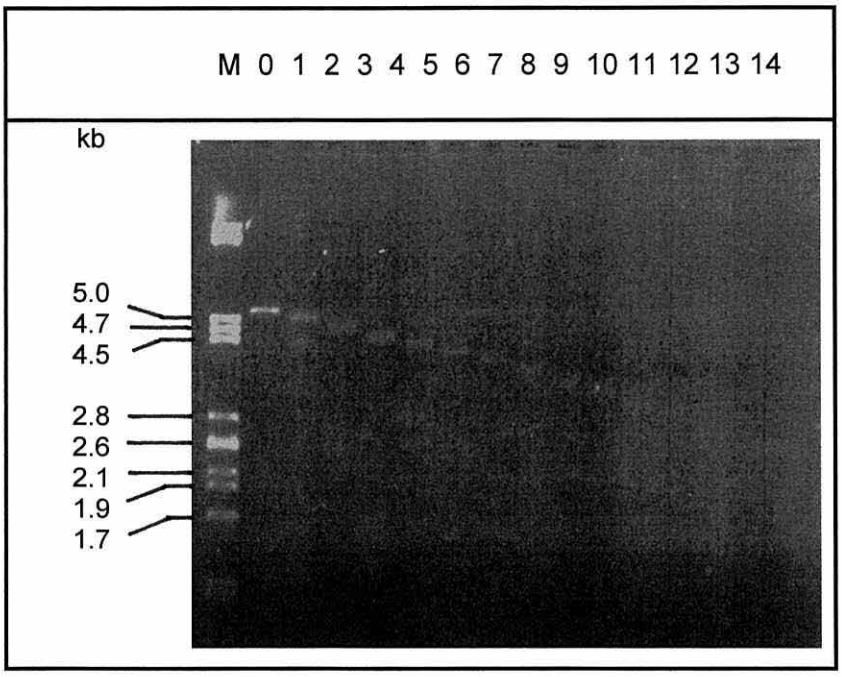


Figure 3.21. An agarose gel analysis of the deleted fragments produced by exonuclease III forward deletion (Section 2.10.11) of pNS1.



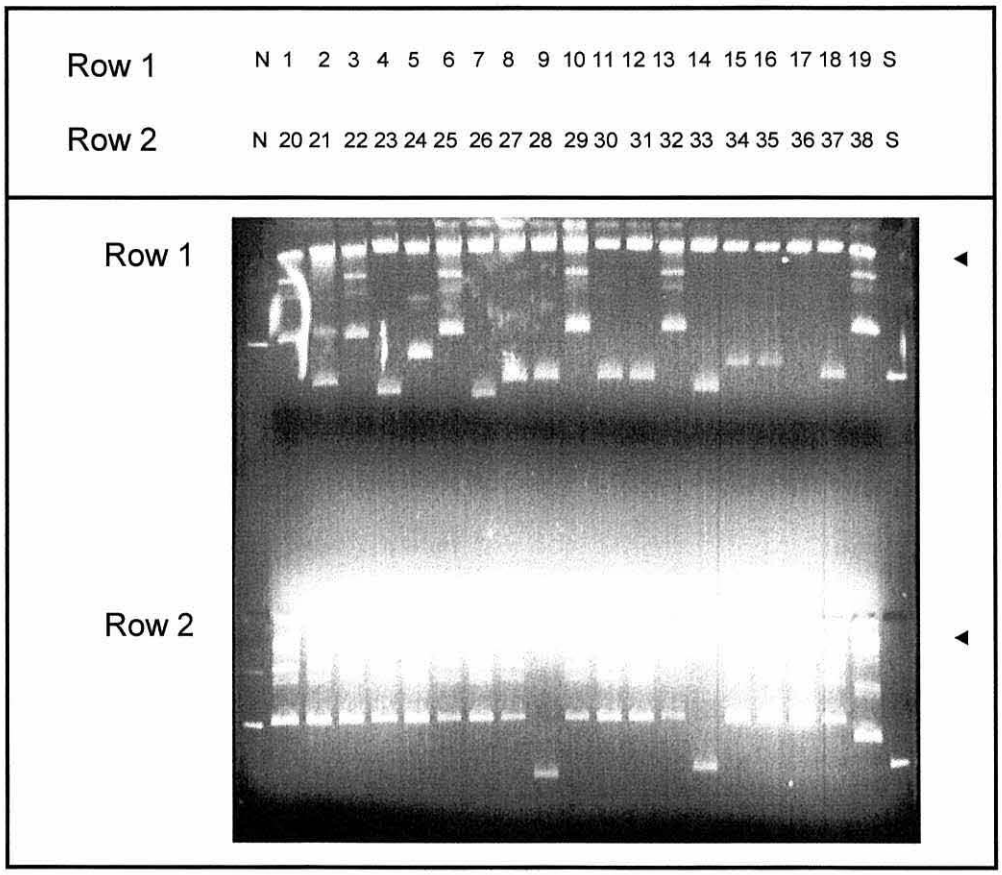
Time points used between each sample were 3 minutes. Half of each fraction was used to run on the gel. Undeleted linearised pNS1 served as a 0 minute time point sample.

The lanes contained:

M, λ-*Pst* I DNA marker; **0**, undeleted pNS1; **1-14**, fraction number.

Figure 3.22. Agarose gel analysis of the nested deletion clones. Plasmids were isolated from transformants by a quick miniprep method (Section 2.10.1.4). All samples were loaded uncut. Uncut pSK$^+$ was included to serve as a "no insert control", while uncut pNS1 served as a "no deletion control" control.

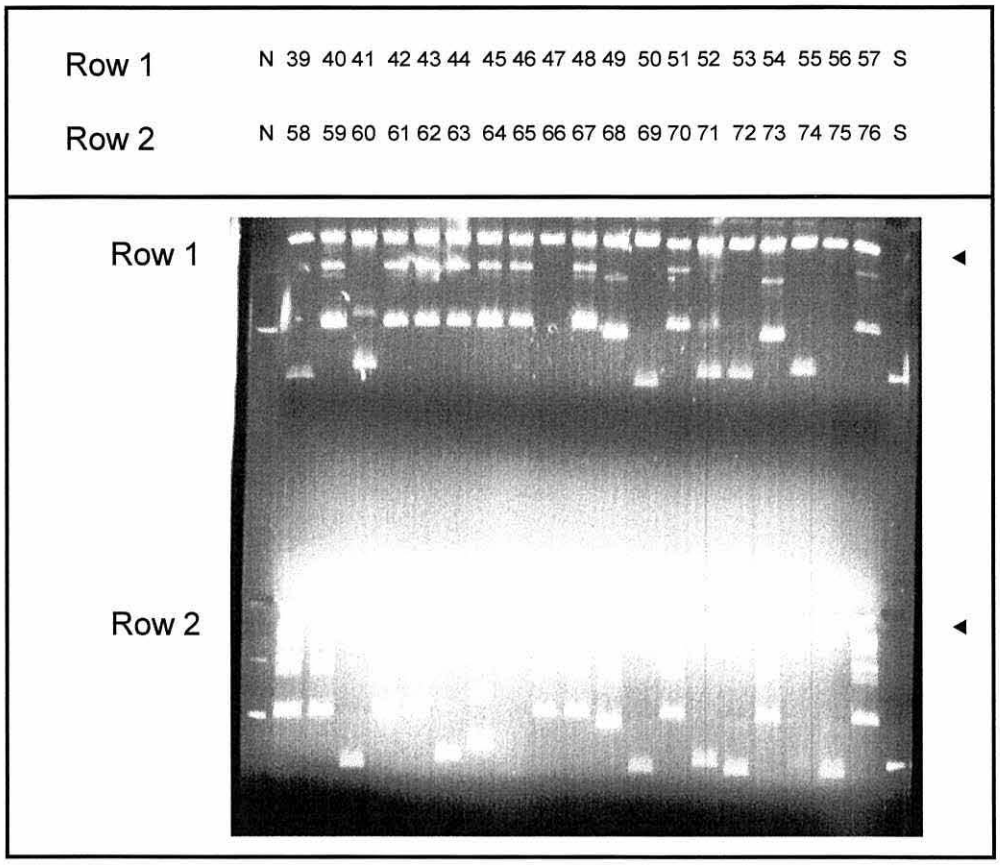
a.



The lanes contained:

N, uncut pNS1; **1-38**, clone number; **S**, uncut pSK$^+$. ◂ - bacterial DNA material.

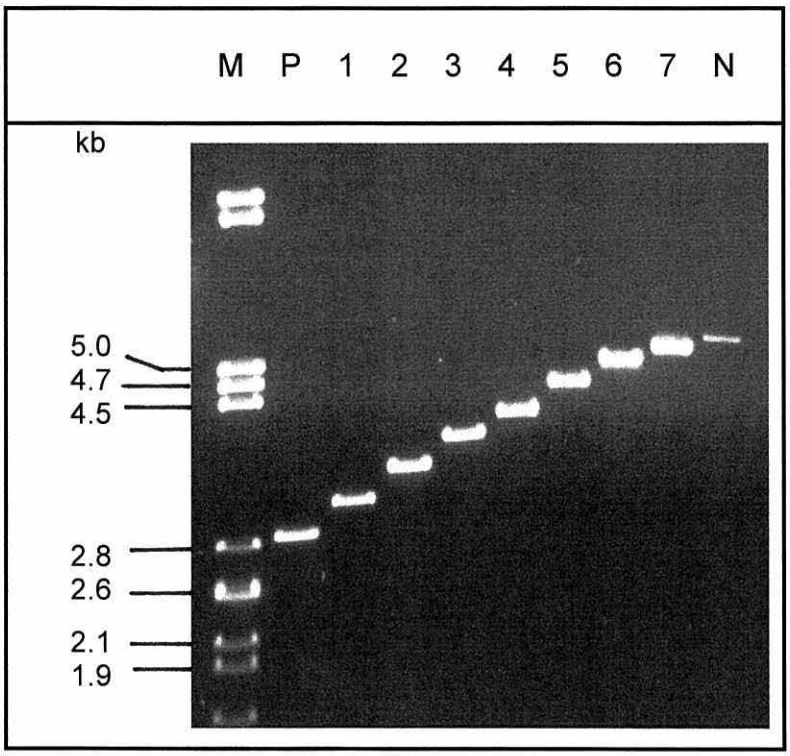
Figure 3.22b. Agarose gel analysis of the nested deletion clones.



The lanes contained:

N, uncut pNS1; **39-76**, clone number; **S**, uncut pSK^+; ◂ - bacterial DNA material.

Figure 3.23. An agarose gel analysis of the selected forward deletion clones.

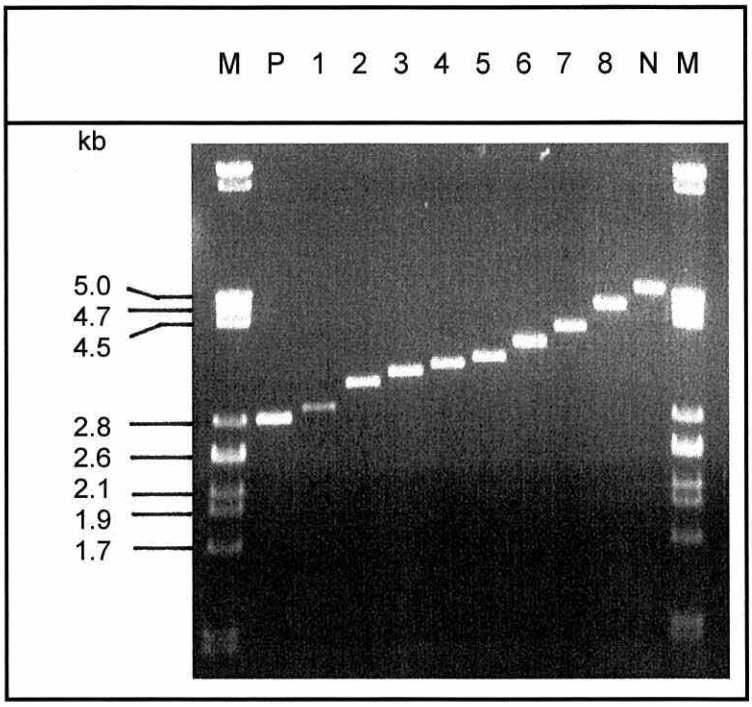


All clones were subjected to *Hind* III restriction before they were loaded onto the gel. 200 ng of each sample were loaded onto the gel. 200 ng of *Hind* III-digested pSK$^+$ were included as a control. 100 ng of *Bam*H I-cut pNS1 was also included.

The lanes contained:

M, λ-*Pst* I DNA marker; **P**, pSK$^+$; **1,** pNS16F; **2**, pNS15F; **3**, pNS14F; **4**, pNS13F; **5**, pNS12F; **6**, pNS11F; **7** pNS10F; **N**, pNS1.

Figure 3.24. An agarose gel analysis of the selected reverse deletion clones.

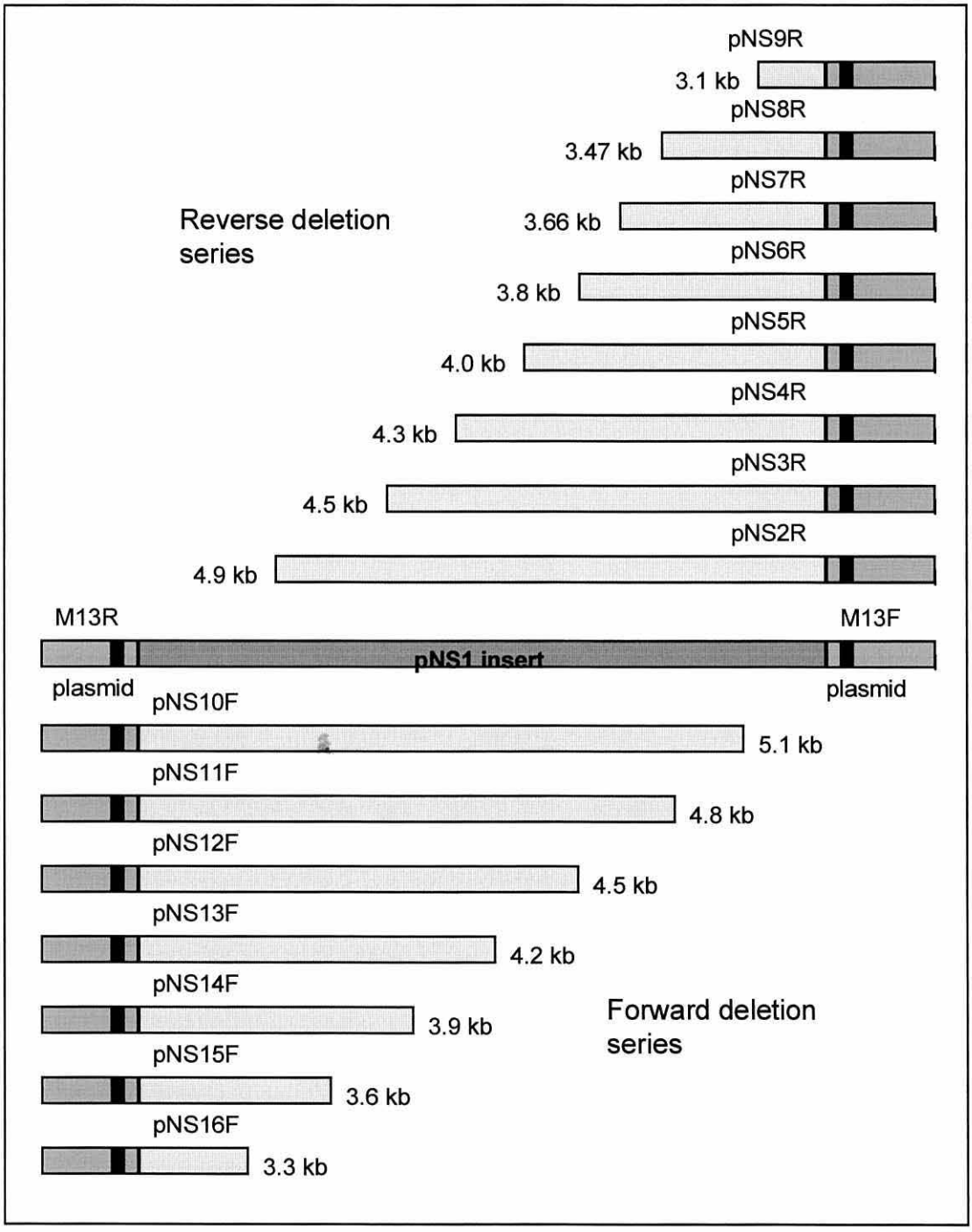


All clones were subjected to *Hind* III restriction before they were loaded onto the gel. 200 ng of each sample (100 ng for clone 1) were loaded onto the gel. 200 ng of *Hind* III-digested pSK^+ were included as a control. 200 ng of *Bam*H I-cut pNS1 was also included.

The lanes contained:

M, λ-*Pst* I DNA marker; **P**, pSK^+; **1**, pNS9R; **2**, pNS8R; **3**, pNS7R; **4**, pNS6R; **5**, pNS5R; **6**, pNS4R; **7**, pNS3R; **8,** pNS2R; **N**, pNS1.

Figure 3.25. The clones produced by the forward and reverse deletion series of pNS1.



The designation of the clone is shown on top of each clone. The size of the clones (in kb) is shown at the end each clone. M13R and M13F show the position of the reverse and forward M13 primer relative to the pNS1 insert.

Table 3.2. Forward deletion clones of pNS1.

Clone name	Estimated size (kb)
pNS10F	5.10
pNS11F	4.80
pNS12F	4.50
pNS13F	4.20
pNS14F	3.90
pNS15F	3.60
pNS16F	3.30

Table 3.3. Reverse deletion clones of pNS1.

Clone name	Estimated size (kb)
pNS2R	4.90
pNS3R	4.50
pNS4R	4.30
pNS5R	4.00
pNS6R	3.80
pNS7R	3.66
pNS8R	3.47
pNS9R	3.10

3.12 Sequencing of the pNS1 insert

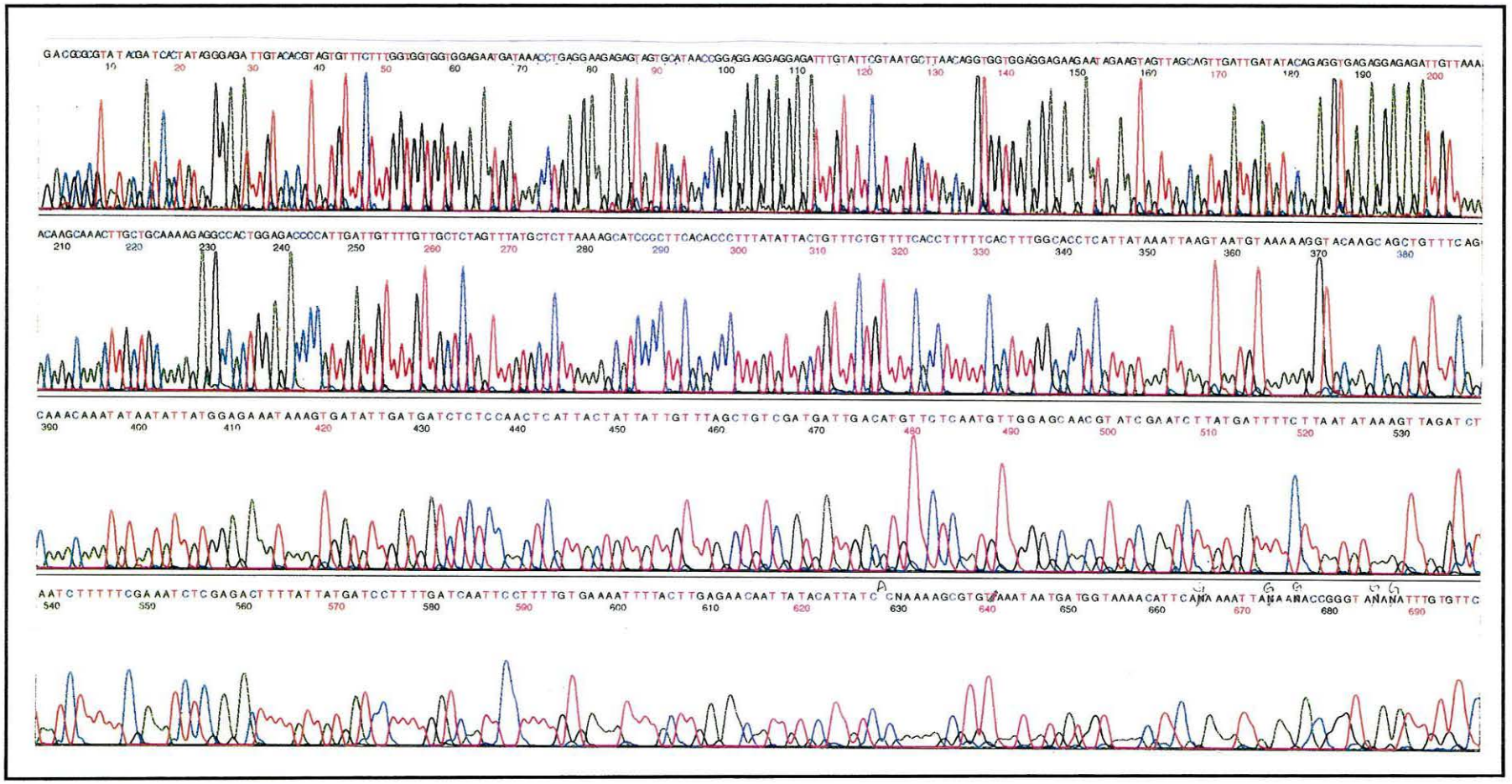
The clones pNS10F, pNS11F, pNS12F, pNS13F, pNS14F, pNS15F and pNS16F were sequenced using automated sequencing technology (ABI automated sequencer). The primer used was the M13 universal forward primer. An example of the readout from the automated sequencing run is shown in Figure 3.26. For each clone, approximately 500 nucleotides of

readable sequence were obtained. Two types of anomaly were normally observed when reading the sequences derived from automated sequencing. The first was that the nucleotide was registered to a position but the identity of the nucleotide could not be confirmed. This happened when more than one nucleotide of the same type occupied a position where the peak for that type of nucleotide was broad. The second anomaly was that the nucleotides within the sequences could not be identified because the peak of that nucleotides was not so obvious (very small) and overlapped with its neighbouring nucleotide peaks (the `N' nucleotide will be registered at this position). In both cases, the anomalies were examined by manual sequencing of the appropriate clones once more (or more than once if necessary).

Manual sequencing was also performed for all the reverse deleted clones - pNS2R, pNS3R, pNS4R, pNS5R, pNS6R, pNS7R, pNS8R and pNS9R - using the M13 reverse primer. Approximately 400 to 500 nucleotides were read from each clone by manually sequencing these clones. The sequences were then aligned by using the Gene Jockey program to give a total sequence of pNS1 insert of 2459 bp (Figure 3.27). The sequence contained an open reading frame of 780 nucleotides that encodes a polypeptide of 259 amino acids with a molecular weight of 29704 Daltons. Amino acid sequences of the polypeptide are shown in Figure 3.28.

The polypeptide contained numerous Ser-Pro-Pro-Pro-Pro repeat motifs which are characteristic of extensin. The gene contained within the pNS1 insert was therefore designated *extC* (two genes coding for extensins were previously isolated from an oilseed rape plant designated *extA* [Evans *et al.*, 1990] and *extB* [Gatehouse *et al.*, 1990]). There is no dominant amino acid repeat motif found in the polypeptide sequence except that the repeat motifs Ser-Pro-Pro-Pro-Pro-Val-Met-His-Tys-Ser-Leu-Pro-Gln-Val-Tyr-His, Ser-Pro-Pro-Pro-Pro-Val-Lys-Gln-Tyr-Ser and Ser-Pro-Pro-Pro-Pro-Val-Lys-Gln-Tyr-Ser are repeated twice (Table 3.4). All the remaining amino acid sequence motifs are only repeated once. A putative `TATA' box and `CAAT' box are located at 58 and 180 respectively upstream from the translation start

Figure 3.26. A read out sequence of pNS12F clone obtained using the ABI automated sequencer.



```
TGGATTAGTTACTAGATTAAAATTCACACCTTGTGCGAGACAAATATTCGGTATACAAAC   -1387
ACTTGGGTTGTATGTTAGTATTTAACTATTTATAGTTAATTTTTTTCATTTTTTTCAATT   -1327
ATTTAATATATATTTATTATTCTGTTAGTTTATAATATGTAAAAACATAAAATAAGTAAT   -1267
AGTACGTAAAACTAATTTTATATATAATGATCATTCCACAAAAGACGCGGATTTTAAACT   -1207
AGTTTATTATATTATAGATGATTATTGAATAAAGAAACATTTGGTAATTTAAAAAAAAAG   -1147
ATAAATTAGTGGACATTTGGTCAACCATTTGAATTAAGACGCTGTTTCTTAACAGTTATT   -1087
TCATACTCAGAATAATTTTATTTTTCAACTTTTCTTAGGAAAAAAAAGCACAAGGTGCCA   -1027
ACAAGAACTGTATCCGAAAAAAAAACCCAACTCTCAAAAGCATTAAAAAATCAACCGTTC   -967
TCATTTGAAAGGTATGGATGGATTCCCATCAAATTTGTTCTTACATTTACGGATGTACGA   -907
ATAGAGTTGGAACATACACTGTTCCAACCGCATATGTTTGCGTGGGCTTCCAACTTTGCT   -847
GACATGAATGGTTACCAATAGACCAATCCAATAATACCACAGAAACCTCCTGATATTCCT   -787
CAGATTCATTAGATCTTCTGAAGATCTGAAGTTAAAAATACCATCTTCAACTAAAAAGGC   -727
GTAGCTGTAAGGGTTGAAACTGAAACTATGAACAGAAGTATGGTTGGAATAGCTATAGAA   -667
AATATCAGAAAAGATGCAAATGTACTTTAACAAAAAAAAGATGTAAAAGTAGAAAGGAGG   -607
CGACAATTTAATAACTTTGTTAAGTAAAAACTTTCTCAAAAAAAAAAAACTCTGTTAAGT   -547
AAAATACATGATCGATAGATTTGAAATAAGTGGTCTCGTTGGTTTTGGCTACTAATATTG   -487
GATCAAAGATCTACAAACGTGAAACATCTTGTATAGGAACACAAATCTCTACCCGGTCTT   -427
CTAATTTTCTGAATGTTTTACCATCATTATTTACACGCTTTTTGTGATAATGTATAATTG   -367
TTCTCAAGTAAAATTTTCACAAAAGGAATTGATCAAAAGGATCATAATAAAAGTCTCGAG   -307
ATTTCGAAAAAGATTAGATCTAACTTTATATTAAGAAAATCATAAGATTCGATACGTTGC   -247
TCCAACATTGAGAACATGTCAATCATCGACAGCTAAACAATAATAGTAATGAGTTGGAGA   -187
GATCATCAATATCACTTTATTTCTCCATAATATTATATTTGTTTGCTGAAACAGCTGCTT   -127
GTACCTTTTTACATTACTTAATTTATAATGAGGTGCCAAAGTGAAAAAGGTGAAAACAGA   -67
AACAGTAATATAAAGGGTGTGAAGGGGATGCTTTTAAGAGCATAAACTAGAGCAACAAAA   -7
CAATCAATGGGGTCTCCAGTGGCCTCTTTTGCAGCAAGTTTGCTTGTTTTAACAATCTCT   54
CCTCTCACCTCTGTATATCAATCAACTGCTAACTACTTCTATTCTTCTCCTCCACCACCT   114
GTTAAGCATTACGAATACAAATCTCCTCCTCCTCCGGTTATGCACTACTCTCTTCCTCAG   174
GTTTATCATTCTCCACCACCACCAAAGAAACATACGGTGAACAAATCTCCTCCTCCTCCG   234
GTTAAACAATATTCTCCTCCTTGGTTACGGTCTCCACCACCTCCCAGGAAAGACTACGTG   294
TACAAATCTCCACCCCCACCGGTTAAGCAATATTCTTCACCACCACCCAATAAATATTAC   354
GTATACCAATCTCCTCCTCCTCCGGTTAAGCACTACTCACCCCCTTCAGTTTACCATTCT   414
CCACCCCCACCTAAAAATCAGTACGTCTACAAATCTCCACCACCACCAGTTAAACACTAT   474
ACTCCCCCGGTTTACCACTCTGGACCACCACCAAAGAAACACTACATGTACAAATCTCCT   534
CCTCCTCCGGTTATGCACTACTCTCTTCCTCAGGTTTACCACTCTCCACCACCACCAAAG   594
AAGCATTACGTATACAAATCCCCTCCTCCTCCGGTTAAGCACTATTCTCCTCGTCTAGTT   654
TACCATTCCCCACCACCACCAAAGAAAAAGTACGTATATAAATCACCTCCTCCTCCAGTC   714
CGCCACTACTTTCCCCCACACCATCTTTACCTCTACAAATCTCCTCCTCCTCCATACCAC   774
TATTAGAATTCTTTGTCACGGTAATAACTCTCCCTATACTTACATATCTACACACCTCAT   834
TAAGTTAACAAGTAAAAACCCTACATGGATGCATGCATGAGAATATTTATATGTTTATAT   894
CGTATATTGTATATATACTTTGCAGGAGAACACGATGTGCGCAAGAAGACAATCAACAT   954
GGAATCCATAATAAGAGAAACATAGAGTGACAAGAGAATCCTTATCCAAACATAAAGCT   1113
```

Figure 3.27. The sequence of the pNS1 insert. Nucleotides are numbered from the A of the start codon for translation. An open reading frame is shown in red. A putative `TATA' box is shown in green. A putative `CAAT' box is shown in purple. Putative poly-A+ signals are shown in blue.

Figure 3.28. The deduced amino acid sequences of the *extC* extensin polypeptide. The SPPPP motifs are shown in red. The potential intramolecular isodityrosine (IDT) cross-links-forming sequence motifs are shown in blue.

```
MGSPVASFAASLLVLTISPLTSVYQSTANYFYSSPPPPVKHYEYKSPPPP    50
VMHYSLPQVYHSPPPPKKHTVNKSPPPPVKQYSPPWLRSPPPPRKDYVYK    100
SPPPPVKQYSSPPPNKYYVYQSPPPPVKHYSPPSVYHSPPPPKNQYVYKS    150
PPPPVKHYTPPVYHSGPPPKKHYMYKSPPPPVMHYSLPQVYHSPPPPKKH    200
YVYKSPPPPVKHYSPRLVYHSPPPPKKKYVYKSPPPPVRHYFPPHHLYLY    250
KSPPPPYHY                                              259
```

Table 3.4. Consensus amino acid repeat sequences of the polypeptide encoded by *extC*.

Group repeat	Number of repeats
SPPPPVKHYEYK	1
SPPPPVMHYSLPQVYH	2
SPPPPKKHTVNK	1
SPPPPVKQYS	2
SPPPPRKDYVYK	1
SPPPNKYYVYQ	1
SPPPPVKHY	2
SPPPPKNQYVYK	1
SPPPPVKHYTPPVYH	1
SGPPPKKHYMYK	1
SPPPPKKHYVYK	1
SPPPPKKKYVYK	1
SPPPPVRHYFPPHHLYLYK	1
SPPPPYHY	1

codon. Hydropathy plots (Kyte and Doolittle, 1982) of the amino acid sequence showed that the major part of the polypeptide is hydrophilic except for a region on the N-terminal of the polypeptide (Figure 3.29).

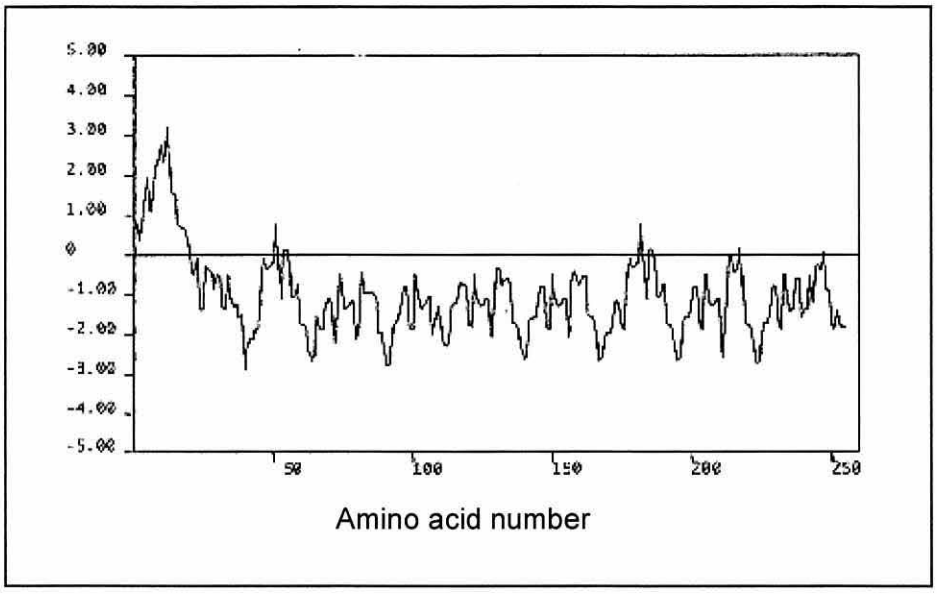


Figure 3.29. Hydropathy plot (Kyte and Doolittle, 1982) of *extC* extensin. Values above the horizontal line indicate the hydrophobic regions, and values below the horizontal line indicate the hydrophilic regions.

3.13 Studies on the expression of the *extC* gene and accumulation of transcripts corresponding to *extC* under different treatments

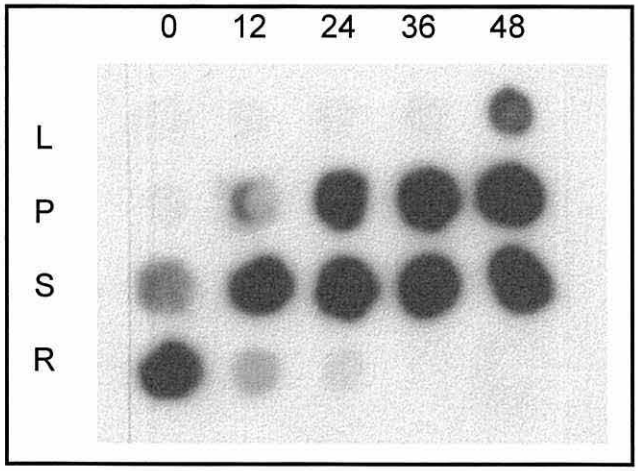
The effect of wounding (either by punching out leaf discs using a cork borer or by the application of dialysis clamps, and by slicing of petioles, stems and roots using a scalpel blade), abscisic acid (ABA) treatment, sodium salicylate treatment and methyl jasmonate (MeJ) treatment on the expression of the *extC* gene and accumulation of transcripts corresponding to *extC* in oilseed rape plant tissues were investigated by dot blot and Northern blot hybridisation analyses. Total RNA was extracted from tissues that had been wounded or incubated in ABA solution, sodium salicylate solution and MeJ solution through cut petioles for different times. Northern blot hybridisation analyses were performed against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 covering the coding sequence of the gene, and also against the radiolabelled *extA* coding sequence probe for comparison.

a) Effect of wounding

Wounding of leaves by punching out leaf discs using a cork borer or slicing of petioles, stems and roots with a scalpel blade did not induce the expression of the *extC* gene. No signal was detected after the dot blot of total RNA samples from wounded tissue was probed with the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 and washed to a high stringency, 0.1x SSC/0.1% SDS at 65^{0}C (results not presented). No signal was detected in untreated leaves, petioles, stems and roots. However, when the blot was washed to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C), strong signals were seen in unwounded root, in 24 h, 36 h and 48 h wounded petioles, and in all wounded stems (Figure 3.30). Significant levels of signals were also seen in 48 h wounded leaf, 12 h wounded petiole and in unwounded stem. A low level of signal was also seen in the 12 h wounded root. In contrast, significant level of *extA* signal was seen 24 h after leaves were wounded (Figure 3.31). The signal reached a maximum level at 36 h and decreased at 48 h. In wounded petioles, a significant level of signal was detected 12 h after the tissues were wounded. The signal increased sharply at 24 h and continued to increase until 48 h. In roots, wounding of tissues did not induce expression of the *extA* gene. However, strong signals were seen in unwounded root (0 h). No signal was detected in unwounded leaves and petioles.

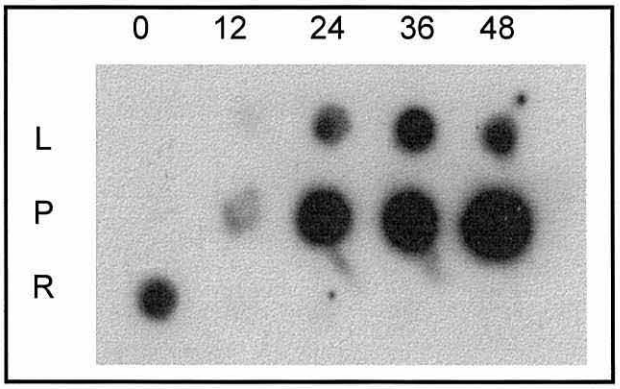
Wounding of leaves by the application of dialysis clamps and subsequent incubation in sterile distilled water for 12 h, 24 h, 36 h and 48 h also failed to induce accumulation of transcripts corresponding to *extC*. No signals were detected after total RNA samples from the heavily wounded leaves were probed with the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 and the blot was washed to a high stringency, 0.1x SSC/0.1% SDS at 65^{0}C (results not presented). When the blot was washed to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C), two transcripts of sizes 1.26 kb and 1.45 kb were, however, detected in all the heavily wounded leaves (Figure 3.32). The 1.26 kb transcript was present at almost the same level in 12 h, 24 h, 36 h and 48 h wounded leaves. The level of the 1.45 kb transcript increased to a maximum at 24 h, decreased at 36 h and remained constant at

Figure 3.30. Dot blot of total RNA extracted from wounded tissues hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 and washed to a medium stringency.



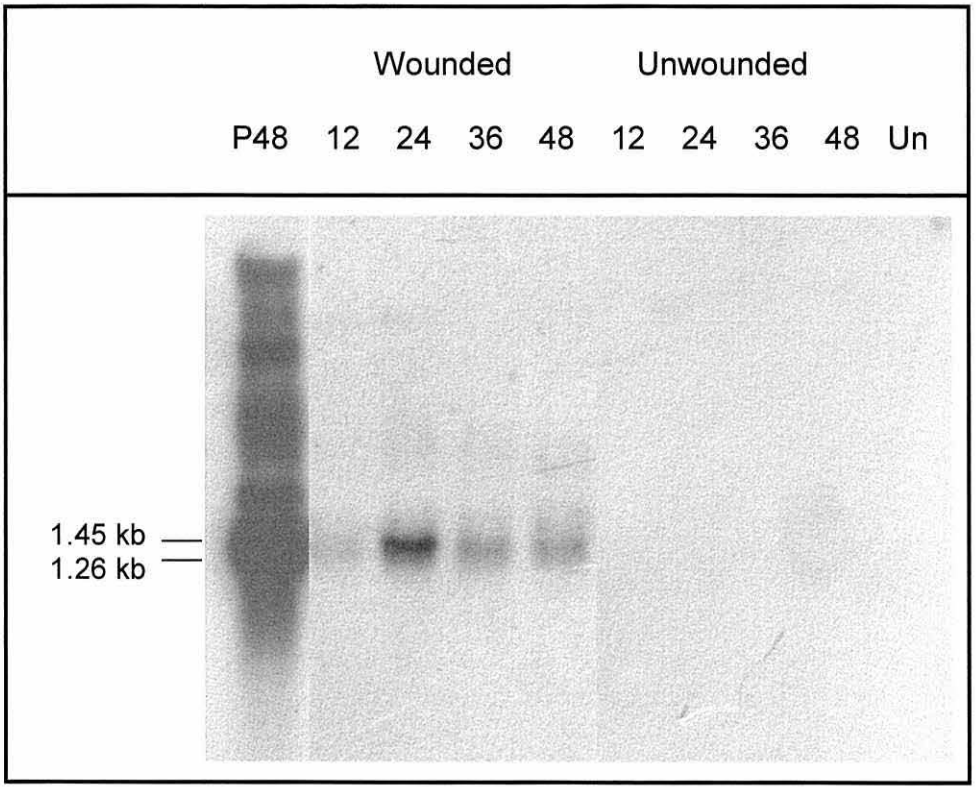
Total RNA was extracted from wounded leaf, petiole, stem and root at 0 h, 12 h, 24 h, 36 h and 48 h (Section 2.13.1) after application of the wounding stimulus, and hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 in a dot blot. 0 h root RNA sample served as a positive control for the hybridisation. A standard procedure for dot blot hybridisation (Section 2.11.5) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^{0}C. **L**, leaf; **P**, petiole; **S**, stem; **R**, root. 0 refers to RNA extracted from unwounded tissues (0 h).

Figure 3.31. Dot blot of total RNA extracted from wounded tissues hybridised against the radiolabelled *extA* coding sequence.



Total RNA was extracted from wounded leaf, petiole and root at 0 h, 12 h, 24 h, 36 h and 48 h (Section 2.13.1) after application of the wounding stimulus, and hybridised against the radiolabelled *extA* coding sequence probe in a dot blot. 0 h root RNA sample served as a positive control for the hybridisation. A standard procedure for dot blot hybridisation (Section 2.11.5) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 65^0C. **L**, leaf; **P**, petiole; **R**, root. 0 refers to RNA extracted from unwounded tissues (0 h).

Figure 3.32. Northern blot of total RNA extracted from wounded leaves hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1.



Total RNA was extracted from leaves that had been wounded by the application of dialysis clamps and subsequently incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h (Section 2.13.1). Total RNA was also extracted from untreated leaf and from unwounded leaves that had been incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h and used as controls for the treatment. Total RNA from 48 h wounded petioles (P48, see Figure 3.30) was also included as a positive control for the hybridisation. Northern hybridisation was performed using the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 according to standard procedures (Section 2.11.6). The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C. **Un**, untreated leaf.

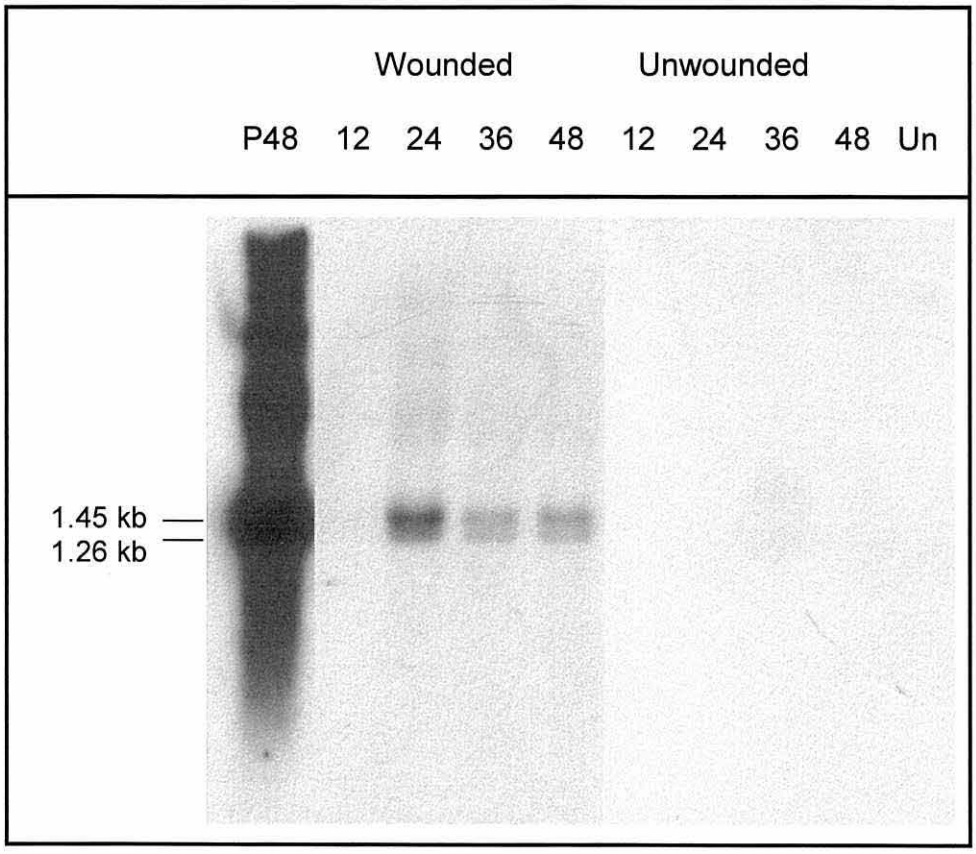
48 h. No transcripts were detected in unwounded leaves and in untreated leaf (Un).

Probing of the same total RNA samples with the radiolabelled *extA* coding sequence probe however, resulted in high levels of accumulation of the 1.26 kb and 1.45 kb transcripts in 24 h wounded leaf (Figure 3.33). The level of transcripts decreased at 36 h and increased again at 48 h. In all cases, the 1.45 kb transcript accumulated to a higher level compared to the 1.26 kb transcript. In control experiments where leaves were incubated in sterile distilled water through their cut petioles and also in untreated leaves, no transcripts were detected (Figure 3.33). However, after the blot was exposed for a long period (2 weeks), a low level of transcript was detected in both the control experiments (results not presented). A graphical representation of the northern blot hybridisation (Figure 3.44a) showed that at 24 h the level of the 1.45 kb transcript was doubled compared to the 1.26 kb transcript. There was not much difference in the level of the two transcripts at 36 h. At 48 h, however, the level of the 1.45 kb transcript was doubled again compared to the 1.26 kb transcript even though the levels of the two transcripts were lower compared to the level at 24 h. Washing the blot to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C) however, resulted in the accumulation of significant levels of the two transcripts in 12 h wounded leaf. The level of the transcripts increased to a maximum at 24 h, decreased and remained constant between 36 h and 48 h (Figure 3.34). In 24 h, 36 h and 48 h wounded leaves, the level of the 1.45 kb transcript was higher compared to the 1.26 kb transcript. In 12 h wounded leaf however, the two transcripts accumulated to almost the same level. Significant levels of the transcripts were also detected in all unwounded leaves and in untreated leaf.

b) Effect of ABA treatment

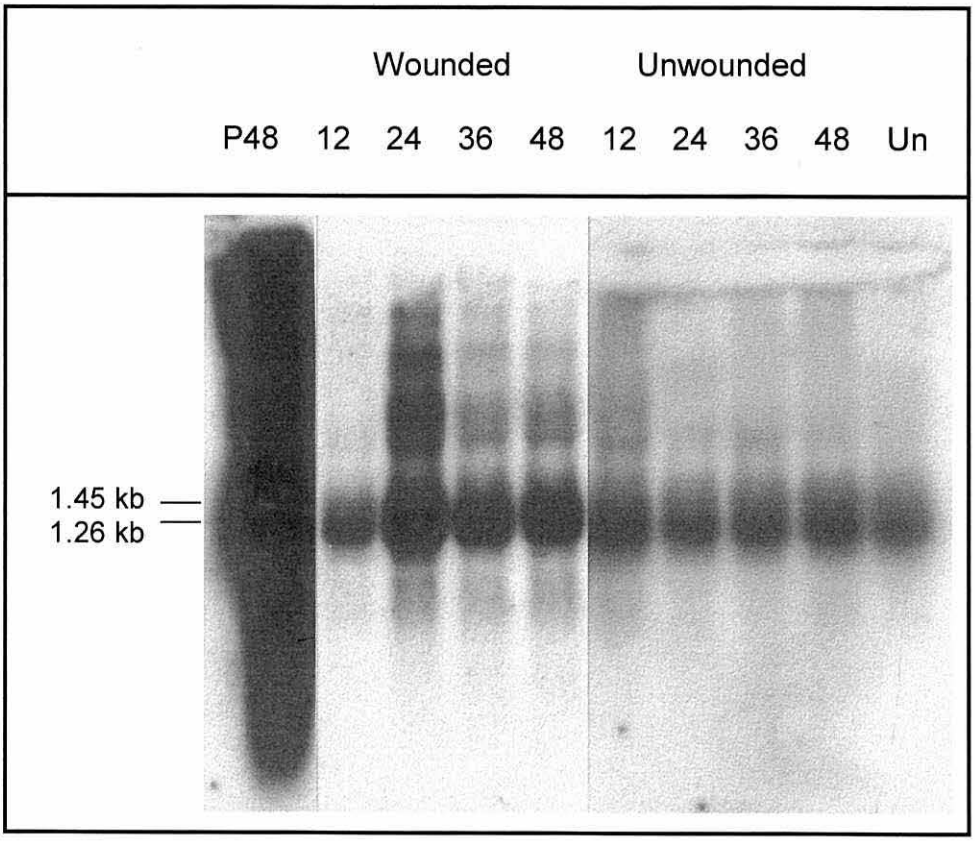
Treatment of detached leaves with ABA was performed by immersing their cut petioles in a 100 μM solution for 12 h, 24 h, 36 h and 48 h. This did not induce the accumulation of transcripts corresponding to *extC*. No signals were detected after the blot was probed with the 1.32 kb *extA*-hybridising

Figure 3.33. Northern blot of total RNA extracted from wounded leaves hybridised against the radiolabelled *extA* coding sequence.



Total RNA was extracted from leaves that had been wounded by the application of dialysis clamps and subsequently incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h (Section 2.13.1). Total RNA was also extracted from untreated leaf and from unwounded leaves that had been incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h and used as controls for the treatment. Total RNA from 48 h wounded petioles (P48, see Figure 3.31) was also included as a positive control for the hybridisation. Northern hybridisation was performed using the radiolabelled *extA* coding sequence probe according to standard procedures (Section 2.11.6). The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 65^{0}C. **Un**, untreated leaf.

Figure 3.34. Northern blot of total RNA extracted from wounded leaves hybridised against the radiolabelled *extA* coding sequence and washed to a medium stringency.

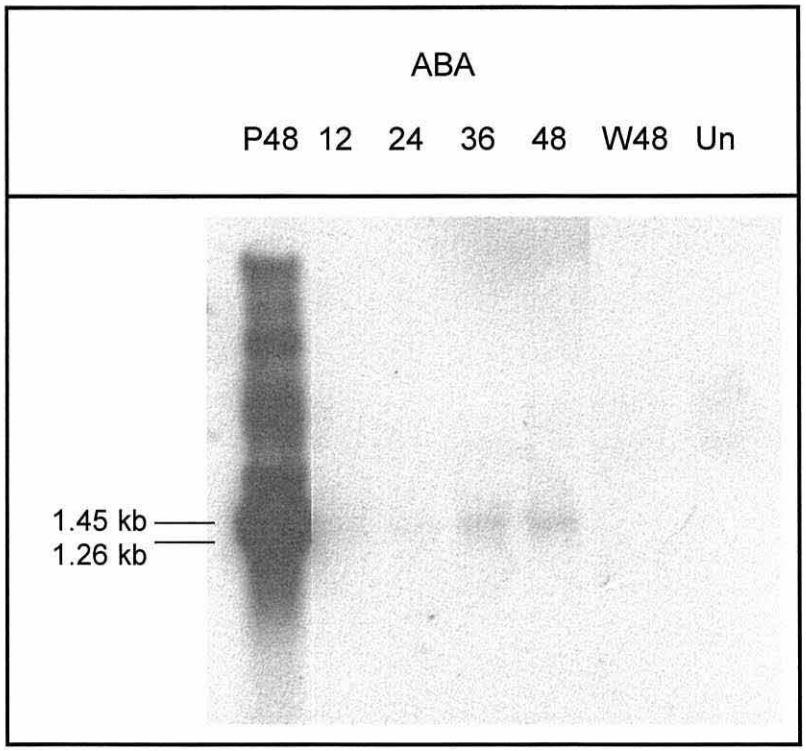


Total RNA was extracted from leaves that had been wounded by the application of dialysis clamps and subsequently incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h (Section 2.13.1). Total RNA was also extracted from untreated leaf and from unwounded leaves that had been incubated in sterile distilled water for 12 h, 24 h, 36 h and 48 h and used as controls for the treatment. Total RNA from 48 h wounded petioles (P48, see Figure 3.31) was also included as a positive control for the hybridisation. Northern hybridisation was performed using the radiolabelled *extA* coding sequence probe according to standard procedures (Section 2.11.6). The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C. **Un**, untreated leaf.

fragment of pNS1 and washed to a high stringency, 0.1x SSC/0.1% SDS at 65^{0}C (results not presented). However, when the blot was washed to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C), significant levels of the 1.26 kb and 1.45 kb transcripts were seen in 36 h and 48 h ABA-treated leaves (Figure 3.35). The level of the transcripts in both samples was almost the same. However, the 1.45 kb transcript accumulated to a high level compared to the 1.26 kb transcript in both samples. In 12 h and 24 h ABA-treated leaves, almost no signals were detected.

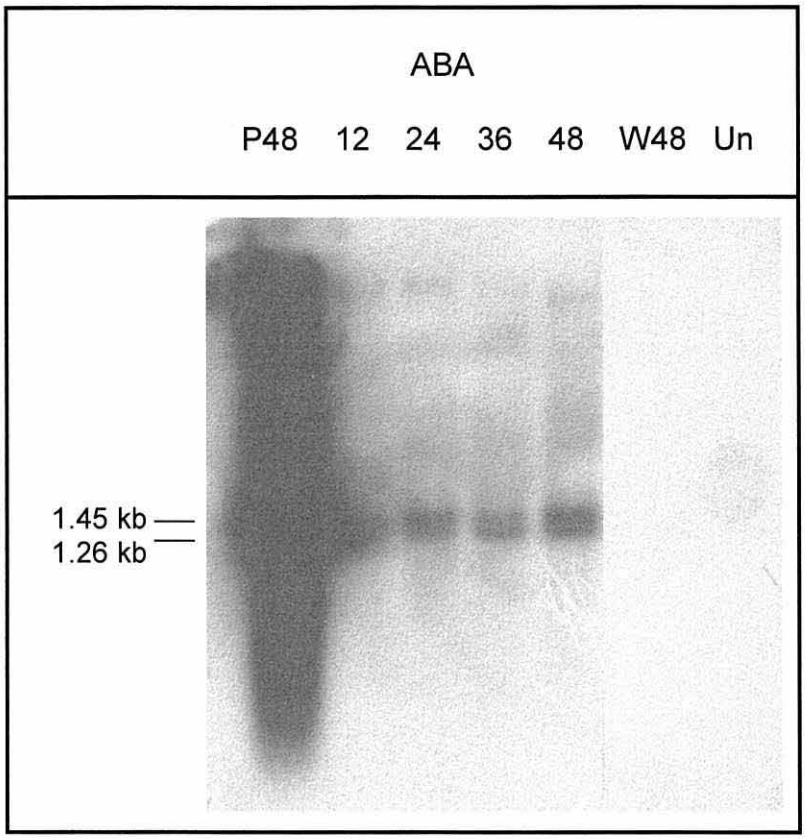
Probing of the same total RNA samples with the coding sequence of the *extA* gene however, resulted in the detection of the 1.26 kb and 1.45 kb transcripts in all the ABA-treated leaves (Figure 3.36). Significant levels of transcripts were seen 12 h after the leaves were treated, increased at 24 h, decreased again at 36 h and increased again at 48 h. In the 24 h and 48 h ABA-treated leaves, the 1.45 kb transcript accumulated to a relatively high level compared to the 1.26 kb transcript, whereas in the 12 h and 36 h ABA-treated leaves, both transcripts accumulated almost to the same level. A graphical representation of the northern blot hybridisation (Figure 3.44b) showed that there was not much difference in the level of hybridisation between the 1.26 kb and 1.45 kb transcripts, even though the level of the 1.45 kb transcript was higher than the 1.26 kb transcript at 24 h and 48 h. Washing the blot to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C) however resulted in the accumulation of the two transcripts in different patterns compared to the accumulation pattern produced when the blot was washed to a high stringency (Figure 3.37). The two transcripts accumulated to almost the same level in 24 h and 36 h ABA-treated leaves whereas the highest level of transcript accumulation was detected in 48 h ABA-treated leaves. In addition, the 1.45 kb transcript accumulated to a higher level compared to the 1.26 kb transcript in 48 h ABA-treated leaf. In 12 h ABA-treated leaves, the levels of the transcripts could not be seen due to the extremely high signal produced by the control (P48) sample.

Figure 3.35. Northern blot of total RNA extracted from abscisic acid (ABA) treated leaves hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1.



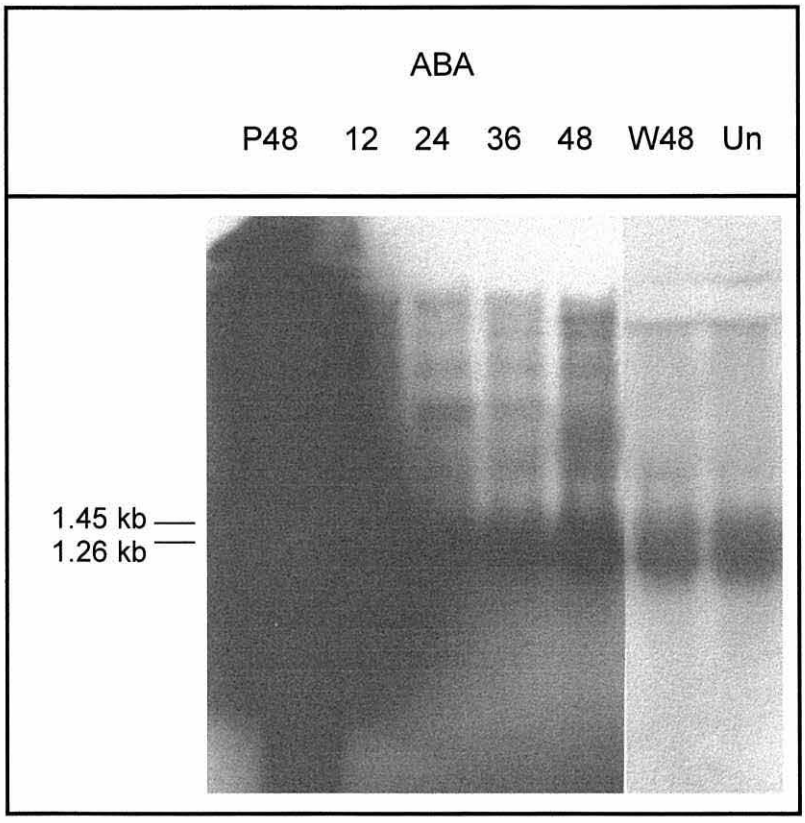
Total RNA was extracted from leaves that had been incubated in a 100 μM abscisic acid solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.32 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.30) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C.

Figure 3.36. Northern blot of total RNA extracted from abscisic acid (ABA) treated leaves hybridised against the radiolabelled *extA* coding sequence.



Total RNA was extracted from leaves that had been incubated in a 100 μM abscisic acid solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 65^{0}C.

Figure 3.37. Northern blot of total RNA extracted from abscisic acid (ABA) treated leaves hybridised against the radiolabelled *extA* coding sequence and washed to a medium stringency.



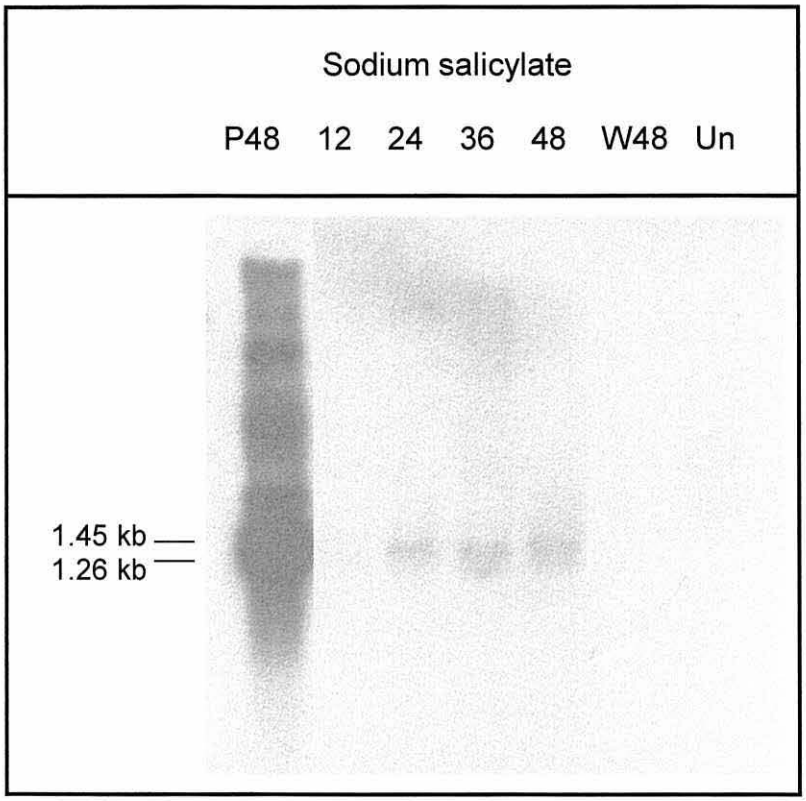
Total RNA was extracted from leaves that had been incubated in a 100 μM abscisic acid solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^{0}C.

c) Effect of sodium salicylate treatment

In response to sodium salicylate, transcripts hybridising to pNS1 did not accumulate after leaves were incubated in a 1 mM solution of sodium salicylate for 12 h, 24 h, 36 h and 48 h through their cut petioles. No transcripts were detected after the blot was probed with the 1.32 kb *extA*-hybridising fragment of pNS1 and washed to a high stringency, 0.1x SSC/0.1% SDS at 65^{0}C (results not presented). However, significant levels of the 1.26 kb and 1.45 kb transcripts were seen in 24 h, 36 h and 48 h sodium salicylate-treated leaves when the blot was washed to a medium stringency, 0.1x SSC/0.1% SDS at 42^{0}C (Figure 3.38). The 1.45 kb transcript accumulated to almost the same level in 24 h, 36 h and 48 h sodium salicylate-treated leaves. The levels of the 1.26 kb transcript were lower than the levels of the 1.45 kb transcript at 36 h and 48 h while there was almost no 1.26 kb transcript detected at 24 h.

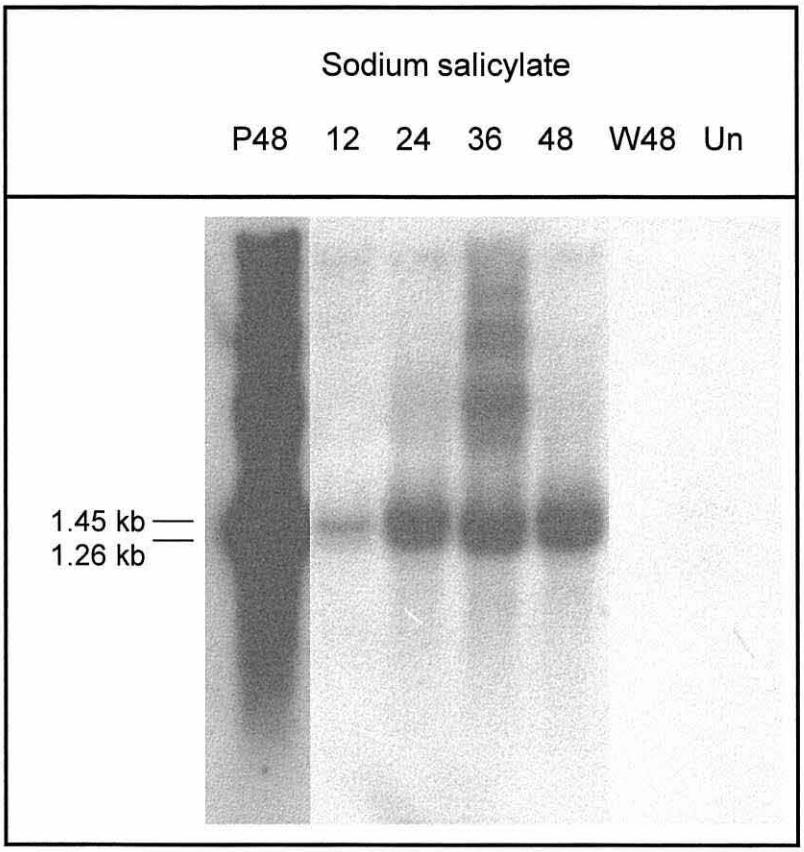
Probing of the same RNA samples with the *extA* coding sequence probe however resulted in significant levels of the 1.26 kb and 1.45 kb transcripts being detected 12 h after the tissues were treated (Figure 3.39). The level of the transcripts increased sharply at 24 h and continued to increase until 48 h (except for the 1.45 kb transcript where it decreased from 36 h to 48 h). In the 12 h, 24 h and 36 h samples the level of the 1.45 kb transcript was relatively high compared to the 1.26 kb transcript. In the 48 h sample however, both transcripts accumulated to the same level. A significant level of higher molecular weight transcripts were also seen 36 h after the leaves were treated (Figure 3.39). A graphical representation of the northern blot hybridisation (Figure 3.44c) showed that the level of the 1.45 kb transcript was almost three times higher than the 1.26 kb transcript and continued to be expressed more than the 1.26 kb transcript at 24 h and 36 h. At 48 h the level of the two transcripts was, however, the same. Washing of the blot to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C), resulted in the same pattern of accumulation of the transcripts as the pattern produced when the blot was washed to a high stringency (Figure 3.40). The signals were, however, more intense in the medium stringency-washed blot compared to the high stringency-washed blot.

Figure 3.38. Northern blot of total RNA extracted from sodium salicylate treated leaves hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1.



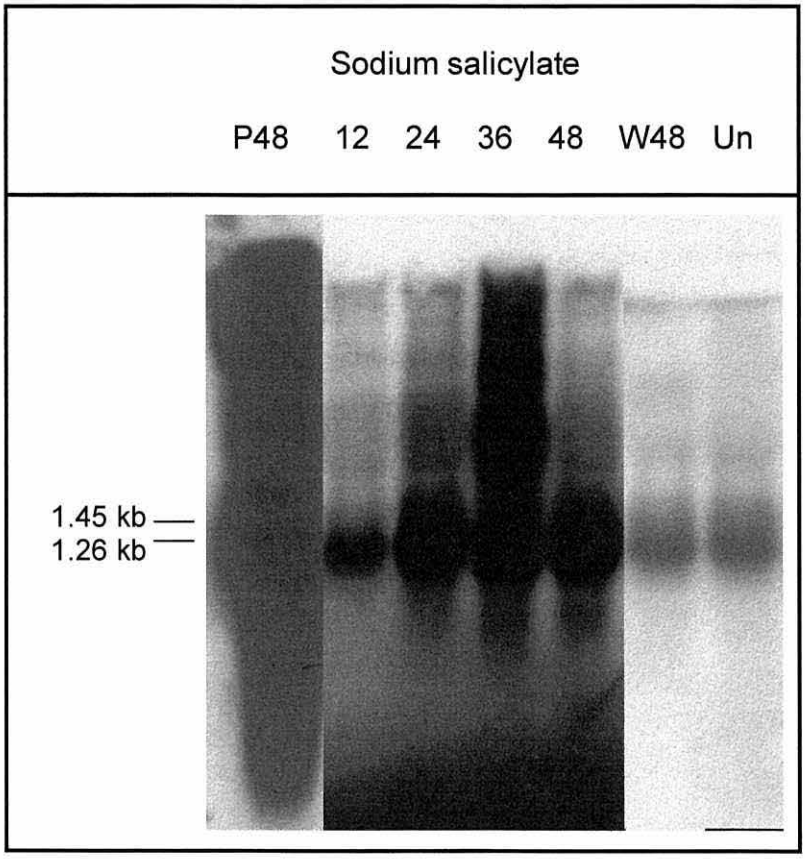
Total RNA was extracted from leaves that had been incubated in a 1 mM sodium salicylate solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.32 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.30) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C.

Figure 3.39. Northern blot of total RNA extracted from sodium salicylate treated leaves hybridised against the radiolabelled *extA* coding sequence.



Total RNA was extracted from leaves that had been incubated in a 1 mM sodium salicylate solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 65^0C.

Figure 3.40. Northern blot of total RNA extracted from sodium salicylate treated leaves hybridised against the radiolabelled *extA* coding sequence and washed to a medium stringency.



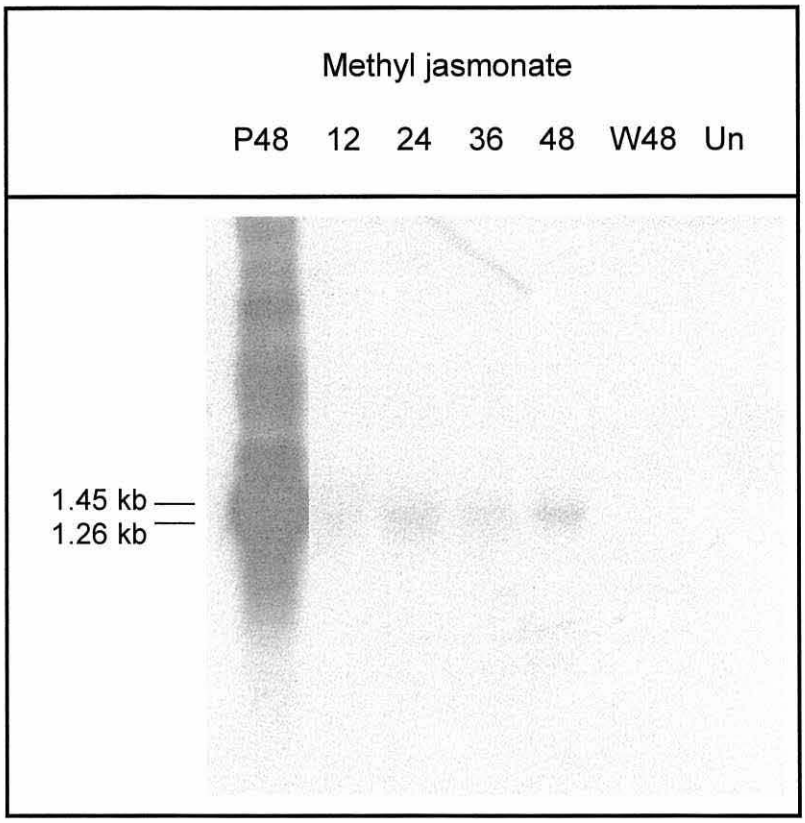
Total RNA was extracted from leaves that had been incubated in a 1 mM sodium salicylate solution through their cut petioles for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C.

d) Effect of methyl jasmonate (MeJ) treatment

Treatment of leaves with MeJ by immersing their cut petioles in a 50 μM solution for 12 h, 24 h, 36 h and 48 h also failed to induce accumulation of transcripts corresponding to *extC*. No transcripts were detected after the total RNA from MeJ-treated leaves was probed with the 1.32 kb *extA*-hybridising fragment of pNS1 and the blot was washed to a high stringency, 0.1x SSC/0.1% SDS at 65^{0}C (results not presented). However, when the blot was washed to a medium stringency, low levels of the 1.45 kb transcript were detected in 12 h MeJ-treated leaf (Figure 3.41). The levels of the transcript increased at 24 h, decreased at 36 h and increased again at 48 h. Almost no 1.26 kb transcript was detected in all MeJ-treated leaves.

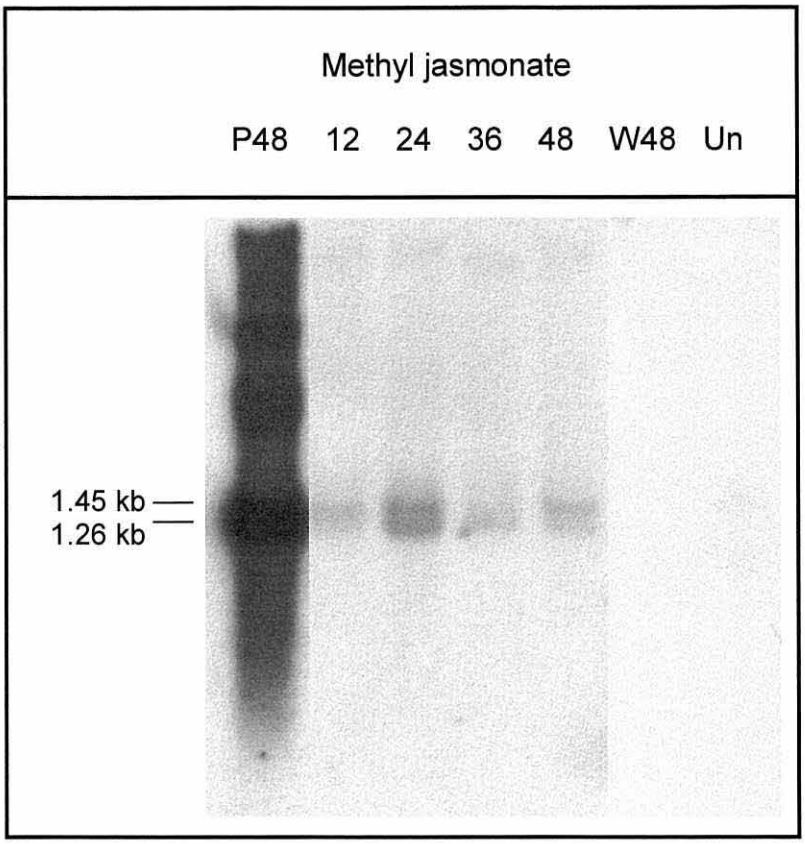
When the same RNA samples were probed with the coding sequence of the *extA* gene, a very low level of the 1.26 kb and 1.45 kb transcripts was detected 12 h after the tissues were treated (Figure 3.42). The level of transcripts increased and reached a maximum level at 24 h, decreased at 36 h before they slightly increased again at 48 h. A graphical representation of the northern blot hybridisation (Figure 3.44d) showed that there was not much difference in the level of these two transcripts during the treatment except at 48 h, where the level of the 1.45 kb transcript was almost doubled compare to the 1.26 kb transcript. At 12 h, the level of the 1.45 kb transcript was just slightly higher than the 1.26 kb transcript. Washing of the blot to a medium stringency (0.1x SSC/0.1% SDS at 42^{0}C) however resulted the accumulation of the two transcripts with the same pattern as that produced by the high stringency-washed blot (Figure 3.43). The signals were, however, more intense in the medium stringency-washed blot compared to the high stringency-washed blot.

Figure 3.41. Northern blot of total RNA extracted from MeJ treated leaves hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1.



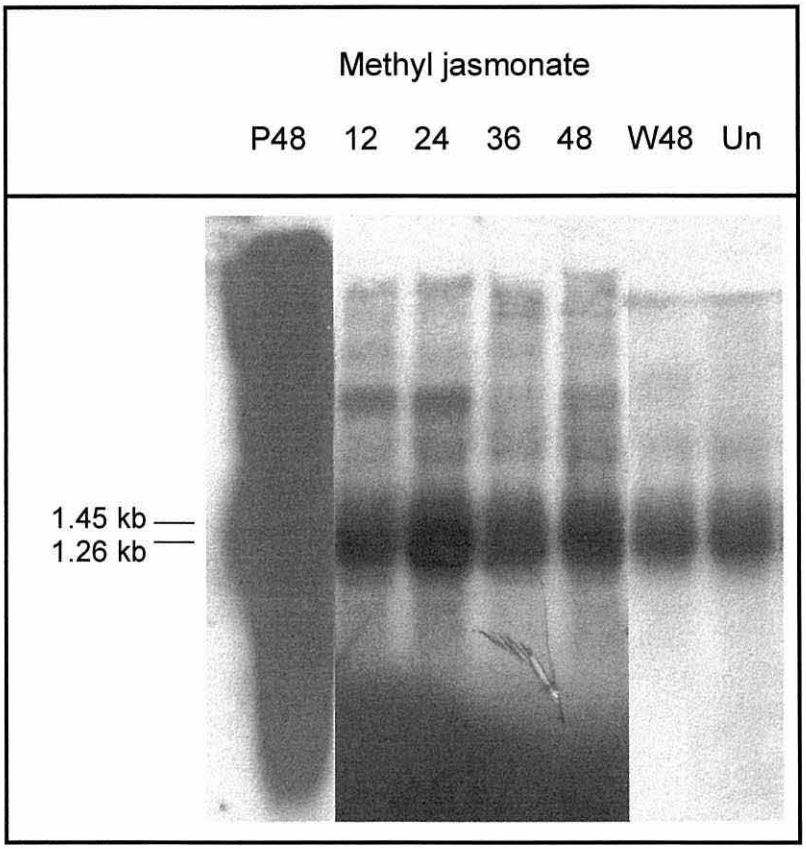
Total RNA was extracted from leaves that had been incubated in a 50 μM MeJ solution for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.32 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.30) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C.

Figure 3.42. Northern blot of total RNA extracted from MeJ treated leaves hybridised against the radiolabelled *extA* coding sequence.



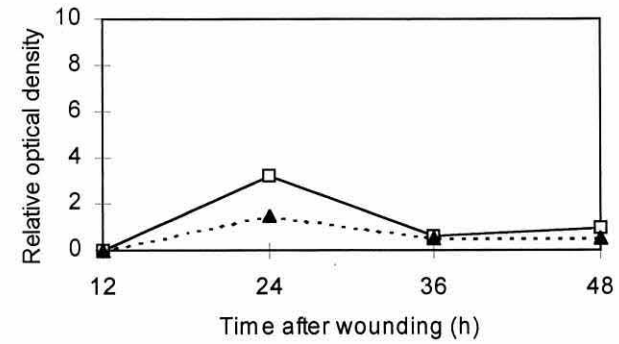
Total RNA was extracted from leaves that had been incubated in a 50 μM MeJ solution for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 65^0C.

Figure 3.43. Northern blot of total RNA extracted from MeJ treated leaves hybridised against the radiolabelled *extA* coding sequence and washed to a medium stringency.

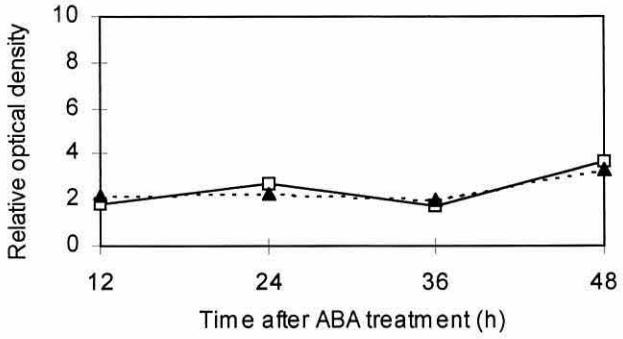


Total RNA was extracted from leaves that had been incubated in a 50 μM MeJ solution for 12 h, 24 h, 36 h and 48 h (Section 2.13.2) and hybridised against the radiolabelled *extA* coding sequence probe in a northern blot. Total RNA from untreated leaf (Un) and from leaves that had been incubated in sterile distilled water for 48 h (W48, see Figure 3.33 legend) were included to serve as controls for the treatment. P48 total RNA (see Figure 3.31) served as a positive control for the hybridisation. A standard procedure for northern blot hybridisation (Section 2.11.6) was followed. The final post-hybridisation washing stringency was 2X 10 minutes in 0.1X SSC/0.1% SDS at 42^0C.

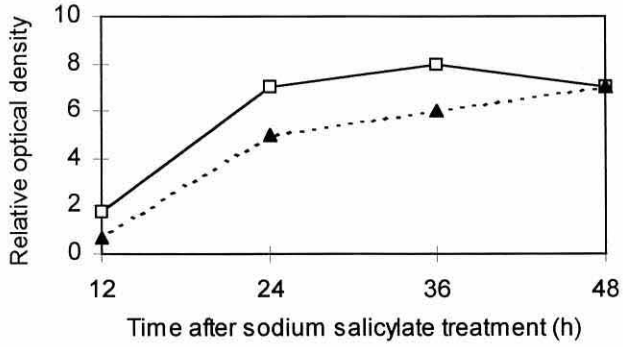
a)



b)



c)



d)

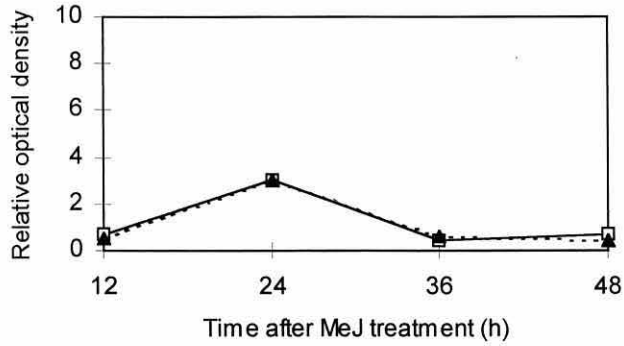


Figure 3.44. Graphical representations of the northern blot hybridisation of RNA from Figures 3.33, 3.36, 3.39 and 3.42. ▲ - 1.26 kb transcript; □ - 1.45 kb transcript.

Chapter 4
Discussion

Chapter 4
Discussion

4.1 Isolation of an oilseed rape genomic clone encoding the cell wall protein extensin

Southern blot hybridisation of the *Hin*d III-digested rape genomic DNA against the radiolabelled *extA* coding sequence probe revealed 5 hybridising bands. The sizes of the fragments were approximately 2.5 kb, 3.0 kb, 4.7 kb, 6.0 kb and 10.0 kb respectively. These results suggest that *extA* is one member of a complex multigene family coding for extensin proteins in the oilseed rape genome. Cell wall extensin genes in other plants have also been shown to belong to multigene families such as those found in soybean (Ahn *et al.*, 1996; Hong *et al.*, 1989; Ye and Varner, 1991), maize (Hood *et al.*, 1988; Stiefel *et al.*, 1990) and tobacco (Memelink *et al.*, 1993; Parmentier *et al.*, 1995; Hirsinger *et al.*, 1997).

Selection of the vector to be used in cloning is very important. It is determined by the size of the foreign DNA to be cloned. Generally, bacteriophage λ vectors are the most efficient vectors when the size of the DNA insert to be cloned is large. Most of the bacteriophage λ vectors can accept foreign DNA up to 15 kb and some can accept up to 24 kb of foreign DNA (Sambrook *et al.*, 1989). Two other types of vectors that have been used in cloning large fragments are cosmids and single-stranded, filamentous bacteriophage vectors (Sambrook *et al.*, 1989). Cosmid vectors and filamentous bacteriophage vectors are, however not widely used because of limitations in reliability, and are only infrequently used to clone DNA inserts larger than 24 kb. In this work, plasmid vectors were chosen because of their reliability and ease of handling. In addition, plasmid vectors are more efficient than any other cloning vector when the DNA to be cloned is less than 10 kb in size (Sambrook *et al.*, 1989). These characteristics of plasmid vectors are appropriate to this work because the size of DNA fragments to be cloned was between 2.5 - 10.0 kb.

The library was screened against the radiolabelled *extA* coding sequence probe. One positive signal was detected after the filters were washed at high stringency at 0.5X SSC/0.1% SDS for 1 hour at 68^{0}C. After the positive clone was purified and recombinant plasmid isolated, *Hin*d III restriction digest of the plasmid revealed an insert of size 2.5 kb. The clone was then designated as pNS1 (plasmid Nik Sidik 1, Figure 3.15).

A restriction endonuclease digestion map of the clone (Figure 3.15) showed that the smallest fragment from the insert that gave the strongest hybridisation to the *extA* coding sequence probe was the 1.32 kb fragment produced by the *Bst*E II/*Xho* I double digest of the clone (lane 3, Figure 3.17). Removal of 0.19 kb from this fragment by double digestion of pNS1 with *Xho* I and *Pvu* II restriction enzymes (lane 4, Figure 3.16) resulted in the remaining 1.14 kb fragment hybridising to the *extA* probe to a lesser degree (lane 4, Figure 3.17). Consequently, when 0.24 kb was removed from the 1.14 kb DNA fragment by double digestion of pNS1 with *Pvu* II and *Eco*R I (lane 6, Figure 3.16), the remaining 0.90 kb of the fragment hybridised to the *extA* probe to a lesser degree compared to the 1.14 kb fragment (lane 6, Figure 3.17). Also, when another 0.34 kb was removed from the 0.90 kb fragment by double digestion of pNS1 with *Pvu* II and *Acc* I (lane 5, Figure 3.16), the remaining 0.57 kb DNA fragment hybridised to the *extA* probe to a lesser extent compared to the 0.90 kb fragment (lane 5, Figure 3.17).

These results suggest that there is no specific region of pNS1 that is highly homologous to the coding sequence of *extA*. Homology of pNS1 to the coding sequence of *extA* resides within a 1.31 kb region between the *Xho* I restriction enzyme site on the pNS1 insert and the *Hin*d III cloning site on the pSK$^+$ vector (Figure 3.15). The complete sequence of pNS1 is however needed before this can be proved. However, one must remember that because pSK$^+$ itself contains another *Xho* I restriction enzyme site 15 nucleotides to the right of the *Hin*d III cloning site (Figure 3.15), the 1.32 kb fragment produced by the *Xho* I/*Pvu* II double digestion of the pNS1 contains 15 nucleotides of the pSK$^+$ vector plasmid. The presence of 15 nucleotides of the pSK$^+$ sequence within the 1.32 kb of pNS1 DNA fragment did, however, not contribute to the strong signals produced by the 1.32 kb fragment as a

result of hybridisation of the fragment to the radiolabelled *extA* coding sequence probe. This is because a test experiment showed that no hybridisation was detected after the *Hind* III-digested rape genomic DNA was probed with the radiolabelled pSK^+ at a washing stringency of 0.5X SSC/0.1% SDS for 1 hour at 68^0C (results not presented).

In order to discover whether the pNS1 insert hybridised to the same genomic DNA fragment as shown by the coding sequence probe of the *extA* gene, oilseed rape genomic DNA was digested with *Hind* III and hybridised against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1. The autoradiograph produced (Figure 3.19) showed that out of six *Hind* III fragments which hybridised to the probe, only the 2.5 kb fragment was of the same size as the one produced by using the *extA* coding sequence probe (Figure 3.1). These results suggest that pNS1 represents a gene member of a complex multigene family which is different from the *extA* multigene family. A complex pNS1 multigene family may represent another type of extensin gene family in the oilseed rape genome different from the *extA* gene family. The fact that pNS1 was isolated from the screening of the library with the *extA* probe and that the 2.5 kb *Hind* III fragment was detected in the Southern blot hybridisation of the *Hind* III-digested oilseed rape genomic DNA with the *extA* probe suggest that it shares homology with the probe. A complete sequence of pNS1 is however necessary for this possibility to be investigated.

4.2 Sequence analysis of *extC*

Sequencing of *extC* showed that the gene contained an open reading frame encoding a polypeptide. The characteristics of the polypeptide - rich in proline residues (30.5%) and the presence of the Ser-Pro-Pro-Pro-Pro repeat motifs - confirms that the open reading frame represents a gene encoding the cell wall protein extensin. Arrangement of the proline residues within the protein sequence is important in determining the degree of post-translational hydroxylation of proline residues to hydroxyprolines. According to Kielisziewski *et al.* (1990), proline residues within the repeat motifs Ser-Pro-Pro-Pro-Pro are always hydroxylated. The proline residues within the dipeptide sequences Lys-Pro, Tyr-Pro and Phe-Pro are never hydroxylated.

However, the proline residue within the Pro-Val dipeptide sequence is invariably hydroxylated. Therefore, out of 79 proline residues within the protein sequence of *extC*, 78 (98.7%) are hydroxylated (one proline residue is located within the Phe-Pro dipeptide sequence).

By comparing the consensus amino acid sequence repeats between the *extC* extensin and the extensin encoded by the *extA* gene, only the Ser-Pro-Pro-Pro-Pro-Val-Lys-His-Tyr motif out of 17 *extC* extensin amino acid sequence motifs was similar to the *extA* extensin amino acid sequence motifs. Three more amino acid sequence motifs in the *extC* extensin are however, also comparable to the *extA* extensin amino acid sequence motif (Ser-Pro-Pro-Pro-Pro-Lys-Lys-His-Tyr-Glu-Tyr-Lys) but with one or two changes in the amino acid residues. Those three amino acid sequence motifs of the *extC* extensin are the Ser-Pro-Pro-Pro-Pro-Lys-Lys-His-Tyr-Val-Tyr-Lys, the Ser-Pro-Pro-Pro-Pro-Val-Lys-His-Tyr-Glu-Tyr-Lys and the Ser-Pro-Pro-Pro-Pro-Lys-Lys-Lys-Tyr-Val-Tyr-Lys. The rest of the *extC* extensin amino acid sequence motifs are completely different (except the Ser-Pro-Pro-Pro-Pro motifs) from the *extA* extensin amino acid sequence motifs. The difference in the amino acid sequence motifs between the two extensin proteins suggest that the *extC* extensin is different from the *extA* extensin. The *extC* gene therefore represents a gene member of another extensin multigene family group, which is different from the *extA* multigene family group in *B. napus*. This supports the suggestion made earlier (page 5 - Section 4.1), based on the result of Southern hybridisation of the *Hind* III-digested oilseed rape genomic DNA against the pNS1 probe which showed that different sizes and patterns of band appeared (Figure 3.19) compared to the result when the coding sequence of *extA* was used as a probe (Figure 3.1). Comparison of the nucleotide sequences and protein sequences between *extC* and *extA* showed that the *extC* nucleotide sequences were 79.6% similar to the *extA* nucleotide sequences (Figure 4.1), while the *extC* protein sequences were 76.1% similar to the *extA* protein sequences (Figure 4.2). These results suggest that even though the *extC* multigene family is different from the *extA* multigene family, they are closely related.

```
extA  ATGGCCTCTTTGGCAGCAACTTTGCTTGTCTTAGCACTTTCT-CTTG   46
extC  ****GG***CCA*TG**---**C*-T***C---****AG**-*G****  40

extA  GTTTTGTATCTGAAACCACCGCAAATTACTACTACTCTTCTCC-TCC   92
extC  T***---------***-------***--**-**C****-*A**-**T  66

extA  -TCCACCGGTCAAACACTACACTCCTCCGGTGTACAAGTCTCCTCCA  138
extC  G*AT*T*AA****CTG***-***A**T*--*A*T*T--*********  107

extA  CCACCGGTAAAGCACTACTCTCCTCCGGTTTACAAATCCCCACCTCC  185
extC  *****T**T*****T***--------------------------    125

extA  ACCAAAGAAGGACTACGAGTACAAATCACCTCCACCACCGGTCAAGC  232
extC  ----------------**A*******T*****T**T*****T*T**  156

extA  ATTACTCTCCTCCTCCA-GTTTA-CAAGTCTCCACCTCCTCCCAAGA  277
extC  *C*******T*****-*G*****T**-T********A**A**A****  201

extA  AACATTACGA-GTACAAATCACCTCCTCCACCGGTTTA-CAAATCTC  322
extC  *****-***GT*A*******T********T******A*A*-***A*T  246

extA  CTCCTCCTCCGGTTTACCACTCACCTCCACCACCTAAAAAACACTAC  369
extC  ********T-****-**-GG**T**A*****T**C*GG***G*****  290

extA  GAGTACAAATCACCTCCTCCACCGGTTTACAAGTCTCCTCCCCCTCC  416
extC  *T*********T**A**C*********---***-*----------   321

extA  GGTCTACCACTCTCCTCCACCACCTAAGAAACACTACG-AGTACAAA  462
extC  -----*AT*T***T*A********C**T***T*T****T*-***C**  362

extA  TCTCCTCCTCCACCGGTTTA-CAAGT-CTCCTCCTCCTCCAGTCTAC  507
extC  **********T******A*G*-*C*A**-*A**C***T****T***  407

extA  CACTCTCCTCCACCACCTAAGAAA-CACTACGAGTACAAATCACCTC  553
extC  **T*****A**C********-***T**G****TC********T**A*  453

extA  CTCCACCGGTTTACAAGTCTCC-TCCTCCTCCAGTCTACCACTCTCC  599
extC  *A*****A***---**A-*A*TA*A****C**G**T*********GG  496

extA  CCCACCACCTAAGAAACACTACGA-GTACAAATCTCCTCCTCCACCG  645
extC  A********A***********-*T******************T***  542

extA  GTTTA--CA-AGTCTCCTCCTCCTCCGGTTTACCACTCTCCTCCACC  689
extC  ***-*TG*--*C*ACT***T*****A***************A*****  586

extA  ACCTAAGAAGCATTACG-AGTACAAATCTCCTCCTCCTCCGGTTTAC  735
extC  ***A*************T*-********C***************A*G  632

extA  CAGTCTCCTCCCCCTCCGGTTTACCACTCTCCCCCACCACCAAAGAA  782
extC  **C*A*T****T*G**TA********T**C**A**************  679

extA  ACACTACGAATACAAATCGCCGCCACCTCCCGTCTATTCTCCTCCTC  829
extC  *A*G****T***T*****A**T**T*****A****---*G*------  717

extA  CACCGGTTCACTACTCACCTCCTCACCACCCCTACCTTTACAAATCT  876
extC  --------*******TT**C**A*****T*TT*****C*********  756
```

pre-protein was compared with the N-terminal amino acid sequences of the *extA* extensin (Evans *et al.*, 1990), the extensin protein encoded by a carrot genomic clone pDC5A1 (Chen and Varner, 1985b) and the *atExt1* extensin from *Arabidopsis* (Genbank accession number U43627).

Figure 4.5. Amino acid sequences of the leader peptide of the *extC* extensin showing the three possible cleavage sites (▾). ⩓ - shows hydrophobic amino acid. • - shows small neutral amino acids.

The cleavage site for these three extensins has already been determined (Figure 4.6). Based on these similarities in the amino acid sequences between these four leader peptides, the cleavage site of the leader peptide in the *extC* extensin is expected to be located between the A and N residues at positions 28 and 29. The expected mature *extC* extensin therefore begins with an asparagine (N) residue at position 29, and contains 231 amino acid residues with a molecular weight of 26909 Daltons.

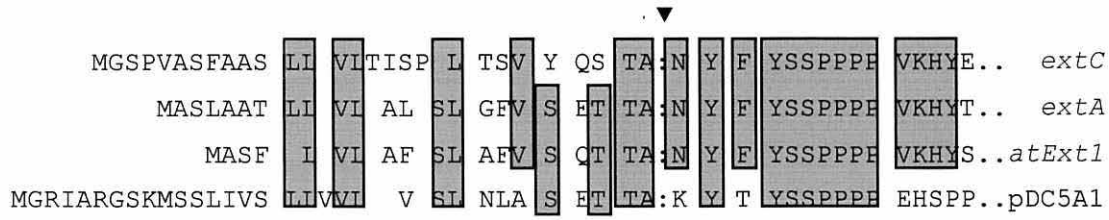


Figure 4.6. Comparison of the leader peptides from four extensin molecules. Residues that are the same in all four sequences are boxed. ▾ - shows the expected position of the leader peptide cleavage site.

Cleavage of the leader peptide from the mature protein is known to occur co-translationally in the endoplamic reticulum (ER) (Bennett and Oysteryoung, 1991). Extensin mRNAs are known to be translated in the cytoplasm and then transported and insolubilised into the cell wall. To allow transport to take place every protein molecule must have a sorting signal to direct the process, and these signals are the function of the leader peptide (Bennet and Oysteryoung, 1991).

Because experiments to determine the transcription start site (TCS) were not performed, the exact location of the TCS could not be determined. However, the TCS location was predicted by comparing the nucleotide sequences upstream from the translation start codon of the *extC* open reading frame with the consensus TCS sequence of the *extA* gene. Figure 4.7 shows that the sequence 5'TAAGAGCATAAACT3' found at the location between 19 and 32 nucleotide upstream from the translation start codon of *extC* is very similar to the 5'TAAGAGCATCAAAC3' TCS consensus sequence of the *extA* gene. Because the transcription start site of the *extA* gene has been determined - to begin at the adenine residue (underlined) - the transcription start site of *extC* is predicted also to start at the adenine residue (A - Figure 4.7) located within the tri-peptide sequence CAT, 25 nucleotides upstream from the translation start codon. This position agrees with the results from analysis of 79 plant genes which showed that the TCS of 85% of these genes starts at the adenine residue (Joshi, 1987b).

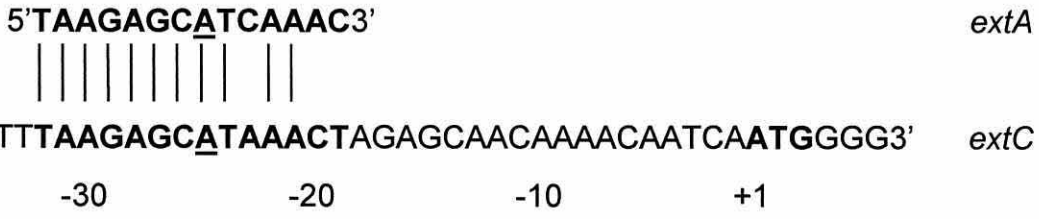


Figure 4.7. Comparison of the TCS consensus sequence of the *extA* gene with the sequence of *extC* upstream of the translation start codon. The translation start codon for the *extC* gene (in bold) is marked as position +1. The TCS for the *extA* gene is underlined (A - upper panel). The expected TCS for the *extC* gene is also underlined (A - lower panel).

Upstream from the translation start codon is 1446 bp of promoter sequence. A putative `TATA' box, TATAAA was found at location 33 nucleotides upstream from the expected TCS, and is different from the consensus `TATA' box sequence suggested for plant genes, TATATATA (Joshi, 1987b). However, one should remember that out of 79 genes analysed, only 16 have the consensus `TATA' box TATATATA. The remaining genes contain variations of TATATATA sequence (Joshi, 1987b). In addition, the same putative `TATA' box sequence as the one found in the *extC* promoter was also found in the soybean extensin gene, *SbHRGP3* sequence (Ahn *et al.*, 1996). Moreover, the location of the TATAAA sequence agrees with the suggested location for the `TATA' box in plant genes, i.e. 32 ± 7 nucleotides upstream from the TCS (Joshi, 1987b).

A putative `CAAT' box was found 122 nucleotides upstream from the `TATA' box. This location is considered a little bit further upstream from the suggested location for the `CAAT' box which is within approximately 35 nucleotides upstream from the `TATA' box (Robinson *et al.*, 1993). However, the *Arabidopsis* extensin gene, *atExt1* has also been shown to have a `CAAT' box further upstream (159 nucleotides) from the `TATA' box (Genbank accession number U43627). Another gene which also has a `CAAT' box further upstream (134 nucleotides) is the extensin gene *HRGP4.1* from kidney bean (Corbin *et al.*, 1987; Wycoff *et al.*, 1995).

4.3 Expression studies

Previous studies have shown that *extA* gene is specifically expressed in the roots of healthy oilseed rape plants (Evans *et al.*, 1990). After roots were wounded, the expression level of the gene decreased rapidly until it reached a basal level 10 h after application of the wounding stimulus (Shirsat *et al.*, 1996b). However, application of the wounding stimulus to oilseed rape leaves and petioles significantly induced expression of the *extA* gene (Figure 3.31). Significant levels of signal were seen in leaf and petiole 24 h and 12 h respectively after the wounding stimulus was applied to the tissues. Total RNA samples from wounded stem were not included in the dot blot experiment. However, Shirsat *et al.* (1996b) have shown that significant levels

of the *extA* hybridising transcripts were detected in stem 17 h after the tissue was wounded and continued to increase, reaching a maximum between 36 h and 48 h. Expression of *extA* in reponse to wounding suggests that the gene may play a very important role in the plant defense response to mechanical injury in leaf, petiole and stem. Other defense-related genes which are expressed in plant tissues after wounding include the genes for the proteinase inhibitor I (*pin1*) and II (*pin2*) in tomato (Sanchez-Serrano *et al.*, 1986; Pena-Cortes *et al.*, 1989) and aspartate and cysteine proteinase inhibitors in potato (Hildman *et al.*, 1992).

It is known that extensins are synthesized as soluble precursors and are immobilised (cross-linked) in the cell wall by a hydroperoxidase/peroxidase-mediated process (Cooper and Varner, 1983, 1984) even though the nature of the cross-linkage remains unresolved. Increase in extensin deposition and extensin cross-linking should therefore lead to a more impenetrable cell wall barrier, thus toughening the wall and impeding pathogen infection. Observations that extensin insolubilisation is enhanced within just a few minutes of wounding, elicitor treatment, or glutathione treatment (Bradley *et al.*, 1992) and that extensin can react as an agglutination agent for plant pathogens (Leach *et al.*, 1982; Mellon and Helgeson, 1982; van Holst and Varner, 1984), further supports the involvement of extensins in the plant defense system. The observation that the *extA* gene is systemically induced in oilseed rape leaf by wounding the root (Shirsat - personal communication) strongly supports this suggested function for the involvement of extensins in plant defense.

Wound-induced expression of extensin transcripts has also been seen in other plant systems such as the carrot storage root (Chen and Varner, 1985b; Ecker and Davis, 1987; Tierney *et al.*, 1988), bean hypocotyl (Corbin *et al.*, 1987), maize leaves and coleoptiles (Ludevid *et al.*, 1990), soybean leaves and stems (Ahn *et al.*, 1996) and tobacco leaves (Hirsinger *et al.*, 1997). An increase in the levels of transcripts corresponding to extensin genes as a result of pathogen infection and elicitor treatment has also been seen in bean hypocotyls (Corbin *et al.*, 1987; Showalter *et al.*, 1985; Templeton *et al.*, 1990) and sunflower stems (Mouly *et al.*, 1992).

Because *extC* was isolated from the screening of the oilseed rape mini genomic library using the coding sequence of *extA* as a probe, experiments were set up in order to see if *extC* would also respond to the same wounding treatment as shown by the *extA* gene. Dot blot hybridisation of the same total RNA samples as used in the *extA* dot blot experiment plus total RNA samples from wounded stem were hybridised with the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1. Results from this experiment showed that there was no signal corresponding to the probe detected in either unwounded root or in wounded leaf, petiole, stem and root after the blot was washed to a high stringency, 0.1X SSC/0.1% SDS at 65^{0}C (results not presented). However, when the blot was washed to a medium stringency (0.1X SSC/0.1% SDS at 42^{0}C), strong signals were seen in all wounded leaf, stem and petiole, and in unwounded root (Figure 3.30) with the pattern similar to that produced by *extA*. These results showed that *extC* did not respond to the wounding treatment and was not expressed in the roots of healthy plants. The signals seen when the blot was washed to a medium stringency suggest that the pNS1 probe non-specifically hybridised to the mRNA transcripts which were the product of *extA* gene expression. This suggestion was made based on the similarity between the pattern of signals seen in the *extA* dot blot (Figure 3.31) and *extC* dot blot (washed to a medium stringency - Figure 3.30). The signals may also be from hybridisation of the pNS1 probe to the other mRNA transcripts, which share a certain degree of sequence homology to the probe. Washing the blot to a high stringency resulted in the non-specific hybridisation of the probe to disappear and strongly supports the suggestion.

It is possible that *extC* is expressed at a low level in unwounded root and in wounded leaf, petiole and stems. In order to investigate this, the dot blot membrane filter washed to a high stringency was exposed to an autoradiograph film for 2 weeks. Results from the longer exposure time proved that the *extC* gene did not respond to wounding treatments in leaf, petiole, stem and root and was not expressed in unwounded root as no signal was detected from all RNA samples despite the long photographic exposure (results not shown). Northern blot experiments of the same RNA samples confirmed that *extC* was not expressed in response to wounding as there was

no transcript detected even after the membrane was exposed for 2 weeks (results not published).

Creelman and Mullet (1991) have reported that accumulation of extensin mRNA requires more severe wounding. In their experiments the elongation regions of soybean seedling hypocotyls were excise wounded and RNA extracted. Northern blots of wounded hypocotyl RNA using the carrot extensin genomic clone pDC5A1 as a probe showed that there was no increase in the level of mRNA corresponding to the pDC5A1 probe. However, when the hypocotyls were extensively wounded by slicing the tissue into 1 to 2 mm sections, mRNA levels corresponding to the pDC5A1 increased dramatically. Therefore, experiments were set up in order to see if more severe wounding of oilseed rape tissues would induce the expression of the *extC* gene. Detached oilseed rape leaves in sterile distilled water were severely wounded by applying dialysis clamps extensively and total RNA was extracted from the tissues 12 h, 24 h, 36 h and 48 h after application of the clamps. Northern blot hybridisation of total RNA against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 showed that there was no transcript corresponding to the probe detected even after 2 weeks exposure (results not published). These results indicated that *extC* was not activated by mechanical injury in leaf and was almost certainly not involved in the wound healing process.

Washing the blot to a medium stringency (0.1x SSC/0.1% SDS at 42^0C), however resulted in two transcripts of sizes 1.26 kb and 1.45 kb appearing in all wounded leaf RNA (Figure 3.32) with a pattern similar to that produced when the RNA samples were probed with the coding sequence of *extA* (discussed below). These results support the earlier suggestion that the signals seen on the dot blot of wounded RNA samples probed with the 1.32 kb *extA*-hybridising fragment of pNS1 probe (Figure 3.30) were the result of non-specific hybridisation of the pNS1 probe to the transcripts from the expression of the *extA* gene.

When the same RNA samples were hybridised against the radiolabelled *extA* coding sequence probe, two transcripts of 1.26 kb and 1.45 kb were detected 24 h after the leaves were wounded (Figure 3.33). The

levels of the two transcripts decreased and remained almost constant between 36 h and 48 h. Because a comparison of northern blots of the wounded leaf (with a cork borer) RNA against the *extA* probe was not done, a direct comparison of the *extA* expression pattern between the two different wounding treatments could not be done. However, based on the RNA dot blot results (Figure 3.31), it is clear that the expression pattern of *extA* between the two wounding experiments is different. After wounding of leaf by punching leaf discs with a cork borer, significant levels of *extA* signal were first seen at 24 h, reached a maximum level at 36 h and decreased at 48 h. In addition, Shirsat *et al.* (1996b) showed that the 1.26 kb and 1.45 kb transcripts accumulated to the same extent in response to wounding of leaves with a cork borer. However, results in Figure 3.33 showed that the 1.45 kb transcript accumulates to a higher level compared to the 1.26 kb transcript 24 h and 48 h after the leaves were wounded by application of dialysis clamps. Scanning of the autoradiographs with a laser densitometer (Figure 3.44a) clearly showed that the level of the 1.45 kb transcript was more than doubled compared to the 1.26 kb transcript at 24 h. At 36 h, the levels of the 1.45 kb transcript were just slightly higher than the 1.26 kb transcript, and were doubled again at 48 h even though the levels for both the transcripts were lower compared to the level at 24 h.

Evans *et al.* (1990) have confirmed that the 1.26 kb transcript is the product of the *extA* gene, and suggested that the 1.45 kb transcript was a product of another unknown expressed gene. Because results from these studies showed that the 1.45 kb transcript was present in both wounding experiments, the gene encoding the 1.45 kb transcript must therefore respond to the same wounding stimuli as the *extA* gene. It was proposed that different extensin genes are expressed in different tissues to fulfill different functions. Therefore, it is possible that the 1.45 kb transcript is a product of a gene closely related to *extA* and a member of the *extA* multigene family, and may probably play the same function as suggested for *extA* in plant defense.

In order to further examine whether the *extC* gene responds to different physiological treatments, several experiments were performed. Detached leaves were treated with abscisic acid (ABA), sodium salicylate and methyl

jasmonate (MeJ) for different times before total RNA were extracted. Northern blot hybridisation of the total RNA from ABA-, sodium salicylate-, MeJ-treated leaves were then performed against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1. These compounds were chosen because the ability of ABA, salicylic acid (SA) and its sodium salt (sodium salicylate), jasmonic acid (JA) and its methyl ester (MeJ) to induce expression of several genes especially those involved in plant defense has been proven previously (Delaney *et al.*, 1995; Enyedi *et al.*, 1992; Heitz *et al.*, 1997; Hildman *et al.*, 1992; Malamy *et al.*, 1990; Pena-Cortes *et al.*, 1989, 1996; Ward *et al.*, 1991; Yalpani *et al.*, 1991). The same experiments were also repeated using the radiolabelled coding sequence of *extA* as a probe.

Northern blot hybridisations of the total RNA samples from ABA-, sodium salicylate- and MeJ-treated leaves against the radiolabelled 1.32 kb *extA*-hybridising fragment of pNS1 showed that there were no transcripts corresponding to the probe detected after the blots were washed to a high stringency (0.1X SSC/0.1% SDS at 65^0C) (results not presented). *extC* was therefore not induced by ABA, sodium salicylate or MeJ in the leaves of oilseed rape plants, and possibly is not involved in plant defense. Detection of low levels of the 1.26 kb and 1.45 kb transcripts in the northern blots of RNA from ABA- (Figure 3.35), sodium salicylate- (Figure 3.38) and MeJ-treated leaves (Figure 3.41) when the blots were washed to a medium stringency (0.1X SSC/0.1% SDS at 42^0C) were the results of non-specific hybridisation of the probe to the mRNA product of *extA* expression and also to the 1.45 kb transcript. This indicates that regulation of expression of the *extC* gene is different from the regulation of the *extA* gene. *extC* may respond to some other stresses such as cold injury, heat shock, gravity, developmental events, elicitor treatment, fungal infections, as well as bacterial and viral infections as has been shown for many extensin genes (Showalter, 1993). The gene may also be expressed in tissues other than those assayed for expression, or at other developmental stages in the oilseed rape plant life cycle. However, because of time limitations, these experiments were not performed. These treatments should be done in future in order to further characterise the nature of expression of *extC*. The possibility also exists that

extC may express in regions of the plant which experience tensile stress as has been shown by the *extA* gene (Shirsat *et al.*, 1996a). In order for this possibility to be tested, plants carrying a *extC* promoter-GUS gene fusion need to be constructed, and experiments similar to that of Shirsat *et al.* (1996a) need to be performed.

Results from northern blot experiments of total RNA samples probed with the radiolabelled *extA* coding sequence showed that treatment of leaves with ABA (Figure 3.36), sodium salicylate (Figure 3.39) and MeJ (Figure 3.42) induced the accumulation of both the 1.26 kb and 1.45 kb transcripts. Within just 12 h of treatment, significant levels of transcripts were seen in all ABA-, sodium salicylate- and MeJ-treated leaves. Compared to the wounded leaf (Figure 3.33), significant levels of the transcripts were first seen after only 24 h. The expression pattern of the two transcripts in each treatment was also different. In wounded, ABA- and MeJ-treated leaves, the levels of the two transcripts fluctuated within the whole period of the experiments. Significant levels of transcripts were detected at 12 h, reached a maximum at 24 h (except for the ABA-treated leaves where a maximum level of transcripts were detected at 48 h), decreased at 36 h and increased again at 48 h (Figure 3.44a, b, and d). In most cases, the levels of the 1.45 kb transcript were either higher or equal compared to the 1.26 kb transcript except in the ABA-treated leaves (12 h and 36 h, Figure 3.44b) and the MeJ-treated leaves (36 h, Figure 3.44d) where the levels of the 1.45 kb transcript were slightly lower than the levels of the 1.26 kb transcript. In sodium salicylate-treated leaves (Figure 3.44c), expression of the 1.26 kb transcript gradually increased until 48 h. The 1.45 kb transcript level however, gradually increased until 36 h before it slightly decreased to the 48 h time point. These results showed that treatment of leaves with ABA, sodium salicylate and MeJ induced accumulation of the same transcripts in the same manner as they were induced by wounding. Observations that the 1.26 kb and 1.45 kb transcripts accumulated faster in ABA-, sodium salicylate- and MeJ-treated leaves compared to the wounded leaves may suggest that induction of *extA* and the gene coding for the 1.45 kb transcript by wounding may require synthesis of endogenous ABA, salicylic acid and MeJ. This is because when these

intermediates are provided exogenously, transcript accumulation is faster. Because experiments to determine the levels of ABA, salicylic acid and MeJ in leaves were not performed, the differences in the levels of ABA, salicylic acid and MeJ in leaves before and after the leaves were wounded could not be compared.

The stronger signals of the two transcripts observed in the northern blots of total RNA from dialysis clamping-wounded-leaves, ABA-, sodium salicylate- and MeJ-treated leaves probed with the coding sequence of the *extA* gene and washed to a medium stringency (Figure 3.34, 3.37, 3.40, and 3.43 respectively) compared to the results from the high stringency washing (Figure 3.33, 3.36, 3.39 and 3.42 respectively) may be due to non-specific hybridisation of the probe to other mRNAs of the same sizes or the products of other genes expressed in response to these treatments. This is very likely because *extA* is a member of a multigene family consisting of at least six other genes (Evans *et al.*, 1990). Therefore, it is possible that these genes are also induced by the same treatments and mRNA transcripts from these expressed genes non-specifically hybridise to the *extA* probe. However, this hypothesis can not be tested until the rest of the *extA* gene family members are isolated and characterised.

Very few results have been published regarding the induction of cell wall protein genes (especially extensins) as a result of the treatment of plant tissues with ABA, sodium salicylate (or SA) and MeJ (or JA). However, a study on the root specific extensin from tobacco using a genomic clone pEXT2 as a probe showed that extensin transcript levels in leaves were slightly increased 48 h after salicylic acid treatment (Tire *et al.*, 1994). Studies on the wound-inducible Class III cDNA clone encoding the glycine-rich protein (GRP) in tomato showed that treatment of tomato stems with ABA resulted in the marked accumulation of the Class III GRP gene mRNA (Showalter *et al.*, 1992). The GRP gene in maize was also induced by the treatment of embryos with ABA (Gomez *et al.*, 1988). Studies by Creelman *et al.* (1992) using the carrot cDNA clone pDC16 (coding for the proline-rich cell wall protein gene) showed that addition of MeJ to soybean suspension cultures induced

accumulation of PRP mRNA. In addition, it was shown that wounding of soybean stem rapidly induced the accumulation of MeJ and jasmonic acid.

Several questions can now be asked: (1) If it is true that ABA, sodium salicylate and MeJ are involved in the wound-inducible expression of the *extA* gene, can the signal transduction pathway be elucidated? (2) Do these hormones individually induce expression of the *extA* gene or do any of these compounds induce the synthesis of the other compounds (for example, sodium salicylate inducing synthesis of ABA and/or JA) and subsequently inducing expression of the *extA* gene? (3) Does each of these hormones follows its own signal transduction pathway in the induction of the *extA* gene or do they share the same signal transduction pathway?

Many studies have shown that ABA, SA (or sodium salicylate) and JA (or MeJ) are important signalling factors in the induction of plant gene expression especially those involved in plant defense mechanisms (Delaney *et al.*, 1995; Enyedi *et al.*, 1992; Farmer and Ryan, 1990; Heitz *et al.*, 1997; Herde *et al.*, 1996; Malamy *et al.*, 1990; Pena-Cortes *et al.*, 1989, 1993, 1995; Schweizer *et al.*, 1997; Shah *et al.*, 1997; Ward *et al.*, 1991; Wu *et al.*, 1997; Yalpani *et al.*, 1991; Yu *et al.*, 1997). It is known that ABA is present in probably all higher plants, and has been implicated in the control of a wide range of essential physiological processes including seed development and the plant adaptation to environmental stresses. Evidence for the involvement of ABA in the induction of the wound-inducible defense-related *pin2* gene has been obtained (Pena-Cortes *et al.*, 1989). In their studies in tomato and potato, Pena-Cortes *et al.* (1989) found that *pin2* gene expression was induced in tomato and potato leaves after the tissues were wounded. When ABA-deficient mutant plant leaves were wounded, expression of the *pin2* gene was not detected. However, when unwounded leaves from ABA-deficient mutant potato and tomato were sprayed with ABA, expression of the *pin2* gene was induced in all treated leaves showing a pattern identical to that shown as a result of wound induction. They also found that not only the *pin2* gene was induced in the treatment of potato and tomato leaves with ABA, but two other defense-related protein genes (leucine amino peptidase and

threonine deaminase) were also induced, showing a pattern similar to that produced by wounding (Hildman *et al.*, 1992).

It has been suggested that ABA and MeJ share the same signal transduction pathway in inducing expression of a wound-inducible *pin2* gene (Hildman *et al.*, 1992; Pena-Cortes *et al.*, 1989, 1995). This suggestion was made as a result of studies on the effect of ABA and MeJ on *pin2* gene expression in tomato and potato. In these studies, it was found that application of ABA to wild-type plants induced accumulation of JA in addition to the induction of the *pin2* gene. Furthermore, application of MeJ did not induce accumulation of ABA but induced expression of the *pin2* gene. They therefore concluded that ABA and MeJ shared the same signalling pathway in inducing *pin2* gene expression and that the site of action of JA was located downstream of the site of action of ABA (Pena-Cortes *et al.*, 1995).

Several sets of experiments have suggested that JA/MeJ might be a central component of intracellular signalling in response to wounding or pathogen attack. MeJ has been shown to stimulate the accumulation of wound-inducible vegetative storage proteins in soybean plants and suspension cultures (Masson and Mullet, 1990; Staswick, 1990). In addition, airborne MeJ was able to trigger the accumulation of *pin1* and *pin2* proteins in the leaves of potato plants to even higher levels than those induced by wounding (Farmer and Ryan 1990). Likewise, Hildmann *et al.* (1992) demonstrated that JA strongly induced the accumulation in potato leaves of all the isolated ABA-responsive/wound-induced genes including *pin2*, leucine aminopeptidase and threonine deaminase. More interestingly, they found that incubation of potato leaves with JA resulted in similar levels of mRNA accumulation in both wild-type and ABA-deficient mutant plants. Other wound- and pathogen-inducible genes and proteins which were also induced by the JA/MeJ treatment included the accumulation of the products of the papain inhibitor genes (Bolter, 1993), a ribosome-inactivating protein (Chaudhry *et al.*, 1994), phenyl ammonia lyase (Gundlach *et al.*, 1992), *pin2* (Herde *et al.*, 1996; Pena-Cortes *et al.*, 1992), PR-1b and osmotin (PR-5) (Chang *et al.*, 1997), β-1,3-glucanase, chitinase, thaumatin-like and peroxidase (Schweizer *et al.*, 1997) and lipoxygenase (Heitz *et al.*, 1997).

Although the relevance of this response *in vivo* is not yet known, the fact that all the ABA/wound-responsive genes are also induced by JA and that both wild-type and ABA-deficient plants respond to treatment with JA strongly indicates that JA may act as an intermediate step in the signalling pathway that leads to the activation of these genes. As such, JA could bypass the initial recognition events requiring ABA and thus trigger the induction of the genes even in the absence of ABA.

The exact mechanism on how JA/MeJ is involved in the signal transduction pathway leading to the activation of the wound- and pathogen-inducible genes is still unknown. However, it is known that JA is located downstream of ABA in the signal transduction pathway (Pena-Cortes *et al.*, 1995). Several lines of evidence support a role for the JA biosynthetic pathway (octadecanoid pathway) in the signalling of the wound response. Application to tomato leaf surfaces of linolenic acid (precursor for the biosynthesis of JA), as well as the biosynthetic intermediates between linolenic acid and JA, results in the induction of defense gene expression (Farmer and Ryan, 1992). Mechanical wounding or supplying systemin (a peptide released by plant tissues upon wounding and able to move throughout the vascular tissue of the plant [Pearce *et al.*, 1991]) to young tomato plants through their cut stems results in a rapid and transient accumulation of linolenic acid (Conconi *et al.*, 1996) and JA (Doares *et al.*, 1995). Inhibitors of the octadecanoid pathway block the induction of defense genes by systemin and linolenic acid (Doares *et al.*, 1995; Farmer *et al.*, 1994). A tomato mutant impaired in the octadecanoid pathway, called *def1* (Howe *et al.*, 1996), only weakly expresses defense genes following wounding or supplying excised plants with systemin, linolenic acid, or carbohydrate elicitors. Study on the JA biosynthesis pathway showed that JA was synthesised from linolenic acid (Vick and Zimmermann, 1983). It was therefore suggested that the activation of the octadecanoid pathway was initiated by the release of linolenic acid from membrane lipids by activating phospholipase A, the enzyme which catalyses the conversion of lipid to linolenic acid, possibly by ABA (Chandra *et al.*, 1996).

Due to the similarity between the response of *extA* and other wound- and pathogen-inducible genes to the same treatments (wounding, ABA and MeJ) as discussed, a hypothesis can now be made that induction of *extA* by wounding, ABA and JA follows the same signalling pathway as suggested for these defense genes. However, this hypothesis remains to be tested. In addition, the levels of ABA and MeJ were not measured during the wounding experiments, and the unavailability of ABA-deficient mutant oilseed rape plants precluded this hypothesis being tested. Therefore, in order to prove or disprove the hypothesis, the same experiments as performed by Pena-Cortes *et al.* (1995) should be done in the future.

The involvement of SA/sodium salicylate in inducing the expression of the wound-inducible *extA* gene is of interest. Due to the observation that the wound-inducible *extA* gene is expressed to high levels in leaves that have been treated with sodium salicylate, a suggestion can be made that wounding of leaves induced accumulation of SA and subsequently SA induces the expression of the *extA* gene through an yet unidentified signal transduction pathway. The fact that a significant level of expression was detected 12 h after treatment of leaves with sodium salicylate (no expression of *extA* 12 h after leaves were wounded either by using a cork borer or dialysis clamps), may strongly support this suggestion. However, until further experiments are done, this hypothesis can not be proved or disproved. Endogenous levels of SA need to be measured in wounded leaves and the relationship between the accumulation of endogenous SA (if present) with the expression of the *extA* gene need to be established. Furthermore, a SA-deficient mutant plant needs to be identified and the effect of wounding and application of SA/sodium salicylate in this mutant plant needs to be tested.

The ability of SA and its sodium salt to induce expression of pathogenesis-related genes is also evident. Malamy *et al.* (1990) have shown that induction of pathogenesis-related-1 (PR-1) gene expression is parallelled with an increase in endogenous SA levels in tobacco after the resistant Xanthi tobacco plants were inoculated with tobacco mosaic virus (TMV). Inoculation of susceptible tobacco leaves with TMV only caused a small accumulation of PR-1 mRNA and SA. They also observed that the induction of PR-1 genes

and SA not only occurred in infected leaves but also in untreated leaves distal from the treated ones (systemic activation). Interestingly, when excised leaves of TMV-resistant tobacco were fed with SA, an increase in the endogenous SA level as well as in PR-1 mRNA accumulation was also observed (Yalpani *et al.*, 1991). Studies by Ward *et al.* (1991) showed that infection of TMV-resistant tobacco leaves with TMV induced accumulation of 9 pathogenesis-related mRNAs to a high level. The same set of genes was also induced when tobacco leaves were sprayed with sodium salicylate (Ward *et al.*, 1991). Studies in *Arabidopsis* showed that PR-1, PR-2 and PR-3 gene expression was induced after plants were treated with exogenous SA, and subsequently increased the plant resistance to the pathogen *Peronospora parasitica* (Delaney *et al.*, 1995).

Induction of *extA* gene expression by sodium salicylate treatment may further support the hypothesis that the *extA* gene plays an important role in the plant defense system. Even though experiments to prove this hypothesis have yet to be done, several lines of evidence already support this idea. Exogenous application of SA and its sodium salt have been shown to induce the accumulation of several pathogenesis-related mRNAs and proteins which lead to an increase in the plant resistance to pathogen attack in several plant systems including tobacco (Conrath *et al.*, 1997; Enyedi *et al.*, 1992; Ward *et al.*, 1991; Yalpani *et al.*, 1991), *Arabidopsis* (Delaney *et al.*, 1995; Uknes *et al.*, 1992) and cucumber (Rasmussen *et al.*, 1991). In addition, infection of several plants with pathogens induces the accumulation of SA and at the same time enhances the accumulation of mRNAs and proteins corresponding to the pathogenesis-related genes. These relationships have been shown in tobacco (Enyedi *et al.*, 1992; Gaffney *et al.*, 1993; Malamy *et. al.*, 1990; Vernooij *et al.*, 1994; Ward *et al.*, 1991) and cucumber (Metraux *et al.*, 1990; Rasmussen *et al.*, 1991).

The mode of action of SA is still unknown. It is possible that the same signal transduction pathway as suggested for wounding, ABA and MeJ in inducing wound-inducible gene expression is followed by SA. However, there are no results published so far about the relationship between wounding, high levels of accumulation of endogenous SA, and expression of the wound-

inducible genes in plants. Furthermore, no one has so far reported on the relationship between wounding, ABA treatment and JA/MeJ treatment with the accumulation of endogenous levels of SA in plant tissues. Therefore, the possibility that the mode of action of sodium salicylate in inducing the wound-inducible expression of *extA* gene follows a different signal transduction pathway from the one followed by wounding, ABA and MeJ can not be excluded. The differences between the expression pattern of the *extA* gene in ABA-, sodium salicylate- and MeJ-treated leaves (Figure 3.44) may support the latter suggestion.

References

References

Adair W.S., and Apt K.E. (1990). Cell wall regeneration in *Chlamydomonas*: accumulation of mRNAs encoding cell wall hydroxyproline-rich glycoproteins. *Proc. Natl. Acad. Sci. USA*, 87: 7355-7359.

Adair W.S., and Snell W.J. (1990). The *Chlamydomonas reinhardtii* cell wall: structure, biochemistry, and molecular biology. *In* Organisation and Assembly of Plant and Animal Extracellular Matrix (Adair W.S., and Mecham R.P., eds.). New York: Academic Press, pp. 15-84.

Adam C.A., Nelson W.S., Nunberg A.N., and Thomas T.L. (1992). A wound-inducible member of the hydroxyproline-rich glycoprotein gene family in sunflower. *Plant Physiol.*, 99: 775-776.

Ahn J.H., Choi Y., Kwon Y.M., Sang-Gu K., Choi Y.D., and Lee J.S. (1996). A novel extensin gene encoding a hydroxyproline-rich glycoprotein requires sucrose for its wound-inducible expression in transgenic plants. *Plant Cell*, 8: 1477-1490.

Albersheim P. (1976). The primary cell wall. *In* Plant Biochemistry, 3rd edition (Bonner J., and Varner J.E., eds.). New York: Academic Press, pp. 225-271.

Aleith F., and Richter G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. *Planta*, 183: 17-24.

Alexander D.C., McKnight T.D., and Williams B.G. (1984). A simplified and efficient vector – primer cDNA cloning system. *Gene*, 31: 79-89.

Allen A.K. (1983). Potato lectin - a glycoprotein with two domains. *In* Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins (Goldstein I.J., and Etzler M.E., eds.). New York: Alan R. Liss, pp. 71-85.

Allen A.K., Desai N.N., Neuberger A., and Creeth J.M. (1978). Properties of potato lectin and the nature of its glycoprotein linkages. *Biochem. J.*, 171: 665-674.

Anderson R.L., Clarke A.E., Jermyn M.A., Knox R.B., and Stone B.A. (1977). A

carbohydrate-binding arabinogalactan protein from liquid suspension cultures of *Lolium multiflorum* endosperm. *Aust. J. Plant Physiol.*, 4: 143-158.

Anderson J.M., Spilatro S.R., Klauer S.F., and Franceschi V.R. (1989). Jasmonic acid-dependent increase in the level of vegetative storage proteins in soybean. *Plant Sci.*, 62: 45-52.

Arsenijevic-Maksimovic T., Broughton W.J., and Krause A. (1997). Rhizobia modulate root-hair-specific expression of extensin genes. *Mol. Plant-Microbe Interac.*, 10: 95-101.

Ashford D., Desai N.N., Allen A.K., Neuberger A., O'Neil M.A., and Selvendran R.R. (1982). Structural studies of the carbohydrate moieties of lectins from potato (*Solanum tuberosum*) tubers and thormapple (*Datura stramonium*) seeds. *Biochem. J.*, 201: 199-208.

Ashford D., Menon R., Allen A.K., and Neuberger A. (1981). Studies of the chemical modification of potato (*Solanum tuberosum*) lectin and its effect on haemagglutinating activity. *Biochem. J.*, 199: 399-408.

Aspinal G.O. (1980). Chemistry of cell wall polysaccharides. *In* The Biochemistry of Plants - A comprehensive treatise, Volume 3 (Stumpt P.K., and Conn E.E., eds.). New York: Academic Press, pp. 473-497.

Averyhart-Fullard V., Datta K., and Marcus A. (1988). A hydroxyproline-rich protein in the soybean cell wall. *Proc. Natl. Acad. Sci. USA*, 261: 82-94.

Ayers A.R., Ebel J., Finelli F.D., Berger N., and Albersheim P. (1976). Host-pathogen interactions IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytopthora megasperma* var. Sojae. *Plant Physiol.*, 57: 751-759.

Bacic A., Churms S.C., Stephen A.M., Cohen P.B., and Fincher G.B. (1987). Fine structure of the arabinogalactan-protein from *Lolium multiflorum*. *Carbohydr. Res.*, 162: 85-94.

Bacic A., Harris P.J., and Stone B.A. (1988). Structure and function of plant cell walls. *In* The Biochemistry of Plants, Volume 14 (Priess J., ed.). New York: Academic Press, pp. 297-371.

Baldwin T.C., Coen E.S., and Dickinson H.G. (1992). The *ptl1* gene expressed in the transmitting tissue of *Antirrhinum* encodes an extensin-like protein. *Plant J.*, 2: 733-739.

Bao W., O'Malley D.M., and Sederoff R.R. (1992). Wood contains a cell wall structural protein. *Proc. Natl. Acad. Sci. USA*, 89: 6604-6608.

Baron_Epel O., Ghary P.K., and Stone B.A. (1988). Pectins as mediators of wall porosity in soybean cells. *Planta*, 175: 389-385.

Bauer W.P., Talmadge K.W., Keegstra K., and Albersheim P. (1973). The structure of plant cell walls. II. The hemicelluloses of the walls of suspension-cultured sycamore cells. *Plant Physiol.*, 51:174-187.

Benhamou N., Lafontaine P.J., Mazau D., and Esquerre-Tugaye M.T. (1991a). differential accumulation of hydroxyproline-rich glycoproteins in bean root nodule cells infected with a wild-type strain or a C4-dicarboxylic acid mutant of *Rhizobium leguminosarum* bn. *phaseoli. Planta*, 184: 457-467.

Bennet A.B., and Osteryoung K.W. (1991). Protein transport and targeting within the endomembrane system of plants. *In* Plant Genetic Engineering, Volume 1 (Grierson D., ed.). Blackie Academic and Professional, pp. 199 -237.

Bergeron D., Beauseigle D., and Bellemare G. (1993). Sequence and expression of a gene encoding a protein with RNA-binding and glycine-rich domains in *Brassica napus*. *Biochem. Biophys. Acta*, 1216: 123-125.

Bergeron D., Boivin R., Baszczynski C.L., and Bellemare G. (1994). Root-specific expression of a glycine-rich protein gene in *Brassica napus. Plant Science*, 96: 87-98.

Berkel J.V., Salamini F., and Gebhardt C. (1994). Transcripts accumulating during cold storage of potato (*Solanum tuberosum* L.) tuber are sequence related to stress-responsive genes. *Plant Physiol.*, 104: 445-452.

Biggs K.J., and Fry S.C. (1990). Solubilisation of covalently bound extensin from Capsicum cell walls. *Plant Physiol.*, 92: 197-204.

Birecka H., and Miller A. (1974). Cell wall and protoplast isoperoxidases in

relation to injury, indolacetic acid and ethylene affects. *Plant Physiol.*, 53: 569-574.

Boller T. (1987). Hydrolytic enzymes in plant disease resistance. *In* Plant-microbe Interactions (Kosuge T. and Nester E.W., eds.). New York: Macmillan, pp. 385-414.

Bolter C.J. (1993). Methyl jasmonate induces papain inhibitor(s) in tomato leaves. *Plant Physiol.*, 103: 1347-1353.

Boudart G., Decahmp-Guillaume G., Laffite C., Ricart G., Barthe J.P., Mazau D., and Esquerre-Tugaye M.T. (1995). Elicitors and suppressors of hydroxyproline-rich glycoprotein accumulation are solubilised from plant cell walls by endopolygalacturonase. *Eur. J. Biochem.*, 232: 449-457.

Bowles D.J. (1990). Defense-related proteins in higher plants. *Ann. Rev. Biochem.*, 59: 873-907.

Bradley D.J., Kjellbom P., and Lamb C.J., (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*, 70: 21-30.

Brett C., and Waldron K. (1990). Physiology and Biochemistry of Plant Cell Walls. Unwin Hyman, London.

Brownleader M.D., Ahmed N., Trevan M., Chaplin M.F., and Dey P.M. (1995). Purification and partial characterization of tomato extensin peroxidase. *Plant Physiol*, 109: 1115-1123.

Burke D., Kaufman P., McNeil M., and Albersheim P. (1974). The structure of plant cell walls. IV. A survey of the walls of suspension-cultured monocots. *Plant Physiol.*, 54: 109-115.

Caelles N.C., Delseny M., and Puigdomenech P. (1992). The hydroxyproline-rich glycoprotein gene from *Oryza sativa*. *Plant Mol. Biol.*, 18: 617-619.

Carpita N.C. (1987). The biochemistry of the `growing' plant cell wall. *In* Physiology of Cell Expansion During Plant Growth (Cosgrove D.J., and Knievel D.P., eds.). Rockville, M.D: Am. Soc. Plant Physiol., pp. 28-45.

Carpita N.C., and Gibeaut D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.*, 3: 1-30.

Casalongue C., and Pont Lezica R. (1985). Potato lectin: A cell-wall glycoprotein. *Plant Cell Physiol.*, 26: 1533-1539.

Cassab G.I. (1986). Arabinogalactan proteins during the development of soybean root nodules. *Planta*, 168: 441-446.

Cassab G.I., and Varner J.E. (1987). Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. *J. Cell Biol.*, 105: 2581-2588.

Cassab G.I., and Varner J.E. (1988). Cell wall proteins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* 39: 321-353.

Cassab G.I., Nieto-Sotelo J., Cooper J.B., Van-Holst G-J., and Varner J.E. (1985). A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. *Plant Physiol.*, 77: 532-535.

Chandler P.M., and Robertson M. (1994). Gene expression regulated by abscisic acid and its relation to stress tolerance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 45: 113-141.

Chandra S., Heinstein P.F., and Low P.S. (1996). Activation of phospholipase A by plant defense elicitors. *Plant Physiol.*, 110: 979-986.

Chang P.F.L., Xu Y., Narasimhan M.L., Cheah K.T., Durzo M.P., Damsz B., Kononowicz A.K., Abad L., Hasegawa P.M., and Bressan R.A. (1997). Induction of pathogen resistance and pathogenesis-related genes in tobacco by heat-stable *Trichoderma* mycelia extract and plant signal messengers. *Plant Physiol.*, 100: 341-352.

Chaudhry B., Muller-Uri F., Cameron-Mills V., Gough S., Simpson D., Skriver K., and Mundy J. (1994). The barley 60 kDa jasmonate-induced protein (JIP60) is a novel ribosom-inactivating protein. *Plant J.*, 6: 815-824.

Chen C.G., Cornish E.C., and Clarke A.E. (1992). Specific expression of an extensin-like gene in the style of *Nicotiana alata*. *Plant Cell*, 4: 1053-1062.

Chen J., and Varner J.E. (1985a). Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proc. Natl. Acad. Sci. USA*, 82: 4399-4403.

Chen J., and Varner J.E. (1985b). An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin. *EMBO J.*, 4:

2145-2151.

Christoffersen R.E., Tucker M.L., and Laites G.G. (1984). Gene expression during fruit ripening in avocado fruit: the accumulation of cellulase in RNA and protein as demonstrated by cDNA hybridisation and immunodetection. *Plant Mol. Biol.*, 3: 385-391.

Clarke A.E., Anderson R.L., and Stone B.A. (1979). Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, 18: 521-540.

Clarke A.E., Gleeson P.A., Jermyn M.A., and Knox R.B. (1978). Characterization and localization of β-lectins in lower and higher plants. *Aust. J. Plant Physiol.*, 5: 707-722.

Clarke A.E., Knox R.B., and Jermyn M.A. (1975). Localization of lectins in legume cotyledons. *J. Cell Sci.*, 19: 157-67.

Clarke J.A., Lisker N., Lamport D.T.A., and Ellingboe A.H. (1981). Hydroxyproline enhancement as a primary event in the successful development of *Erysiphe graminis* in wheat. *Plant Physiol.*, 67: 188-189.

Clarke A.E., and Stone B.A. (1962). β-1,3-Glucan hydrolases from grape vine (Vitis vinifera) and other plants. *Phytochemistry*, 1: 175-188.

Cleland R.E. (1971). Cell wall extension. *Ann. Rev. Plant Physiol.*, 22: 197-222.

Cline K., and Albersheim P. (1981). Host-pathogen interactions XVI. Purification and characterization of a beta-glucosyl hydrolase/transferase present in the walls of soybean cells. *Plant Physiol.*, 68: 207-220.

Coche Th., Dewez M., and Bakers M.C. (1994). Generation of unlimited supply of a subtracted probe using magnetic beads and PCR. *Nucl. Acids Res.*, 22: 1322-1323.

Conconi A., Miquel M., Browse J.A., and Ryan C.A. (1996). Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. *Plant Physiol.*, 111: 797-803.

Condit C.M (1993). Developmental expression and localization of petunia glycine-rich protein-1. *Plant Cell*, 5: 277-288.

Condit C.M., and Meagher R.B. (1986). A gene encoding a novel glycine-rich structural protein of petunia. *Nature*, 323: 178-181.

Condit C.M., and Meagher R.B. (1987). Expression of a gene encoding a glycine-rich protein in petunia. *Mol. Cell. Biol.*, 7: 4273-4279.

Condit M.C., McLean B.G., and Meagher R.B. (1990). Characterization of the expression of the petunia glycine-rich protein-1 gene product. *Plant Physiol.*, 93: 596-602.

Conrath U., Silva H., and Klessig D.F. (1997). Protein dephosphorylation mediates salicylic acid-induced expression of *PR-1* genes in tobacco. *Plant J.*, 11: 747-757.

Cooper J.B., and Varner J.E. (1983). Insolubilisation of hydroxyproline-rich glycoprotein in aerated carrot root slices. *Biochem. Biophys. Res. Commun.*, 112: 161-167.

Cooper J.B., and Varner J.E. (1984). Cross-linking of soluble extensin in isolated cell walls. *Plant Physiol.*, 76: 414-417.

Cooper J.B., Chen J.A., van Holst G.J., and Varner J.E. (1987). Hydroxyproline-rich glycoproteins of plant cell wall. *Trends Biochem. Sci.*, 12: 24-27.

Cooper J.B., Heuser J.E., and Varner J.E. (1994). 3,4-dehydroproline inhibits cell wall assembly and cell division in tobacco protoplasts. *Plant Physiol.*, 104: 747-752.

Corbin D.R., Sauer N., and Lamb C.J. (1987). Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. *Mol. Cell. Biol.*, 7: 4337-4344.

Cosgrove D.J. (1993). How do plant cell walls extend?. *Plant Physiol.*, 102: 1-6.

Creelman R.A., and Mullet J.E. (1991). Water deficit modulates gene expression in growing zones of seedling. Analysis of differentially expressed cDNAs, a new β-tubulin gene, and expression of genes encoding cell wall proteins. *Plant Mol. Biol.*, 17: 591-608.

Creelman R.A., Tierney M.L., and Mullet J.E. (1992). Jasmonic acid methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. USA*, 89: 4938-4941.

Darvill A., McNeil M., Albersheim P., and Delmer D.P. (1980). The primary cell walls of flowering plants. *In* The Biochemistry of Plants - A comprehensive treatise, Volume 1 (Tolbert N.E., ed.). New York: Academic Press, pp. 91-162.

Datta K., Schmidt A., and Marcus A. (1989). Characterization of two soybean repetitive proline-rich proteins and a cognate cDNA from germinated axes. *Plant Cell*, 1: 945-952.

Delaney T.P., Friedrich L., and Ryals S.A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. USA*, 9: 6602-6606.

Della Penna .D., Alexander D.C., and Bennet A.B. (1986). Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening. *Proc. Natl. Acad. Sci. USA*, 83: 6420-6424.

De Loose M., Gheysen G., Tire C., Gielen J., Villarroel R., Genetello C., van Montagu M., Depicker A., and Inze D. (1991). The extensin signal peptide allows secretion of a heterologous protein from protoplasts. *Gene*, 99: 95-100.

Demura T., and Fukuda H. (1994). Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. *Plant Cell*, 6: 967-981.

de Oliveira D.E., Seurinck J., Inze D., van Montagu M., and Botterman J. (1990). Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins. *Plant Cell*, 2: 427-436.

De S Goldman M.H., De S Pezzotti M., Suerinck J., and Mariani C. (1992). Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. *Plant Cell*, 4: 1041-1051.

de Silva J., Jarman C.D., Arrowsmith D.A, Stronach M.S., Chengappa S., Sidebottom C., and Reid J.S.G. (1993). Molecular characterization of a xyloglucan-specific endo-(1-4)-β-D-glucanase (xyloglucan endo-transglycosylase) from *Nasturtium* seeds. *Plant J.*, 3: 701-711.

Dey P. M., and Campillo E.D. (1984). Biochemistry of the multiple forms of glycosidases in plants. *Adv. Emzymol.*, 56: 141-149.

Doares S.H., Syrovets T., Weiler E.W., and Ryan C.A. (1995).

Oligogalacturonids and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA*, 92: 4095-4098.

Domingo C., Gomez M.D., Canas L., Hernandez-Yago J., Conejero V., and Vera P. (1994). A novel extracellular matrix protein from tomato associated with lignified secondary cell walls. *Plant Cell*, 6: 1035-1047.

Eberhard S., Doubrava N., Marfa V., Mohnen D., Southwick A., Darvill A., and Alberheim P. (1989). Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. *Plant Cell*, 1: 747-755.

Ecker J.R., and Davis R.W. (1987). Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci.* USA, 84: 5202-5206.

Edreva A.M., and Georgieva I.D. (1980). Biochemical and histochemical investigations of α- and β-glucosidase activity in an infectious desease, a physiological disorder and in senescence of tobacco leaves. *Physiol. Plant Pathol.*, 17: 237-243.

Edwards S.M., Dea I.C.M., Bulpin P.V., and Reid J.S.G. (1985). Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeloum majus* L seeds following germination. *Planta*, 163: 133-140.

Enyedi A.J., Yalpani N., Silverman P., and Raskin I. (1992). Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA.*, 89: 2480-2484.

Epstein L., and Lamport D.T.A. (1984). An intramolecular linkage involving isodityrosine in extensin. *Phytochemistry*, 23:1241-1246.

Ertl H., Hallman A., Wenzi S., and Sumper M. (1992). A novel extensin that may organise extracellular matrix biogenesis in *Volvox carteri*. *EMBO J.*, 11: 2055-2062.

Esquerre-Tugaye M.T., and Lamport D.T.A. (1979). Cell surface in plant-microorganism interactions. I. A structural investigation of cell wall hydroxyproline-rich glycoproteins which accumulate in fungus-infected plants. *Plant Physiol.*, 64: 314-319.

Esquerre-Tugaye M.T., Lafitte C., Mazau D., Toppan A., and Touze A. (1979). Cell surfaces in plant-microorganism interactions. II. Evidence for the accumulation of hydroxyproline-rich glycoproteins in the cell wall of

diseased plants as a defense mechanism. *Plant Physiol.*, 64: 320-326.

Evans M., Gatehouse L.N., Gatehouse J.A., Yarwood J.N., Boulter D., and Croy R.R.D. (1990). The extensin gene family in oilseed rape (*Brassica napus*): Characterization of sequences of representative members of the family. *Mol. Gen. Genet.*, 223: 273-287.

Evrard J.L., Jako C., Saint-Guily A., Weil J.H., and Kuntz M. (1991). Anther-specific, developmentally regulated expression of genes encoding a new class of proline-rich proteins in sunflower. *Plant Mol. Biol.*, 16: 271-281.

Farmer E.E., Caldelari D., Pearce G., Walker-simmons M.K., and Ryan C.A. (1994). Diethylthiocarbamic acid inhibits the octadecanoid signalling pathway for the wound induction of proteinase inhibitors in tomato leaves. *Plant Physiol.*, 106: 337-342.

Farmer E.E., and Ryan C.A. (1990). Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. USA*, 87: 7713-7716.

Farmer E.E., and Ryan C.A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell*, 4: 129-134.

Feng Y., and Boersma L. (1995). Kinematics of axial plant root growth. *J. Theor. Biol.*, 174: 109-117.

Fincher G.B., Stone B.A., and Clarke A.E. (1983). Arabinogalactan proteins: Structure, Biosynthesis and Function. *Ann. Rev. Plant Physiol.*, 34: 47-70.

Fong C., Kieliszewski M.J., de Zacks R., Leykam J.F., and Lamport D.T.A. (1992). A gymnosperm extensin contains the serine-tetrahydroxyproline motif. *Plant Physiol.*, 99: 548-552.

Fourney R.M., Miyakoshi J., Day III R.S., and Paterson M.C. Northern blotting: Efficient RNA staining and transfer. Focus 10 (1) BRL Gaithersburg.

Franssen H.J., Nap J-P., Gloudemans T., Stiekema W., Van Dam H., Govers F., Louwerse J., Van Kammen A., and Bisseling T. (1987). Characterization of cDNA for nodulin-75 of soybean: A gene product involved in early stage of root nodule development. *Proc. Natl. Acad.*

Sci. USA, 84: 4495-4499.

Fritz S.E., Hood K.R., and Hood E.E. (1991). Localization of soluble and insoluble fractions of hydroxyproline-rich glycoproteins during maize kernel development. *J. Cell Sci.*, 98: 545-550.

Fry S.C. (1979). Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. *Planta*, 146: 343-351.

Fry S.C. (1980). Gibberellin-controlled pectinic acid and protein secretion in growing cells. *Phytochemistry*, 19: 735-740.

Fry S.C. (1982). Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. *Biochem. J.*, 203: 449-455.

Fry S.C. (1983). Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta*, 157: 111-123.

Fry S.C. (1989a). Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Physiol. Plant.*, 75: 532-536.

Fry S.C. (1989b). The structure and function of xyloglucan. *J. Exp. Botany*, 40: 1-11.

Fry S.C., Smith R.C., Renwick K.F., Martin D.J., Hodge S.K., and Matthews K.J. (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.*, 282: 821-828.

Gaffney T., Friedrich L., Vernooij B., Negratto D., Nye G., Uknes S., Ward E.R., Kessmann H., and Ryals J.A. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, 261: 754-756.

Garcia-Mas J., Messeguer R., Arus P., and Puigdomenech P. (1992). The extensin from *Prunus amygdalus*. *Plant Physiol.*, 100: 1603-1604.

Gaspar T., Penel C., Thorpe T., and Greppin H. (1982). Peroxidases. A Survey of Their Biochemical and Physiological Roles in Higher Plants. Geneva: Univ. Geneva Press.

Gatehouse L.N., Evans M.I., Gatehouse J.A., and Croy R.R.D. (1990). Characterisation of a rape (*Brassica napus* L.) extensin gene encoding a polypeptide relatively rich in tyrosine. *Plant Sci.*, 71: 223-231.

Gleeson P.A., Stone B.A., and Fincher G.B. (1985). Cloning the cDNA for the

arabinogalactan-protein from *Lolium multiflorum* (ryegrass). *AGP news*, 5: 30-36.

Gleeson P.A., McNamara M., Wettenhall E.H., Stone B.A., and Fincher G.B. (1989). Characterization of the hydroxyproline-rich protein core of an arabinogalactan-protein secreted from suspension-cultured *Lolium multiflorum* (Italian ryegrass) endosperm cells. *Biochem. J.*, 264: 857-862.

Godoy J.A., Pardo J.M., and Pintor-Toro J.A. (1990). A tomato cDNA inducible by salt stress and abscisic acid: Nucleotide sequence and expression pattern. *Plant Mol. Biol.*, 15: 695-705.

Gomez J., Sanchez-Martinez D., Stiefel V., Rigau J., Puigdomenech P., and Pages M. (1988). A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature*, 334: 262-264.

Goodwin T.W., and Mercer E.I. (1988). Introduction to Plant Biochemistry, 2nd edition. Pergamon Press.

Grillo S., Leone A., Xu Y., Tucci M., Francione R., Hasegawa P.M., Momti L., and Bressan R.A. (1995). Control of osmotin gene expression by ABA and osmotic stress in vegetative tissues of wild-type and ABA-deficient mutants of tomato. *Physiol. Plant.*, 93: 498-504.

Gundlach H., Muller M.J., Kutchan T.M., and Zenk M.H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. USA*, 89: 2389-2393.

Guo Y., Delseny M., and Puigdomenech P. (1994). mRNA accumulation and promoter activity of the gene coding for a hydroxyproline-rich glycoprotein in *Oryza sativa*. *Plant Mol. Biol.*, 25: 159-165.

Hall M.A. (1976). Plant Structure, Functions and Adaptations. MacMillan, New York.

Hammerschmidt R., Lamport D.T.A., and Muldoon E.P. (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum*. *Physiol. Plant Pathology*, 24: 43-47.

Harms K., Atzorn R., Brash A., Kuhn H., Wasternack C., Willmitzer L., and

Pena-Cortes H. (1995). Expression of the flax allene oxide synthase cDNA leads to increased endogenous jasmonic acid (JA) levels in transgenic potato plants but not to a corresponding activation of JA-responding genes. *Plant Cell*, 7: 1645-1654.

Heckman J.W., Terhune B.T., and Lamport D.T.A. (1988). Characterization of native and modified extensin monomers and oligomers by electron microscopy and gel filtration. *Plant Physiol.*, 86: 848-856.

Heikkila J.J., Papp J.R.T, Schultz G.A., and Bewley J.D. (1984). Induction of heat shock protein messenger RNA in maize mesocotyls by water stress, abscisic acid and wounding. *Plant Physiol.*, 76: 270-274.

Heitz T., Bergey D.R., and Ryan C.A. (1997). A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. *Plant Physiol.*, 114: 1085-1093.

Herde O., Atzorn R., Fisahn J., Wasternack C., Willmitzer L., and Pena-Cortes H. (1996). Localised wounding by heat initiates the accumulation of proteinase inhibitor II in abscisic acid-deficient plants by triggering jasmonic acid biosynthesis. *Plant Physiol.*, 112: 853-860.

Herde O., Fuss H., Pena-Cortes H., and Fisahn J. (1995). Proteinase inhibitor II gene expression induced by electrical stimulation and control of photosynthetic activity in tomato plants. *Plant Cell Physiol.*, 36: 737-742.

Herdenberger F., Evrard J.L., Kuntz M., Tessier L.H., Klein A., Steinmetz A., and Pillay D.T.N. (1990). Isolation of flower-specific cDNA clones from sunflower. *Plant Sci.*, 69: 111-122.

Herman E.M., and Lamb C.J. (1992). Arabinogalactan-rich glycoproteins are localised on the cell surface and in intravacuolar multivesicular bodies. *Plant Physiol.*, 98: 264-272.

Hetherington A.M., and Quatrano R.S. (1991). Mechanisms of action of abscisic acid at the cellular level. *New Phytol.* 119: 9-32.

Hildmann T., Ebneth M., Pena-Cortes H., Sanchez-Serrano J.J., Willmitzer L., and Prat S. (1992). General role of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell*, 4:

1157-1170.

Hirsinger C., Parmentier Y., Durr A., Fleck J., and Jamet E. (1997). Characterization of a tobacco extensin gene and regulation of its gene family in healthy plants and under various stress conditions. *Plant Mol. Biol.*, 33: 279-289.

Hoagland D.R., and Arnon D.I. (1950). The water-culture method for growing plants without soil. *Calif. Agric. Exp. Circ.*, 347: 1-32.

Hong J.C., Cheong Y.H., Nagao R.T., Bahk J.D., Cho M.J., and Key J.L. (1994). Isolation and characterization of three soybean extensin cDNAs. *Plant Physiol.*, 104: 793-796.

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

Hong J.C., Nagao R.T., and Key J.L. (1989). Developmentally regulated expression of soybean proline-rich cell wall protein genes. *Plant Cell*, 1: 937-943.

Hong J.C., Nagao R.T., and Key J.L. (1990). Characterization of a proline-rich cell wall protein gene family of soybean. A comparative analysis. *J. Biol. Chem.*, 265: 2470-2475.

Hood E.E., Shen Q.X., and Varner J.E. (1988). A developmentally regulated hydroxyproline-rich glycoprotein in maize pericarp cell walls. *Plant Physiol.*, 87: 138-142.

Hoson T., Nishitani K., Miyomoto K., Ueda J., Kamisaka S., Yamamoto R., and Masuda Y. (1996). Effects of hypergravity on growth and cell wall properties of cress hypocotyls. *J. Expt. Botany*, 297: 513-517.

Howe G.A., Lightner J., Browse J., and Ryan C.A. (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signalling for defense against insect attack. *Plant Cell*, 8: 2067-2077.

Iuchi S., Yamaguchi-Shinozaki K., Urao T., Terao T., and Shinozaki K. (1996). Novel drought-inducible genes in the highly drought-tolerant cowpea: cloning of cDNAs and analysis of the expression on the corresponding genes. *Plant Cell Physiol.*, 37: 1073-1082.

Jarvis M.C. (1984). Structure and properties of pectin gels in plant cell walls.

Plant Cell Environ., 7: 153-164.

Jarvis M.C., Hall M.A., Threlfall D.R., and Friend J. (1981). The polysaccharide structure of potato cell walls - chemical fractionation. *Planta*, 152: 93-100.

Jermyn M.A. (1980). The nature of the protein moiety of the arabinogalactan proteins. *AGP News*, 3: 26-31.

Jose-Estanyol M., Ruiz-Avila L., and Puigdomenech P. (1992). A maize embryo-specific gene encodes a proline-rich hydrophobic protein. *Plant Cell*, 4; 413-423.

Jose M., and Puigdomenech P. (1993). Structure and expression of genes coding for structural proteins of the plant cell wall. *New Phytol.*, 125: 259-282.

Joshi C.P. (1987a). Putative polyadenylation signals in nuclear genes of higher plants : a compilation and analysis. *Nuc. Acids Res.*, 15: 9627-9640.

Joshi C.P. (1987b). An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nuc. Acids Res.*, 15: 6643-6653.

Kawalleck P., Schmelzer E., Hahlbrock K., and Somssich I.E. (1995). Two pathogen-responsive genes in parsley encode a tyrosine-rich hydroxyproline-rich glycoprotein (HRGP) and an anionic peroxidase. *Mol. Gen. Genet.*, 247: 444-452.

Kawasaki S. (1991). Extensin secreted into the culture medium of tobacco cells. II. Extensin subfamilies of similar composition. *Plant Cell Physiol.*, 32: 721-728.

Keegstra K., Talmadge K.W., Bauer W.D., and Albersheim P. (1973). The structure of plant cell walls. III - A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.*, 51: 188-196.

Keller B., and Baumgartner C. (1991). Vascular-specific expression of the bean GRP 1.8 gene is negatively regulated. *Plant Cell*, 3: 1051-1061.

Keller B., and Lamb C.J. (1989). Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes*

Dev., 3: 1639-1646.

Keller B., Sauer N., and Lamb C.J. (1988). Glycine-rich cell wall protein s in bean: Gene structure and association of the protein with the vascular system. *EMBO J.*, 7: 3625-3633.

Keller B., Schmid J., and Lamb C.J. (1989a). vascular expression of a bean cell wall glycine-rich protein-β-glucuronidase gene fusion in transgenic tobacco. *EMBO J.*, 8: 1309-1314.

Keller B., Templeton M.D., and Lamb C.J. (1989b). Specific localization of a plant cell wall lgycine-rich protein in protoxylem cells of the vascular system. *Proc. Nayl. Acad. Sci. USA*, 86: 1529-1533.

Kieliszewski M., De Zacks R., Leykam J.F., and Lamport D.T.A. (1992). A repetitive proline-rich protein from the gymnosperm Douglas Fir is a hydroxyproline-rich glycoprotein. *Plant Physiol.*, 98: 919-926.

Kieliszewski M., and Lamport D.T.A. (1987). Purification and partial characterization of a hydroxyproline-rich glycoprotein in a graminaceous monocot, *Zea mays*. *Plant Physiol.*, 85: 823-827.

Kieliszewski M., and Lamport D.T.A. (1988). Tying the knots in the extensin network. *In* Self-assembling Architecture (Varner J.E., ed.). New York: Alan R. Liss, pp. 61-76.

Kieliszewski M.J., and Lamport D.T.A. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant J.*, 5: 157-172.

Kieliszewski M., Leykam J.F., and Lamport D.T.A. (1990). Structure of threonine-rich extensin from *Zea mays*. *Plant Physiol.*, 92: 316-326.

Kleis-San Francisco S.M., and Tierney M.L. (1990). Isolation and characterization of a proline-rich cell wall protein from soybean seedling. *Plant Physiol.*, 94: 1897-1902.

Klis F.M. (1976). Glycosylated seryl residues in wall protein of elongating pea stems. *Plant Physiol.*, 57: 224-226.

Knox R.B., Clarke A., Harrison S., Smith P., and Marchalonis J.J. (1976). Cell recognition in plants: determinants of the stigma surface and their pollen interactions. *Plant Physiol.*, 73: 2788-2792.

Labavitch J.M., and Ray P.M. (1974). Relationship between promotion of

xyloglucan metabolism and induction of elongation by indoleacetic acid. *Plant Physiol.*, 54: 499-502.

Labavitch J.M. (1981). Cell wall turnover in plant development. *Ann. Rev. Plant Physiol.*, 32: 385-406.

Lagrimini L.M., and Rothstein S. (1987). Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.*, 84: 438-442.

Lamport D.T.A. (1967). Hydroxyproline-O-glycosidic linkage of the plant cell wallglycoprotein extensin. *Nature*, 216: 1322-1324.

Lamport D.T.A. (1969). The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymatic degradation of primary cell walls. *Biochemistry*, 8: 1155-1163.

Lamport D.T.A. (1986). The primary cell wall: a new model. *In* Cellulose: Structures, Modification, and Hydrolysis (Young R.A., and Rowell R., eds.). New York: Wiley Interscience, pp. 77-89.

Lamport D.T.A., and Catt J.W. (1981). Glycoproteins and enzymes of the cell wall. *In* Encyclopaedia of Plant Physiology (new. ser.). Plant carbohydrate. II: (Tanner W., and Holmes F.A. eds.). New York: Springer-Verlag, pp. 133-165.

Lamport D.T.A., and Epstein L. (1983). A new model for the primary cell wall: a concatenated extensin-celloluse network. *In* Current Topics in Plant Biochemistry and Physiology, Volume II (Randa D.D., Blevins D.G., Larson R.S., and Rapp B.S., eds.). Columbia: University of Missouri, pp. 73-83.

Lamport D.T.A., and Miller D.H. (1971). Hydroxyproline arabinosides in the plant kingdom. *Plant Physiol.*, 48: 454-456.

Lamport D.T.A., Katona L., and Roering S. (1973). Galactosylserine in extensin. *Biochem. J.*, 133: 125-131.

Larkin P.J. (1977a). Plant protoplast agglutination and membrane-bound β-lectins. *J. Cell Sci.*, 26: 31-46.

Larkin P.J. (1977b). Plant protoplast agglutination by artificial carbohydrate antigens. *J. Cell Sci.*, 30: 283-292.

Lau J.M., McNeil M., Darvill A.G., and Albersheim P. (1985). Structure of the

backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary walls of plants. *Carbohydr. Res.*, 137: 111-125.

Lawton M.A., and Lamb C.J. (1987). Transcriptional activation of plant defense genes by elicitor, wounding and infection. *Mol. Cell. Biol.*, 7: 335-341.

Leach J.E., Cantrell M.A., and Sequeira L. (1982). Hydroxyproline-rich bacterial agglutinin from potato: Extraction, purification, and characterization. *Plant Physiol.*, 70: 1353-1358.

Lei M., and Wu R. (1991). A novel glycine-rich cell wall protein gene in rice. *Plant Mol. Biol.*, 16: 187-198.

Levy S., Yorh W.S., Stuike-Prill R., Meyer B., and Staehelin L.A. (1991). Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *Plant J.*, 1: 195-215.

Li S.X., and Showalter A.M. (1996). Immunolocalization of extensin and potato tuber lectin in carrot, tomato and potato. *Physiol. Plant.*, 97: 708-718.

Li X-B., Kieliszewski M., and Lamport D.T.A. (1990). A chenopod extensin lacks repetitive tetrahydroxyrpoline blocks. *Plant Physiol.*, 92: 327-333.

Linthorst H.J.M., van Loon L.C., Memelink J., and Bol J.F. (1990). Characterization of cDNA clones for a virus-inducible, glycine-rich protein from petunia. *Plant Mol. Biol.*, 15: 521-523.

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

Lora J.M., Cruz J.D.L., Benitez T., Llobell A., Pintor-Toro J.A. (1994). A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. *Mol. Gen. Genet.*, 242: 461-466.

Ludevid M.D., Ruiz-Avila L., Valles M.P., Steifel V., Torrent M., Torne J.M., and Puigdomenech P. (1990). Expression of genes for cell-wall proteins in dividing and wounded tissues of *Zea mays* L. *Planta*, 180: 524-529.

Malamy J., Carr J.P., Klessig D.F., and Raskin I. (1990). Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral

infection. *Science*, 250: 1002-1004.

Marfa V., Gollin D.J., Eberhard S., Mohnen D., Darvill A., and Alberheim P. (1991). Oligogalacturonidases are able to induce flowers to form ion tobacco explants. *Plant J.*, 1: 217-225.

Mason H.S., and Mullet J.S. (1990). Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. *Plant Cell*, 2: 569-579.

Matile P. (1975). The Lytic Compartment of Plant Cells. Vienna: Springer-Verlag.

Matsumoto I., Jimbo A., Mizuno Y., Seno N., and Jeanloz R.W. (1983). Purification and characterization of potato lectin. *J. Biol. Chem.*, 258: 2886-2891.

Mauch F., Hadwiger L.A., and Boller T. (1984). Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. *Plant Physiol.*, 76: 607-611.

Mazau D., and Esquerre-Tugaye M.T. (1986). Hydroxyproline-rich glycoprotein accumulation in the cell walls of plants infected by various pathogens. *Physiol. Mol. Plant Pathol.*, 29: 147-157.

Mazau D., Rumeau D., and Esquerre-Tugaye M.T. (1988). Two different families of hydroxyproline-rich glycoproteins in melon callus. Biochemical and immunochemical studies. *Plant Physiol.*, 86: 540-546.

McCann M.C., Wells B., and Roberts K. (1990). Direct visualisation of cross-links in the primary plant cell wall. *J. Cell Sci.*, 96: 323-334.

McCann M.C., Wells B., and Roberts K. (1992b). Complexity in the spatial localization and length distribution of plant cell wall matrix polysaccharides. *J. Microscopy*, 166: 123-136.

McDougall G.J., and Fry S.C. (1990). Xyloglucan oligosaccharides promote growth and activate cellulase: Evidence for a role of cellulose in cell expansion. *Plant Physiol.*, 93: 1042-1048.

McNeil M., Darvill A.G., Fry S.C., and Albersheim P. (1984). Structure and function of the primary cell walls of plants. *Ann. Rev. Biochem.*, 53: 625-663.

McQueen-Mason S., Durako D.M., and Cosgrove D.J. (1992). Two

endogenous proteins that induce cell wall extension in plants. *Plant Cell*, 4: 1425-1433.

McQueen-Mason S.J. (1995). Expansins and cell wall expansion. *J. Exp. Botany*, 46: 1639-1650.

Mellon J.E., and Helgeson J.P. (1982). Interaction of a hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. *Plant Physiol.*, 70: 401-405.

Memelink J., Hoge J.H.C., and Schilperoort R.A. (1987). Cytokinin stress changes the developmental regulation of several defense-related genes in tobacco. *EMBO J.*, 6: 3579-3583.

Memelink J., Swords K.M.M., de Kam R.J., Schilperoort R.A., Hoge J.H.C., and Staehelin L.A. (1993). Structure and regulation of tobacco extensin. *Plant J.*, 4:1011-1022.

Metraux J.P., Signer H., Ryals J.A., Ward. E.R., Wyss-Benz M., Gaudin J., Raschdorf K., Schmid E., Blum W., and Inverardi B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, 250: 1004-1006.

Millane R.P., and Narasaiah T.V. (1992). The molecular structure of tamarind seed polysaccharide. *In* Gums and Stabiliser for the Food Industry 6 (Phillips G.O., William P.A., and Wedlock D.J., eds.). Oxford: IRL Press, pp. 535-539.

Molina A., Mena M., Carbonero P., and Garcia-Olmedo F. (1997). Differential expression of pathogen-responsive genes encoding two types of glycine-rich proteins in barley. *Plant Mol. Biol.*, 33: 803-810.

Monro J.A., Penny D., and Bailey R.W. (1976). The organization and growth of primary cell walls of lupine hypocotyls. Phytochemistry, 15: 1193-1198.

Mouly A., Remeau D., and Esquerre-Tugaye M.T. (1992). Differential accumulation of hydroxyproline-rich glycoprotein transcripts in sunflower plants infected with Sclerotinia sclerotiorum or treated with oxalic acid. *Plant Sci.*, 85: 51-59.

Mundy J., and Chua N.H. (1988). Abscisic acid and water stress induce the expression of a novel rice gene. *EMBO J*, 7: 2279-2286.

Niebel A., de Almeida Engler J., Tire C., Engler G., Montagu M.V., and Gheysen G. (1993). Induction patterns of an extensin gene in tobacco upon nematode infection. *Plant Cell*, 5: 1697-1710.

Nicklas K.J. (1992). Plant Biomechanics - An Engineering Approach to Plant Form and Function. The University of Chicago Press, pp. 234-260.

Norman P.M., Kjellbom P., Bradley D.J., Hahn M.G., and Lamb C.J. (1990). Immunoaffinity purification and biochemical characterization of plasma membrane arabino-galactan-rich glycoproteins of *Nicotiana glutinosa*. *Planta*, 181: 365-373.

Northcote D.H. (1972). Chemistry of the plant cell wall. *Ann. Rev. Plant Physiol.*, 23: 113-132.

Obokata J., Ohme M., and Hayashida N. (1991). Nucleotide sequence of a cDNA clone encoding a putative glycine-rich protein of 19.7 kDa in *Nicotiana sylvestris*. *Plant Mol. Biol.*, 19: 953-95.

O'Neill M.A., and Selvendran R.R. (1983). Isolation and partial characterization of a xyloglucan from the cell walls of *Phaseolus coccineus*. *Carbohyd. Res.*, 111: 239-255.

Palmieri S., Odoardi M., Soressi G.P., and Salamini F. (1978). Indolacetic acid oxidase activity in two high-peroxidase tomato mutants. *Physiol. Plant.*, 42: 85-90.

Parmentier Y., Durr A., Marbach J., Hirsinger C., Criqui M.C., Fleck J., and Jamet E. (1995). A novel wound-inducible extensin gene is expressed early in newly isolated protoplasts of *Nicotiana sylvestris*. *Plant Mol. Biol.*, 29: 279-292.

Pearce G., Strydom D., Johnson S., and Ryan C.A. (1991). A polypeptide from tomato leaves induces the synthesis of wound-inducible proteinase inhibitor proteins. *Sciences*, 253: 895-898.

Pena-Cortes H., Albrech T., Prat S., Weiler E.W., and Willmitzer. L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by bloking jasmonic acid biosynthesis. *Planta*, 191: 123-128.

Pena-Cortes H., Fisahn J., and Willmitzer L. (1995). Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. *Proc. Natl. Acad. Sci. USA*, 92: 4106-4113.

Pena-Cortes H., Liu X., Sanchez-Serrano J., Schmidt R., and Willmitzer L. (1992). Factors affecting gene expression of patatin and proteinase-inhibitor-II gene families in detached potato leaves: implication for their co-expression in developing tubers. *Planta*, 186: 495-502.

Pena-Cortes H., Prat S., Atzorn R., Wasternack C., and Willmitzer L. (1996). Abscisic acid-deficient plants do not accumulate proteinase inhibitor II following systemin treatment. *Planta*, 198: 447-451.

Pena-Cortes H., Sanchez-Serrano J.J., Mertens R., Willmitzer L., and Prat S. (1989). Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. *Proc. Natl. Acad. Sci. USA*, 86: 9851-9855.

Pena-Cortes H., Willmitzer L., and Sanchez-Serrano J.J. (1991). Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family. *Plant Cell*, 3: 963-972.

Pennel R.I., and Roberts K. (1990). Sexual development in the pea is presage by altered expression of arabinogalactan protein. *Nature*, 334: 547-549.

Pennel R.I., Janniche L., Kjellbom P., Scofield G.N., Peart J.M., and Roberts K. (1991). Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *Plant Cell*, 3: 1317-1326.

Pennel R.I., Knox J.P., Scifield G.N., Selvendran R.R., and Roberts K. (1989). A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. *J. Cell Biol.*, 108: 1967-1977.

Peschkle V.M., and Sachs M.M. (1994). Characterization and expression of transcripts induced by oxygen deprivation in maize (*Zea mays* L.). *Plant Physiol.*, 104: 387-394.

Pont-Lezica R.F., Taylor R., and Varner J.E. (1991). *Solanum toberosum* agglutinin accumulation during tuber development. *Plant Physiol.*, 137: 453-458.

Powell D.A., Morris E.R., Gidley M.J., and Rees D.A. (1982). Conformations

and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. *J. Mol. Biol.*, 155: 517-531.

Preston R.D. (1979). Polysaccharide conformation and cell wall function. *Ann. Rev. Plant Physiol.*, 30: 55-78.

Promega Protocol and Application Guide. Second edition (1991). Promega corporation.

Quigley F., Villiot M.L., and Mache R. (1991). Nucleotide sequence and expression of a novel glycine-rich protein gene from *Arabidopsis thaliana*. *Plant Mol. Biol.*, 17: 949-952.

Rackis JJ., Anderson R.L., Sasame H.A., Smith A.K., and Van Etten C.H. (1961). Amino acid in soybean hulls and oil meal fractions. *J. Agric. Food Chem.*, 9: 409-412.

Raghothama K.G., Liu D., Nelson D.E., Hasegawa P.M., and Bressan R.A. (1993). Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. *Plant Mol. Biol.*,. 23: 1117-1128.

Raines C.A., Lloyd J.C., Chao S., John U.P., and Murphy G.J.P. (1991). A novel proline-rich protein from wheat. *Plant Mol. Biol.*, 16: 663-670.

Raskin I. (1992). Role of salicylic acid in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 43: 439-463.

Rasmussen J.B., Hammerschmidt R., and Zook M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after innoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiol.*, 97: 1342-1347.

Raz R., Cretin C., Puigdomenech P., and Martinez-Izquierdo J.A. (1991). The sequence of a hydroxyproline-rich glycoprotein gene from *Sorghum vulgare*. *Plant Mol. Biol.*, 16: 365-367.

Raz R., Jose M., Moya A., Martinez-Izquierdo J.A., and Puigdomenech P. (1992). Different mechanisms generating sequence variability are revealed in distinct regions of the hydroxyproline-rich glycoprotein gene from maize and related species. *Mol. Gen. Genet.*, 233: 252-259.

Rees D.A. (1977). Polysaccharide Shapes. London: Chapman and Hall.

Ring S.G., and Selvendran R.R. (1981). An arabinogalactoxyloglucan from the cell wall of *Solanum tuberosum*. *Phytochemistry*, 20: 2511-2519.

Robinson N.J., Shirsat A.H., and Gatehouse J.A. (1993). Regulation of gene expression. *In* Plant Biochemistry and Molecular Biology (Lea P.J. and Leegood R.C.). John Wiley and Sons, pp. 221-240.

Roby D., Toppan A., Esquerre-Tugaye M.T. (1985). Cell surface in plant-microorganisms interactions. V. Elicitors of fungal and of plant origin trigger the synthesis of ethylene and of cell wall hydroxyproline-rich glycoprotein in plants. *Plant Physiol.*, 77: 700-704.

Rodriguez J.G., and Cardemil L. (1995). Tissue specific expression of cell wall proteins of seedling of *Prosopis chilensis* during development and wound stress. *Physiol. Plant.*, 93: 457-463.

Ruiz-Avila L., Ludevid M.D., and Piugdomenech P. (1991). Differential expression of a hydroxyproline-rich cell wall protein gene in embryogenic tissues of *Zea mays* L. *Planta*, 184: 130-136.

Rushing J.W., and Huber D.J. (1984). *In vitro* characterization of tomato fruit ripening. *Plant Physiol.*, 75: 891-894.

Ryser U., and Keller B. (1992). Untrastructural localization of a bean glycine-rich protein in unlignified primary walls of protoxylem cells. *Plant Cell*, 4: 773-783.

Salts Y., Wachs R., Gruissem W., and Barg R. (1991). Sequence coding for a novel proline-rich protein preferentially expressed in young tomato fruit. *Plant Mol. Biol.*, 17: 149-150.

Sanchez M.C., Smith A.G., and Hackett W.P. (1995). Localized expression of a proline-rich protein gene in juvenile and mature Ivy petioles in relation to rooting competence. *Physiol. Plant.*, 93: 207-216.

Sanchez-Serrano J.J., Schmidt R., Schell J., and Willmitzer L. (1986). Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. *Mol. Gen. Genet.*, 203: 15-20.

Sanger F., Nicklen S., and Coulson A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, 74: 5463-5467.

Scheggia C., Prisco A.E., Dey P.M., Caleo G.R., and Pont Lezica R. (1988). Alteration of pectin pattern in potato tuber by virus X. *Plant Sci.*, 58: 9-14.

Schneiderbauer A., Back E., Sandermann H., and Ernst D. (1995). Ozone induction of extensin mRNA in Scots Pine, Norway Spruce and European Beech. *New Phythol.*, 130: 225-230.

Schweizer P., Buchala A., Silverman P., Seskar M., Raskin I., and Metraux J.P. (1997). Jasmonate-inducible genes are activated in rice by pathogen attack without a concomitant increase in endogenous jasmonic acid levels. *Plant Physiol.*, 114: 79-88.

Sequeira L. (1983). Mechanism of induced resistance in plants. *Ann. Rev. Microbiol.*, 37: 51-79.

Sequeira L., and Graham T.L. (1977). Agglutination of avirulent strains of Pseudomonas solanacearum by potato lectin. *Physiol. Plant Pathol.*, 11: 43-54.

Shah J., Tsui F., and Klessig D.F. (1997). Characterization of a salicylic acid-insensitive mutants (Sai1) of *Arabidopsis thaliana* identified in a selection screen utilizing the salicylic acid-inducible expression of the tms2 gene. *Mol. Plant-Microb Interac.*, 10: 69-78.

Shcherban T.Y., Shi J., Durako D.M., Guiltinam M.J., McQueen-Mason S.J., Shieh M., and Cosgrove D.J. (1995). Molecular cloning and sequencing analysis of expansins - a highly conserved, multigene family of proteins that mediate cell wall extension in plants. *Proc. Natl. Acad. Sci. USA*, 92: 9245-9249.

Sheng J., D'Ovidio R., and Mehdy M.C. (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. *Plant J.*, 1: 345-354.

Shing L., Outlaw W.H., and Epstein L.M. (1995). Expression of an mRNA with sequence similarity to pea dehydrin (Psdhn 1) in guard cells in *Vicia faba* in response to exogenous abscisic acid. *Physiol. Plant.*, 95: 99-105.

Shirsat A.H., Bell A., Spence J., and Harris J.N. (1996a). The *Brassica napus ExtA* extensin gene is expressed in regions of the plant subject to tensile stress. *Planta*, 199: 618-624.

Shirsat A.H., Wieczorek D., and Kozbial P. (1996b). A gene for *Brassica napus* extensin is differentially expressed on wounding. *Plant Mol.*

Biol., 30: 1291-1300.

Shirsat A.H., Wilford N., Evans I.M., Gatehouse L.N., and Croy R.R.D. (1991). Expression of a *Brassica napus* extensin gene in the vascular system of transgenic tobacco and rape plants. *Plant Mol. Biol.*, 17: 701-709.

Short J.M., Fernandez J.M., Sorge J.A., and Huse W.D. (1988). λZAP: a bacteriophage λ expression vector with *in vivo* excision properties. *Nuc. Acid Res.*, 16: 7583-7600.

Showalter A.M. (1993). Structure and function of plant cell wall proteins. *Plant Cell*, 5: 9-23.

Showalter A.M., and Remeau D. (1990). Molecular biology of the plant cell wall hydroxyproline-rich glycoproteins. *In* Organisation and Assembly of Plant and Animal Extracellular Matrix (Adair W.S., and Mecham R.P., eds.). New York: Academic Press, pp. 247-281.

Showalter A.M., and Varner J.E. (1989). Plant hydroxyproline-rich glycoproteins. *In* The Biochemistry of Plants, Volume 15 (Stumpt P.K., and Conn E.E., eds.). New York: Academic Press, pp. 485-520.

Showalter A.M., Bell J.N., Cramer C.L., Bailey J.A., Varner J.E., and Lamb C.J. (1985). Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. *Proc. Natl. Acad. Sci. USA*, 82: 6551-6555.

Showalter A.M., Butt A.D., and Kim S. (1992). Molecular details of tomato extensin and glycine-rich protein gene expression. *Plant Mol. Biol.*, 19: 205-215.

Showalter A.M., Zhou J., Rumeau D., Worst S.G., and Varner J.E. (1991). Tomato extensin and extensin-like cDNAs: structure and expression in response to wounding. *Plant Mol. Biol.*, 16: 547-565.

Shu-xia L., and Showalter A. (1996). Immunolocalization of extensin and potato tuber lectin in carrot, tomato and potato. *Physiol. Plant.*, 97: 708-718.

Silva J.D., Jarman C.D., Arrowsmith D.A., Stronach M.S., Chengappa S., Sidebottom C., and Reid J.S.G. (1993). Molecular characterization of a xyloglucan-specific endo-(1→4)-D-glucanase (xyloglucan *endo*-transglycosylase) from nasturtium seeds. *Plant J.*, 3: 701-711.

Smith C.J. (1993). Carbohydrate chemistry. *In* Plant Biochemistry and Molecular Biology (Lea P.J., and Leegood R.C., eds). John Wiley and Sons Press, pp. 73-111.

Smith J.J., Muldoon E.P., and Lamport D.T.A. (1984). Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. *Phytochemistry*, 23: 1233-1239.

Smith J.J., Muldoon E.P., Willard JJ., and Lamport D.T.A. (1986). Tomato extensin precursors P1 and P2 are highly periodic structures. *Phytochemistry*, 25: 1021-1030.

Smith R.C., and Fry S.C. (1991). Endotransglycosylation of xyloglucans in plant cell suspension cultures. *Biochem. J.*, 279: 529-535.

Stafstrom J.P., and Staehelin L.A. (1986a). Cross-linking patterns in salt-extractable extensin from carrot cell walls. *Plant Physiol.*, 81: 234-241.

Stafstrom J.P., and Staehelin L.A. (1986b). The role of carbohydrate in maintaining extensin in an extended conformation. *Plant Physiol.*, 81: 242-246.

Stafstrom J.P., and Staehelin L.A. (1988). Antibody localization of extensin in cell walls of carrot storage roots. *Planta*, 174: 321-332.

Staswick P.E. (1990). Novel regulation of vegetative storage protein genes. *Plant Cell*, 2: 1-6.

Stermer B.A., and Hammerschmidt R. (1987). Association of heat shock induced resistance to disease with increased accumulation of insoluble extensin and ethylene synthesis. *Physiol. Mol. Plant Path.*, 31: 453-461.

Sticher L., Mauch-Mani B., and Metraux J.P. (1997). Systemic acquired resistance. *Ann. Rev. Phytopathol.*, 35: 235-270.

Stiefel V., Perez-Grau L., Albericio F., Giralt E., Ruiz-Avila L., Ludevid M.D., and Piugdomenech P. (1988). Molecular cloning of cDNAs encoding a putative cell wall protein from *Zea mays* and immunological identification of related polypeptides. *Plant Mol. Biol.*, 11: 483-493.

Stiefel V., Ruiz-Avila L., Raz R., Valles M.P., Gomez J., Pages M., Martinez-Izquierdo J.A., Ludevid M.D., Langdale J.A., Nelson T., and Puigdomenech P. (1990). Expression of a maize cell wall

hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation. *Plant Cell*, 2: 785-793.

Strobel G.A. (1974). Phytotoxins produced by plant parasites. *Ann. Rev. Plant Physiol.*, 25: 541-566.

Stuart D.A., and Varner J.E. (1980). Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. *Plant Physiol.*, 66: 787-792.

Sturm A. (1992). A wound-inducible glycine-rich protein from *Daucus carota* with homology to single-stranded nucleic acid-binding proteins. *Plant Physiol.*, 99: 1689-1692.

Suzuki H., Wagner T., and Tierney M.L. (1993). Differential expression of two soybean (*Glycine max* L.) proline-rich protein genes after wounding. *Plant Physiol.*, 101: 1283-1287.

Tagu D., Walter N., Ruiz-Avila L., Burgess S., Martinez-Izquierdo J.A., Leguay J.J., Netter P., and Puigdomenech P. (1992). Regulation of the maize HRGP gene expression by ethylene and wounding. mRNA accumulation and qualitative expression analysis of the promoter by microprojectile bombardmen. *Plant Mol. Biol.*, 20: 529-538.

Taiz L. (1984). Plant cell expansion: Regulation of cell wall mechanical properties. *Ann. Rev. Plant Physiol.*, 35: 585-657.

Talbott L.D., and Ray P.M. (1992). Molecular size and separability features of pea cell wall polysaccharides. Implications for models of primary wall structure. *Plant Physiol.*, 98: 357-368.

Taylor I.E.P., and Atkins E.D.T. (1985). X-ray diffraction studies on the xyloglucan from tamarind (*Tamarindus indica*) seeds. *FEBS Lett.*, 181: 300-302.

Templeton M.D., Dixon R.A., Lamb C.J., and Lawton M.A. (1990). Hydroxyproline-rich glycoprotein transcripts exhibit different spatial patterns of accumulation in compatible and incompatible interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. *Plant Physiol.*, 94: 1265-1269.

Tierney M.L., Wiechert J., and Pluymers D. (1988). Analysis of the expression of extensin and p33-related cell wall proteins in carrot and soybean.

Mol. Gen. Genet., 211: 393-399.

Tire C., Rycke R.D., Loose M.D., Inze D., Montagu M.V., and Engler G. (1994). Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. *Planta*, 195: 175-181.

Toppan A., Roby D., and Esquerre-Tugaye M.T. (1982). Defense mechanisms of plants against varietal non-specific pathogens. *In* Active Defense Mechanism in Plants (Wood R.K.S., ed.). New York: Plenum Press, pp. 103-117.

Ukness S., Mauch-Mani B., Moyer M., Potter S., Williams S., Dincher S., Chandler D., Slusarenko A., Ward E., and Ryals J. (1992). Acquired resistance in Arabidopsis. *Plant Cell*, 4: 645-656.

Valero P., and Labrador E. (1996). Effect of water stress on the LiCl extracted-cell wall proteins and glycanhydrolytic enzymes during growth of *Cicer arietinium* epicotyls. *Plant Physiol. Biochem.*, 34: 307-313.

van de Wiel C., Scheres B., Franssen H., van Lierop M.-J., van Lammeren A., van Kammen A., and Bisseling T. (1990). The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. *EMBO J.*, 9: 1-7.

van Holst G.J., and Fincher G.B. (1984). Polyproline II conformation in the protein component of arabinogalactan-protein from *Lolium multiflorum*. *Plant Physiol.*, 75: 1163-1164.

van Holst G.J., and Varner J.E. (1984). Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. *Plant Physiol.*, 74: 247-251.

van Huystee R.B., and Zheng X. (1995). Peanut peroxidase, its location and extensin, coniferyl oxidation. *Plant Physiol. Biochem.*, 33: 55-60.

van Kan J.A.L., Cornelissen B.J.C., and Bol J.F. (1988). A virus-inducible tobacco gene encoding a glycine-rich protein shares putative regulatory elements with the ribulose biphosphate carboxylase small subunit gene. *Mol. Plant-Microbe Interact.*, 1: 107-112.

Varner J.E., and Cassab G.I. (1986). A new protein in petunia. *Nature*, 323:

110.

Veluthambi K., Mahadeavn S., and Maheswari R. (1981). Trehalose toxicity in *Cuscuta reflexa*. *Plant Physiol.*, 68: 1369-1374.

Vernooij B., Friedrich L., Morse A., Reist R., Kolditz-Jawhar R., Ward E.R., Uknes S., Kessmann H., and Ryals J.A. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell*, 6: 959-965.

Vick B.A. and Zimmermann D.C. (1983). The biosynthesis of jasmonic acid : A physiological role for plant lipoxygenase. *Biochem. Biophys. Res. Commun.*, 111: 470-477.

von Heijne G. (1985). Signal sequences. The limits of variations. *J. Mol. Biol.*, 184: 99-105.

Walkinshaw M.D., and Arnott S. (1981). Conformations and interactions of pectins. II. Models for junction zones in pectinic acid and calcium pectate gels. *J. Mol. Biol.*, 153: 1075-1085.

Wang Z., and Brown D.D. (1991). A gene expression screen. *Proc. Natl. Acad. Sci. USA.*, 88: 11505-11509.

Ward E.R., Uknes S.J., Williams S.C., Dincher S.S., Wiederhold D.L., Alexander D.C., Ahl-Goy P., Matraux J.P., and Ryals J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, 3: 1085-1094.

Weiser R.L., Wallner S.J., and Waddell J.W. (1990). Cell wall and extensin mRNA changes during cold acclimation of pea seedlings. *Plant Physiol.*, 93: 1021-1026.

Wingate V.P.M., Lawton M.A., and Lamb C.J. (1988). Glutathion causes massive and selective induction of plant defense genes. *Plant Physiol.*, 87: 206-210.

Woessner J.P., and Goodenough U.W. (1989). Molecular characterization of a zygote wall protein: An extensin-like molecule in *Chlamydomonas reinhardtii*. *Plant Cell*, 1: 901-911.

Wojtaszek P., Trethowan J., and Bolwell G.P. (1995). Specificity in the immobilisation of cell wall proteins in response to different elicitor

molecules in suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). *Plant Mol. Biol.*, 28: 1075-1087.

Woo H.H., Hackett W.P., and Das A. (1994). Differential expression of a chlorophyll a/b-binding protein gene and a proline-rich protein gene in juvenile and mature phase English Ivy (*Hedera helix*). *Physiol. Plant.*, 92: 69-78.

Wu G., Shprtt B.J., Lawrence E.B., Leon J., Fitzsimmons K.C., Levine E.B., Raskin I., and Shah D.M. (1997). Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plant. *Plant Physiol.*, 115: 427-435.

Wyatt R.E., Nagao R.T., and Key J.L. (1992). Patterns of soybean proline-rich protein gene expression. *Plant Cell*, 4: 99-110.

Wycoff K.L., Powell P.A., Gonzales R.A., Corbin D.R., Lamb C., and Dixon R.A. (1995). Stress activation of a bean hydroxyproline-rich glycoprotein promoter is superimposed on a pattern of tissue-specific developmental expression. *Plant Physiol.*, 109: 41-52.

Xi X.Y., Behrens B.X., West P.R., and Mort A.J. (1995). Solubilisation and partial characterization of extensin fragments from cell walls of cotton suspension-cultures - evidence for a covalent cross-link between extensin and pectin. *Plant Physiol.*, 108: 1691-1701.

Xu D., Lei M., and Wu R. (1995). Expression of the rice *Osgrp1* promotor-*Gus* reporter gene is specifically associated with cell elongation/expansion and differentiation. *Plant Mol. Biol.*, 28: 455-471.

Xu Y., Buchholz W.G., DeRose R.T., and Hall T.C. (1995). Characterization of a rice gene family encoding root-specific proteins. *Plant Mol. Biol.*, 27: 237-248.

Yalpani N., Silverman P., Wilson T.M.A., Kleier D.A., and Raskin I. (1991). Salicylic acid is a systemic signal and an inducer on pathogenesis-related proteins in virus-infected tobacco. *Plant Cell*, 3: 809-818.

Yasuda E., Ebinuma H., and Wabiko H. (1997). A novel glycine-rich/hydrophobic 16 kDa polypeptide gene from tobacco: similarity to proline-rich protein genes and its wound-inducible and developmentally regulated epxression. *Plant Mol. Biol.*, 33: 667-678.

Ye Z.H., Song Y.R., Marcus A., and Varner J.E. (1991). Comparative localization of three classes of cell wall proteins. *Plant J.*, 1: 175-183.

Ye Z.H., and Varner J.E. (1991). Tissue specific expression of cell wall proteins in developing soybean tissues. *Plant Cell*, 3: 23-37.

Yoshida S., Forno D.A., Cook J.H., and Gomez K.A. (1976). Routine procedure for growing rice plants in culture solution. *In* Laboratory Manual for Physiological Studies in Rice. IRRI, Los Banos, Philippine, pp. 61-66.

Young D.H., and Pegg G.F. (1982). The action of tomato and *Verticillium albo-atrum* glycosidases on the hyphal wall of *V. albo-atrum*. *Physiol. Plant Pathol.*, 21: 411-423.

Yu D., Liu Y., Fan B., Klessig D.F., and Chen Z. (1997). Is the high level of salicylic acid important for disease resistance in potato? *Plant Physiol.*, 115: 343 - 349.

Yu L.X., Chamberland H., Lafontaine J.G., and Tabaeizadeh Z. (1997). Negative regulation of gene expression of a novel proline-threonine, and glycine-rich protein by water stress in *Lycopersicon chilense*. *Genome*, 39: 1185-1193.

Zhou J., Rumeau D., and Showalter A.M. (1992). Isolation and characterization of two wound-regulated of tomato extensin genes. *Plant Mol. Biol.*, 20: 5-17.

Appendix I

Isolation of gene(s) controlling root growth in rice, *Oryza sativa*

1. Introduction

1.1 Rice

Rice is the world's single most important food crop and a primary food for more than a third of the world's population. Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed (David, 1991). In Asia, rice is generally grown by poor farmers on small farms. In most countries rice is a subsistence crop with about half of the harvested rice being retained and consumed by farm households. In addition, most of the rice is consumed within the country where it is produced.

Of the 15 countries growing at least one million hectares of rice in 1990, only two are outside Asia, i.e., Latin America and Africa where the world rice production for both countries is 4% and 2% respectively (Figure 1.1). China and India account for 50% of the world's rice area and 56% of production (David, 1991). Worldwide, rice production has doubled in a 25-year period, from 257 million tons in 1965 to 520 million tons in 1990 (Khush and Toenniessen, 1991).

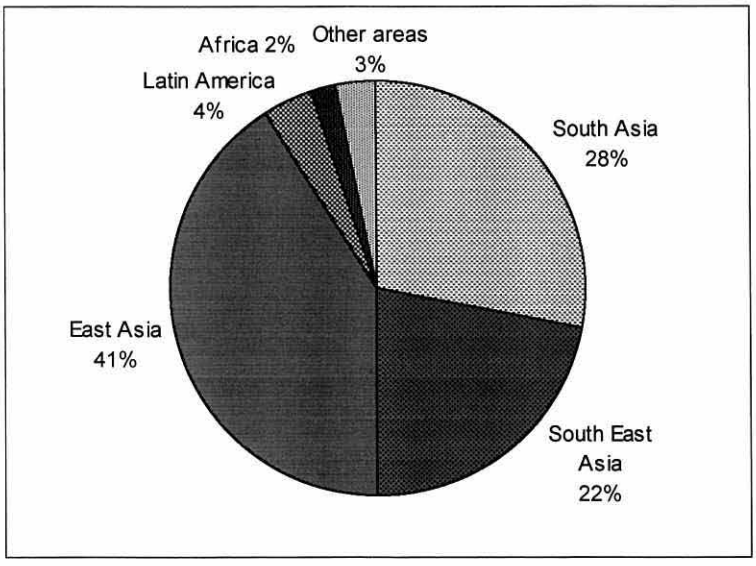


Figure 1.1. Regional distribution of world rough rice production in 1988. (Source: David, 1991).

The annual rate of increase in rice production has now slowed down and there are no new areas that can be brought into rice production. World population which now stands at approximately 6 billion is likely to exceed 8 billion by the year 2020 and during that time the number of rice consumers will probably double. In addition, most of the rice growing countries in Asia have now become self-sufficient in rice production. For example, Asia accounts for 59% of the global population and about 92% of the world rice production, but 90% of the produced rice is consumed by Asian people (IRRI, 1993). World wide, only 3 - 4% of the global rice production was internationally traded from 1980 to 1991 (IRRI, 1993). World rice production must therefore be accelerated at a reasonable rate in order to cope with the increase in the world rice eating population.

1.2 Rice growing area

Rice is grown in a wide range of locations and under a variety of climatic conditions, from the wettest areas to the driest deserts. It is grown along Myanmar's Arakan Coast, where an average of annual rainfall is more than 5100 mm, and at the Al Hasa Oasis in Saudi Arabia, where the annual rainfall is less than 100 mm (IRRI, 1993). Rice is also produced in areas that have a great range of temperature - from 17^0C in Otaru, Japan to 33^0C in the Upper Sindh in Pakistan. The crop is grown at sea level on coastal plains and delta regions throughout Asia, and to a height of 2600 m on the Himalaya in Nepal (IRRI, 1993). In terms of solar radiation, it is grown in the areas which received 25% of potential solar radiation in Myanmar, Thailand, and India's Assam State to those which receive approximately 95% of potential, e.g., Southern Egypt and Sudan (IRRI, 1993).

In South, Southeast and East Asia, which have alternating wet and dry seasonal cycles and also contain many of the world's major rivers and vast deltas, rice occupies an extraordinarily high portion of the total planted area. In these areas, lands are flat and flooded annually during and immediately following the rainy season.

Traditionally, the highest rice yields were obtained from plantings in high-latitude areas. These areas are characterised by having long daylight,

and intensive farming techniques are practised. High yields are also obtained in low-latitude desert areas which receive a high solar energy level such as South-western Australia, Hokkaido in Japan, Spain, Italy, Northern California and the Nile Delta in Egypt (IRRI, 1993). From this we can see that the diversity in geographic, economic, and social conditions under which rice is produced is truly remarkable.

1.3 Rice cultivation systems

Rice is grown in widely diverse production environments in terms of topography, soil type, water regime and climatic factors (David, 1991). There are four types of rice according to the rice cultural systems, i.e., irrigated, rainfed lowland, upland or dryland and deepwater rice (Khush, 1984). The majority of rice ecotypes are semiaquatic plants adapted to saturated soil conditions where it is difficult for other crop species to survive. Figure 1.2 and 1.3 show the distribution of the world's rice area and production by production environments.

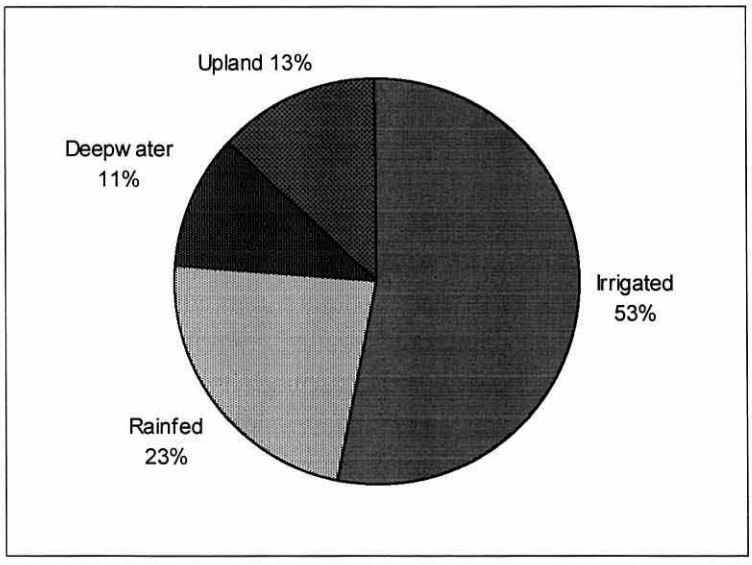


Figure 1.2. Estimated percentage of world rice area by culture type, 1985 - 1987. Rainfed lowland included tidal wetland area (source: David, 1991).

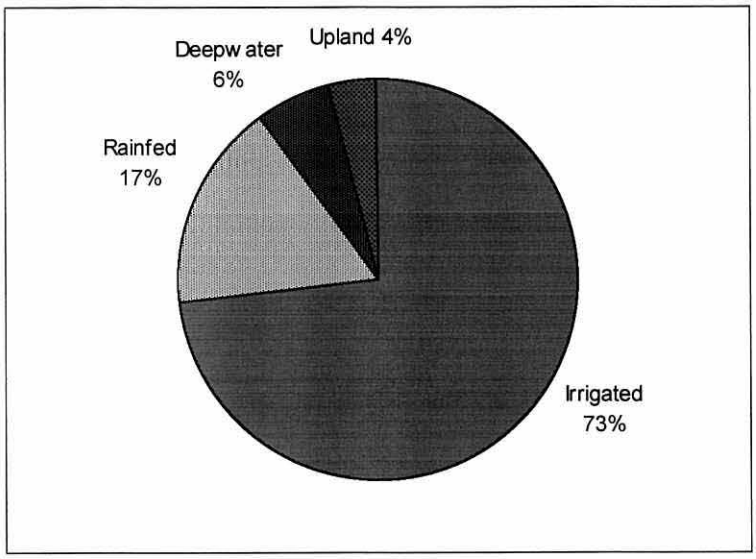


Figure 1.3. Estimated percentage of world rice production by culture type, 1985 - 1987. Rainfed lowland includes tidal wetland production (source: David, 1991).

More than 50% of the world's rice area is irrigated and it contributes to 73% of the world rice production. Irrigated rice is grown in areas that have assured availability of water during its growing season period. It can be normally found in South Asia: Bangladesh, Nepal, Pakistan and Sri Lanka; Southeast Asia: Indonesia, Malaysia, Myanmar, Philippines, Thailand and Vietnam; and East Asia: China (including Taiwan), Japan, Korea (DPR) and The Republic of Korea (IRRI, 1993).

Another 23% of the world's ricelands are rainfed lowlands, characterised by alternating flooding and drying as a result of irregular rainfall patterns (O'Toole and Chang, 1979). In these areas, farmers generally do not have access to irrigation and yield may be seriously jeopardised by drought. In contrast to irrigated rice, rainfed lowland rice only contributes 17% to the world rice production (Figure 1.3). This type of rice is grown in the Philippines, Myanmar, Vietnam, Eastern India, North-eastern Thailand and Bangladesh (David, 1991).

Upland rice represents 13% of the world rice area but only 4% of total production (Figure 1.2 and 1.3). This rice grows in soil with no surface water accumulation and no attempt is made to impound water. It is normally grown on hill slopes and it is almost always exposed to moisture stress during part of the growing season, and its production is seriously jeopardised by drought

(O'Toole and Chang, 1979). The largest upland rice areas are in Brazil and West Africa (David, 1991). However, significant upland rice areas can still be found in India, Indonesia and Laos (David, 1991).

Deepwater rice constitutes 11% of the world's rice area and 6% of production (Figure 1.2 and 1.3). This type of rice represents a unique hydrological situation. It is normally sown on dryland. After a period of 4 - 6 weeks during which the plant establishment is dependent on rainfall, water from watersheds accumulates to as much as 6 metres of depth. Maturing and harvests are timed to occur when floodwater has receded (O'Toole and Chang, 1979). Nearly all deepwater rice areas are located in the low-lying lands on river deltas of South and Southeast Asia such as the Mekong in Vietnam, Chao Phraya in Thailand, Irrawady in Myanmar, and the Ganges and Brahmaputra in Bangladesh and Eastern India (David, 1991).

1.4 Drought

The definition of drought is very subjective. In the context of plant growth, drought is defined on the basis of lack of rainfall, and the resultant soil-moisture deficit (O'Toole and Chang, 1979). It is one of the primary factors responsible for depressing rice yield in chronically dry areas and in destabilising rice production in drought-prone areas. It is a serious yield-limiting factor on almost half of the world's rainfed rice cultures including lowland, upland and deepwater (O'Toole and Chang, 1979; Hanson *et al.*, 1990). Many irrigated rice areas are also prone to drought (Khush, 1984) due to inequitable water distribution systems and deforestation-related reduction in the water yield of many surface irrigation systems (Garrity and O'Toole, 1994). Areas designated as "drought prone" in the classification of rainfed rice environments are widespread geographically in Asia, Africa and Latin America (Garrity and O'Toole, 1994).

The effect of drought on rice yield depends on the timing of drought in relation to plant growth stages. Two critical growth stages have been identified in which the rice yield can be affected by drought, the vegetative stage and the reproductive stage. Drought or water deficit at the vegetative stage appears to be the least detrimental to yield (O'Toole and Chang, 1979).

At this stage, the plant responds to drought by decreasing the number of tillers (O'Toole and Chang, 1979). Drought effects at the vegetative stage may, however, be offset by growth of additional tillers after the water deficit ends. Therefore, the effect is less for cultivars that have a long vegetative phase. The only obvious exception to this case is when water stress is of severe enough intensity and duration to kill some plants and thus reduce the number of plants per unit area and, therefore, reduce the yield (O'Toole and Chang, 1979).

Drought at the reproductive stage is more important and the most damaging to yield for all cereals (Boyer and McPherson, 1975; Begg and Turner, 1976). For rice, the reproductive stage is about the last 55 to 60 days of its life cycle and is characterised by four phases when developmental processes are most sensitive - panicle initiation, meiotic development of gametes, anthesis-fertilisation, and grain filling (O'Toole and Chang, 1979). Water stress at these reproductive stages causes irreparable loss of potential yield (O'Toole and Chang, 1979). It has been reported by Boonjung and Fukai (1996) in their study on water stress on rice that drought at the vegetative stage can cause a reduction in yield of up to 30%. However, when drought occurred during panicle development in the reproductive stage, the reduction of yield increased to up to 60%. When drought occurred during the grain filling stage, the reduction of yield increased to up to 40%.

1.5 Drought avoidance mechanisms

Resistance to, or tolerance of, water stress in crop plants is the combined result of many interacting morphological and physiological characters. Identification of morphological or biochemical characteristics directly related to field drought tolerance has been suggested as a rapid and effective approach to the breeding of drought tolerant crop cultivars (Swindale and Bidinger, 1981). The differential response of rice genotypes to soil and atmospheric water stress has been related to root system characteristics (Chang *et al.*, 1972; Armenta-Soto *et al.*, 1983). In general, rice plants have a shallow root system and only able to extract water from the shallow soil layer. The amount of water extracted from depth is small (Puckridge and O'Toole,

1981; Fukai and Inthapan, 1988). As soil water potential in the surface decreases due to drought, water retained in the deeper layers makes a larger contribution to evapo-transpiration. When most soil water in the shallow layer has been extracted, the plant is unable to extract sufficient water to satisfy evapo-transpiration demands, even though the soil water potential in deep layers is still high. Thus, having a long (or deep) rooting system greatly helps plants to extract water in the deeper soil layers, thus protecting plants from water stress conditions. In addition, plants also have a greater ability to absorb soil nitrogen or fertiliser nitrogen from the deep soil by having long roots (Yoshida and Hasegawa, 1982).

This adaptation mechanism however, gives a great advantage only to upland rice. For the rainfed lowland rice, having a long rooting system does not help plants extracting water from the deep soil layer because the roots are not able to penetrate hard pans which develop close to the soil surface (Fukai and Cooper, 1995). Instead, increasing root branching would probably give a better drought resistance score to this type of rice. Other root-related drought avoidance mechanisms for rice are an increase in the root thickness, and also an increase in the root:shoot ratio (Yoshida and Hasegawa, 1982). It has been shown for rice that there is a high correlation between long and thick roots and field resistance to drought at both vegetative and reproductive growth stages (IRRI, 1983). In fact, it was suggested by O'Toole and Chang (1979) that deep and thick roots are traits that should be incorporated into improved cultivars for upland rice culture. Studies by Mambani and Lal (1983), Wright and Smith (1983), and Lorens *et al.* (1987) have shown that, the increase of width, depth and branching of root systems can decrease plant water-stress in a number of cereal crops including rice, sorghum and maize.

Adaptive mechanisms for drought stress by the shoot have also been shown (O'Toole and Chang, 1979). These include stomata closure, a well-developed cuticle, and leaf rolling. Rice plants adopt these mechanisms in order to reduce water lost via transpiration. Rice plants were also shown to respond to drought stress by reducing the production of new tillers and leaves, reducing leaf elongation, and promoting leaf death (Cutler *et al.*, 1980; O'Toole and Cruz, 1980; Hsiao *et al.*, 1984; Turner *et al.*, 1986). These

responses reduce interception of photosynthetically active radiation (PAR) (Inthapan and Fukai, 1988). Among these mechanisms, it seems that in rice, particularly in upland rice, having a long rooting system is the most important mechanism for the plant to avoid drought stress (Yoshida and Hasegawa, 1982).

2. Materials and methods

2.1 Chemicals and reagents

The chemicals and reagents used in this work were as detailed in Chapter 2 (Section 2.1).

2.2 General stock solutions

The stock solutions used in this work were as detailed in Chapter 2 (Section 2.7).

2.3 General molecular biology methods

The molecular biology methods followed in this work were as detailed in Chapter 2 (Section 2.8).

2.4 DNA procedures

The DNA procedures followed in this work were as detailed in Chapter 2 (Section 2.10).

2.5 RNA procedures

The RNA procedures followed in this work were as detailed in Chapter 2 (Section 2.11).

2.6 Cloning vectors

The vector used for the construction of the rice cDNA library was Uni-ZAP XR (lambda ZAPII vector digested with *Eco*R I/*Xho* I, CIAP treated) from Stratagene.

2.7 Bacterial strains

The *Escherichia coli* strains used in this work were;

Strain	Genotype
SURE	*e14⁻(McrA⁻)* Δ*(mcrCB-hsdSMR-mrr-171 endA1 supE44 Thi-1 gyrAa96 relAa1 lac recB recJ sbcC umuC ::* Tn*5* (Kan^r) *uvrC* [F' *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r)]^c (Stratagene)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r)]^c (Stratagene)

2.8 Bacterial media and growth conditions

Bacterial strains were routinely grown in luria broth (LB) or LB agar at 37^{0}C. When antibiotic selection was required, stock solutions were prepared and an appropriate amount was added to the cooled autoclaved media. Preparations of LB media and antibiotic solutions were as detailed in Chapter 2 (Section 2.4). For bacteriophage transfection of SURE and XL1-Blue *E. coli* cells, 0.6% (w/v) of LB top agar was used. 2X YT media was used for *in vivo* excision of recombinant Uni-ZAPII XR vector packaged into bacteriophage. Preparations of the top agar and 2X YT media were as in Table 2.1

Table 2.1. Preparation of bacterial growth media (g per litre).

	Top agar (0.6%)	2X YT
Bacto tryptone	10	16
Bacto yeast extract	5	10
Nacl	10	10
Agar	6	

2.9 Colour selection by IPTG/X-gal

When the colour selection of the transformant by α-complementation of β-galactosidase was required, IPTG and X-gal were added to the top agar at a final concentration of 2.5 mM and 4.17 mg/ml respectively. IPTG and X-gal were added to the media separately to prevent precipitation. Stock solutions of IPTG and X-gal were prepared as detailed in Chapter 2 (Section 2.5)

2.10 Plant materials

Two varieties of rice plants were used, *O. sativa* L. Azucena and *O. sativa* L. Bala. Rice plants were grown using hydroponics systems according to Yoshida *et al.* (1976) (Table 2.2). Seeds were germinated by placing them on blue paper wetted with sterile distilled water in petri dishes in the dark at 37^0C. After three days, they were transferred to the aerated hydroponics solution in a glass house. Temperature of the glass house was maintained at 25^0C with 14 h day/10 h dark cycle. pH of the solution was maintained at 5.5. Plants were grown for five weeks before tissues were ready for harvesting. The tissues were kept at -70^0C until they were needed. The hydroponics solution was changed everyday. Root growth was measured manually for every 2 days.

Table 2.2. Preparation of hydroponics solution.

Element	Reagent	g per 2 L solution
N	NH_4NO_3	182.8
P	$NaH_2PO_4.2H_2O$	80.6
K	K_2SO_4	142.8
Ca	$CaCl_2$	177.2
Mg	$MgSO_4.7H_2O$	648.0
Mn*	$MnCl_2.4H_2O$	3.0
Mo*	$(NH_4)_6.MO_7O_{24}.4H_2O$	0.148
B*	H_3BO_4	1.868
Zn*	$ZnSO_4.7H_2O$	0.07

Cu*	$CuSO_4.5H_2O$	0.062
Fe*	$FeCl_3.6H_2O$	15.4
	Citric acid monohydrate*	23.8

* Dissolved separately then combined with 100 ml of concentrated H_2SO_4. The volume was made up to 2 litres with dH_2O.

25 ml of each solutions and combination of * solutions were required to prepare 25 litres of the hydroponics solution.

2.11 Preparation of SURE cells and XL-1 Blue competent cells

A single colony of SURE or XL-1 Blue on LB plate was transferred to 100 ml of LB media containing 1.2% (w/v) maltose and 25 μg/ml kanamycin in a sterile 250 ml flask. The flask was incubated at 37^0C and the cells were allowed to grow overnight. Bacterial cells were recovered by centrifuging the growth media at 2500 rpm for 10 minutes at 4^0C in a Mistral 3000i bench centrifuge. The supernatant was discarded and the bacterial pellet resuspended in 0.5 volumes of 10 mM $MgSO_4$. Before the cells were used, they were diluted to an OD_{600} of 0.5 (an OD_{600} of 1 for the bacteriophage *in vivo* excision) with 10 mM $MgSO_4$.

2.12 Construction of the elongation zone of *Oryza sativa* L. Azucena root cDNA library

2.12.1 mRNA isolation

mRNA was isolated from the total RNA (Section 2.11.2, Chapter 2) using the polyATtract mRNA Isolation System III (Promega) according to the instruction manual supplied with the kit. The system uses a biotinylated-oligo (dT) primer to hybridise at high stringency in solution to the 3' poly (A+) region present in most mature eukaryotic mRNA species. The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was then eluted from the solid phase by simple addition of RNAse-free distilled water. Details of the protocol were as stated in the manual.

2.12.2 Construction of the library

The elongation zone of Azucena root cDNA library was constructed using a ZAP-cDNA synthesis Kit (Stratagene). Details of the methods were as explained in the instruction manual supplied with the kit.

2.12.3 Bacteriophage titration

The quality of the packaged cDNA library was tested by titering the packaging reaction. 2 μl of the packaging reaction was taken and serially diluted with SM buffer (1 litre of SM buffer contains 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris.HCl, pH 7.5, 5 ml 2% [w/v] gelatine). 1 μl of diluted and undiluted library were mixed with 200 μl of SURE cells of OD_{600} = 0.5 in small sterile test tubes. The mixtures were incubated at 37^{0}C for 25 minutes with gently shaking. 3 ml of top agar (42^{0}C) was added to the tubes and the mixture was poured immediately onto pre-warmed (37^{0}C) 90 mm LB plates and left to stand at room temperature for 10 - 15 minutes to allow the top agar to set. The plates were then incubated at 37^{0}C overnight. Bacteriophage titer was determined by counting the number of the plaques that appeared on the plate.

2.13 Screening the library

2.13.1 Generation of a subtracted cDNA probes using magnetic beads and PCR

2.13.1.1 Subtraction procedure

Root specific cDNA probes were prepared by a subtraction method and were a modification of a method detailed by Coche *et al.* (1994). 60 μg of total RNA from the elongation zone of Azucena root and 120 μg of total RNA from the elongation zone of Bala root were captured on 0.5 mg magnetic beads (Section 2.12.1) in eppendorf tubes. Beads attached to the elongation zone of Azucena root and the elongation zone of Bala root mRNA were termed (+) beads and (-) beads respectively. The mRNAs were then reverse transcribed on the beads using 200 U of MMLV reverse transcriptase (Promega) with the

presence of 3 μl 10 mM dNTP mix and 1 μl RNasin in a total volume of 50 μl. After one hour at 37^{0}C, the beads were captured and the reaction mixture was discarded. mRNAs were then denatured by adding 100 μl of 0.15 N NaOH and left at room temperature for 5 minutes. The beads were washed once with sterile dH_2O and resuspended in 19.5 μl dH_2O. The (-) beads were stored on ice after this stage. The (+) beads were treated with 5 U of RNAse H for 15 minutes at 37^{0}C prior to random priming synthesis of second cDNA strand. Random priming second strand cDNA synthesis of (+) beads was performed in a 50 μl reaction mixture containing 5 U Klenow polymerase , 2 μl 10 mM dCTP and 10 μl OLB (Section 2.10.7, Chapter 2) for 3 hour at 37^{0}C. The random primed second strand cDNA was denatured with 100 μl 0.15 N NaOH and transferred to a fresh tube, and the (+) beads were resuspended in 50 μl dH_2O and kept on ice. 100 μl of 10X SSC and 5 μl of glycogen were added to the denatured second strand cDNA and the mixture was transferred to the (-) beads. Hybridisation of the random primed second strand (+) cDNA to the first strand cDNA on the (-) beads was performed at 55^{0}C for 24 hours in a Techne hybridisation oven. The (-) beads were then captured and the supernatant was transferred to the (+) beads again (it was first captured and dH_2O was discarded). The un-hybridised (+) sequences were then allowed to re-hybridised to the (+) beads for 6 hours at 55^{0}C and the strands were extended with 2.5 U Klenow polymerase in 0.5X KGB buffer (50 mM potasium glutamate, 12.5 mM Tris.acetate, pH 7.5, 5 mM magnesium acetate, 25 μg/ml BSA, 0.25 mM 2-mercaptoethanol) and 1 mM each dNTP. The beads were washed with 0.5X KGB buffer, resuspended in 0.5X KGB buffer, 1 mM ATP, 10 U Rsa1 , 10 U T_4 DNA ligase, 100 pmoles linker primer (Section 2.13.1.3) and incubated at 37^{0}C for 2 hours to generate short, linker-tailed molecules. The beads were then captured and supernatant was transferred to a fresh tube and used for PCR amplification.

2.13.1.2 PCR amplification

2 μl of the ligation mixture (Section 2.13.1.1) was PCR amplified in a 50 μl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 40 pmoles linker primer and 0.3 U Taq polymerase. Amplification conditions were one

cycle 1' 94^{0}C; thirty cycles 1' 94^{0}C, 2' 57^{0}C, 2' 72^{0}C; and one cycle 5' 72^{0}C. The amplified subtracted probe was then radiolabelled (Section 2.10.7, Chapter 2) and used to screen the library using standard protocols (Section 2.10.10, Chapter 2).

2.13.1.3 Linker primer

The linker primer (Ko *et al.*,, 1990) used was;

GAGATATTAGAATTCTACTC -3'	20 mers
TATAATCTTAAGATGAG -5'	17 mers

They were kinased with 5 U of T_4 polynucleotide kinase (Promega) with the presence of 0.1 mM ATP for each 500 pmoles linker primer at 37^{0}C for 1 hour. The mixture was then heated to 65^{0}C for 1 minute and slowly cooled to room temperature for annealing before it was used.

2.13.2 Plating the library and preparation of the filters

Approximately 1 x 10^5 plaques were plated on each 22 x 22 cm plates using 1 ml of SURE of OD_{600} = 0.5 host cells. The plates were incubated at 37^{0}C until the plaques just about touched each other (approximately 1 mm in diameter). The plates were then refrigerated for 2 hours at 4^{0}C. A piece of Biotrans (+) nylon membrane was placed on each plate. The orientation of the filters was marked and the plaques were allowed to transfer to the membrane filters for 5 minutes. If second filters were required, the second transfer was made for approximately 10 minutes. The filters were peeled from the plates and denatured as detailed in Chapter 2 (Section 2.12.4.2) (excluding the lysis step in 5% SDS). The filters were then baked at 80^{0}C for 1 - 2 hours to fix the DNA.

2.13.3 Hybridisation of the filters

The library was screened against the radiolabelled subtracted cDNA probe using standard protocols for DNA hybridisation (Section 2.10.10, Chapter 2)

2.14 *In vivo* excision of the bacteriophage

Any DNA insert that has been cloned into Uni-ZAP XR vector can be recovered in the form of a phagemid (pBluescript phagemid) containing the insert. The process of this convertion was called *in vivo* excision. *In vivo* excision of the recombinant Uni-ZAP vector was performed according to recommendations given in the instruction manual supplied with the ZAP-cDNA synthesis kit. The plaque of interest was cored from agar plate and transferred to a sterile eppendorf tube containing 500 μl SM buffer and 20 μl of chloroform. The tube was vortexed and then incubated for 1 - 2 hours at room temperature or overnight at 4^0C to release the phage particles. 200 μl of this phage solution was added to 200 μl of SURE cells of OD_{600} = 1.0 in a 50 ml Falcon tube. 1 μl of R408 helper phage was then added and the mixture was incubated at 37^0C for 15 minutes. 5 ml of 2X YT media was then added and the tube was incubated for 3 hours at 37^0C with shaking. The tube was then heated at 70^0C for 20 minutes and spun at 2 500 rpm in a Mistral 3000i centrifuge. Supernatant was collected in a fresh sterile Falcon tube and stored at 4^0C for 1 - 2 months. To plate the rescued phagemid, 200 μl of a serial dilution of the supernatant (phage stock) was combined with 200 μl of SURE cells of OD_{600} = 1.0 in 15 ml tubes. The tubes were then left at 37^0C for 15 minutes and 1 to 100 μl of the mixture was plated onto LB plates containing appropriate antibiotic selection. The plates were then incubated at 37^0C for overnight. Colonies were then isolated and grown for the phagemid isolation.

3. Results

3.1 Root growth

Azucena and Bala showed a difference in root length after they were grown 31 days under a hydroponics system. As shown in Figure 3.1, at the end of the experiment (31 days after transplanting) the roots of Azucena plants reached a maximum length of 41.75 cm (N = 14). This was almost twice that of mean of the Bala roots which reached 27.75 cm (N = 14).

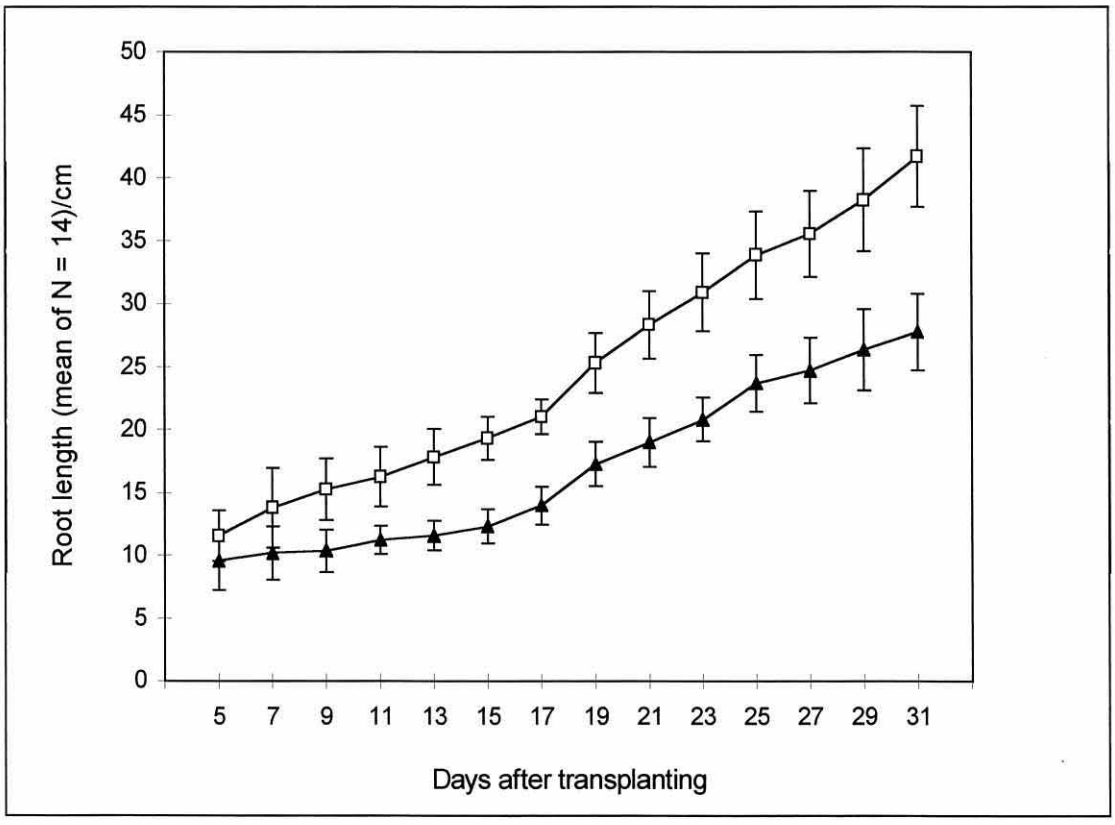


Figure 3.1. Root growth pattern of Azucena and Bala roots. □ - Azucena; ▲ - Bala.

The pattern of root growth for both varieties was similar. During the first 17 days after transplanting, the root length increased at almost a constant rate. From day 17 to the end of experiment, the rate of increase was faster. However, after day 25, the roots of Bala plants appeared to slow down their growth (Figure 3.1). The growth rate of the Azucena roots was faster than the growth rate of the Bala roots overall. The growth rate for the Azucena roots was 0.9553 ± 0.0368 cm/day, whereas the Bala roots grew at 0.5680 ± 0.0289 cm/day.

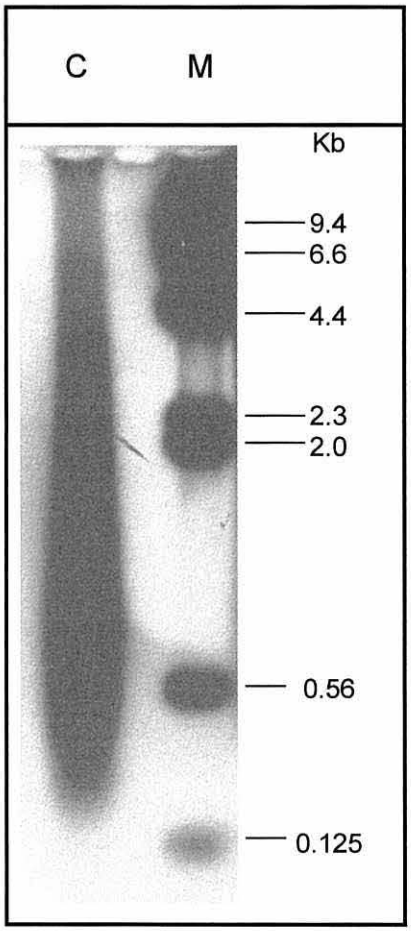
Plant primary roots are divided into three sections, the meristematic zone, the elongation zone and the mature zone (Luxova, 1980). The elongation zone of the roots is the zone where the cells are actively growing and elongating and the major contribution to root length comes from this area (Luxova, 1980). Experiments were performed in order to determine the extent of the elongation zone of the Azucena and Bala roots. Roots from both varieties were marked with insoluble dye markers in a range of 0.5 cm beginning from the root tip, and root growth was observed each day. Results from these experiments showed that the elongation zone of the roots for both Azucena and Bala varieties was the first section of 0.5 cm of the root extending back from the root tip. These sections from Azucena and Bala roots were collected and used to construct a cDNA library and for the preparation of the probe.

3.2 Construction of the elongation zone Azucena root cDNA library

A cDNA library was constructed using the ZAP-cDNA synthesis kit manufactured by Stratagene (Section 2.12.2, Appendix I). The quality of the first strand cDNA was analysed by electrophoresis through a 1.4% agarose gel (Figure 3.2). A continuous smear of bands extending from approximately 0.4 kb to 6.0 kb were detected. The bulk of radioactivity could be detected between 0.5 kb and 4.0 kb. The quality of the second strand cDNA was not analysed.

Approximately 100 ng of the double-stranded cDNA was ligated with 1 μg of the Uni-ZAP XR vector for 2 days at 4^0C before it was packaged using

Figure 3.2. Analysis of the first strand cDNA quality on a normal 1.4% agarose gel.



cDNA and λ DNA markers were denatured in 0.1N NaOH for 15 minutes prior to loading onto the gel. After electrophoresis was complete, the gel was blotted with absorbent paper until it became very thin. It was then wrapped with Saran wrap and exposed to a photographic film at -50^{0}C overnight. ^{32}P-labelled end-filled λ-*Hin*d III fragments to serve as a marker were prepared by including 2.5 μl *Hin*d III 10x buffer, 0.2 mM dATP, 0.2 mM dGTP, 2 μCi [α-^{32}P] dCTP, 1 μg *Hin*d III-digested λ DNA and 1 U Klenow DNA polymerase in a final reaction volume of 25 μl. The reaction was performed at room temperature. After 10 minutes, 2.5 μl of 200 mM EDTA was added to stop the reaction. 2.0 μl was used to run the gel. **C**, first strand cDNA; **M**, λ-*Hin*d III DNA marker.

the Gigapack II Gold packaging extract (Stratagene). The phage cDNA library produced by this packaging had a titer of approximately 4.23 x 10^5 pfu with 96.2% of the plaques being recombinant as judged by plating on LB agar containing IPTG/X-gal. Nine white plaques were randomly chosen for the insert analysis. One blue plaque was picked to serve as a control. *Xho* I/*Eco*R I double-digestion of the phagemid minipreps (Figure 3.3a and b) showed that all of the phagemids obtained from the white plaques contained inserts of sizes ranging in between 0.5 - 1.3 kb. No insert was detected from the phagemid obtained from the blue plaque. Amplification of the library produced a total titer of approximately 1.32 x 10^9 pfu/ml.

3.3 Production of an unlimited supply of cDNA from the elongation zone of Azucena root by PCR amplification

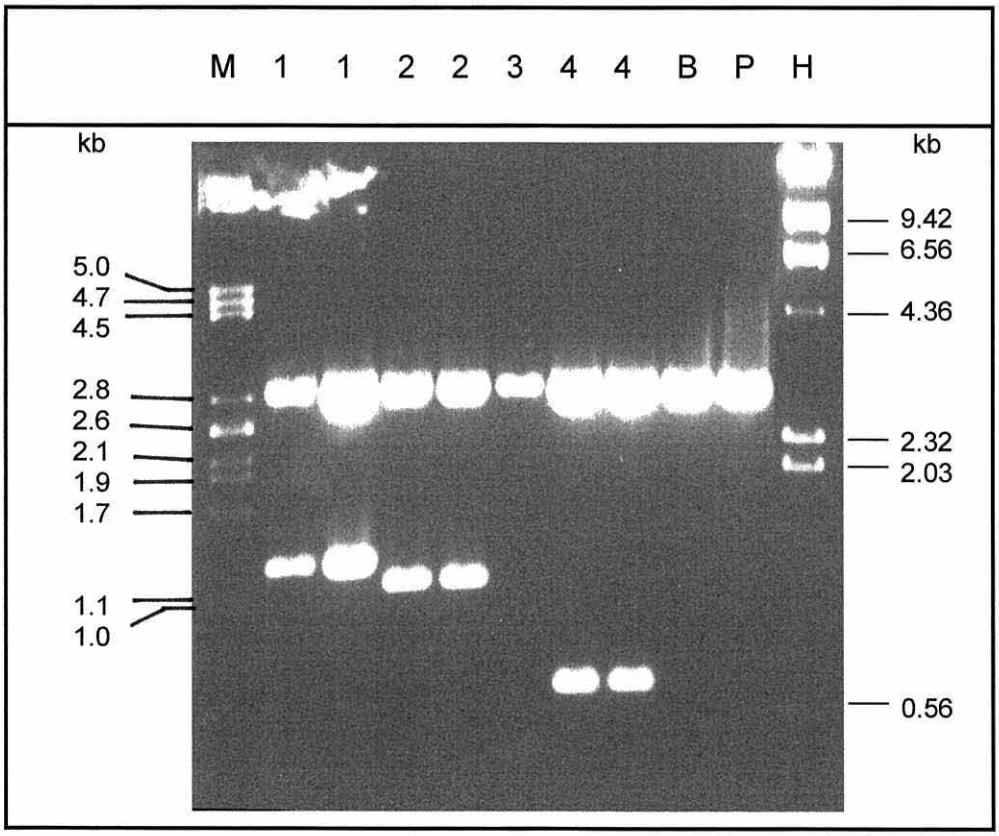
The subtracted cDNA from the elongation zone of Azucena root was prepared as detailed in Section 2.13.1.1 (Appendix I). It was then subjected to PCR amplification in order to generate an unlimited supply of the subtracted probe. Analysis of the PCR amplification of the subtracted cDNA from the Azucena root on a small 1% agarose gel is shown in Figure 3.4. A continuous smear of bands up to approximately 2.1 kb were produced. The bulk of the bands were present between 0.5 kb and 0.8 kb.

3.4 Screening of the elongation zone of Azucena root cDNA library

2.0 μl of the amplified subtracted probe was radiolabelled by random priming and used to screen the cDNA library. None of the cDNA library plaques were found to hybridise to the subtracted probe (results not presented).

Figure 3.3. Analysis of inserts of ten randomly picked plaques from the cDNA library. *In vivo* excision was first performed in order to convert the λ ZAP DNA phage vector to a phagemid (Section 2.14) prior to alkaline miniprep isolation of the plasmid. The plasmids were digested with *Xho* I and *Eco*R I for at least 2 hours at 37^0C before they were analysed on the gels. *Hin*d III-digested pSK^+ was included to serve as a control.

a.

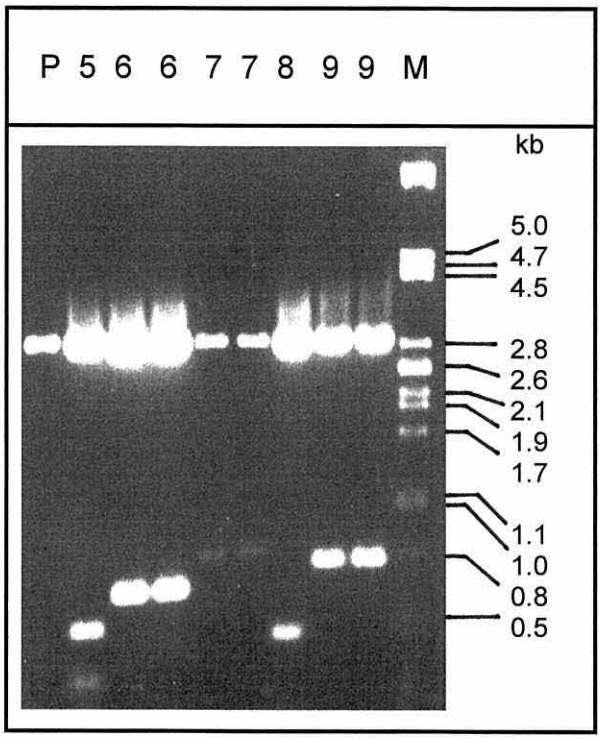


The lanes contained:

M, λ-*Pst* I DNA marker; **1-4**, number of white clones; **B**, blue clone; **P**, pSK^+; **H**, λ-*Hin*d III DNA marker.

Clones **1**, **2** and **4** were duplicates.

b.

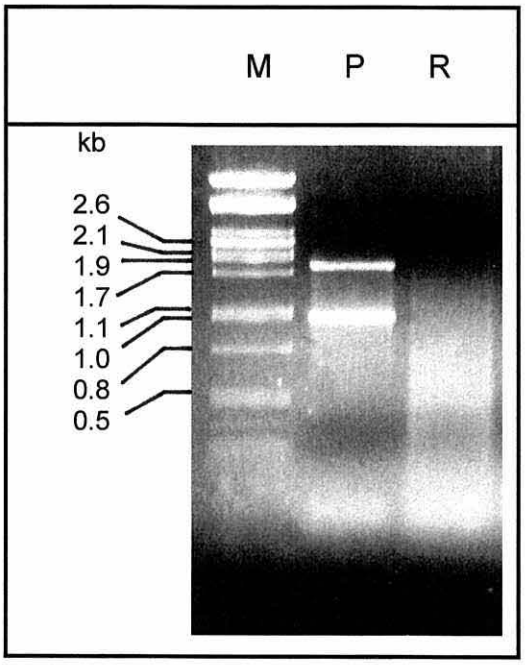


The lanes contained:

M, λ-*Pst* I DNA marker; **P,** pSK$^+$; **5-9**, number of white clones.

Clones **6**, **7** and **9** were duplicates.

Figure 3.4. PCR amplification of subtracted cDNA from the elongation zone of Azucena roots analysed on an agarose gel.



2 μl of the subtracted probe was PCR amplified in a 50 μl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 40 pmoles linker primer and 0.3 U Taq polymerase. Amplification conditions were: one cycle 1' 94^{0}C; thirty cycles 1' 94^{0}C, 2' 57^{0}C and 2' 72^{0}C; one cycle 5' 72^{0}C. As a positive control for the PCR, *Rsa* I-digested pSK$^+$ that had been ligated with the linker-primer was included.

The lanes contained:

M, λ-*Pst* I DNA marker; **P**, *Rsa* I-cut pSK$^+$; **R**, Azucena root subtracted probe.

4. Discussion

Breeding for drought resistance of upland rice is a major component of the research in rice by the International Rice Research Institute's (IRRI) Genetic Evaluation and Utilization (GEU) Program since the early 1970s, aims at countering the adverse affect of drought in unfavoured rice production areas. There are several criteria used in screening and one of them is screening for a deep-root system. Because roots are one of the most important parts of the plant system and possession of a long root system is the most important drought avoidance mechanisms in rice, one might expect that there must be a specific process at the gene level that controls root growth in these long-rooted plant varieties. Because the aim of the study was to isolate a gene (or genes) controlling root growth in rice, two varieties of upland drought resistant rice were used - Azucena and Bala. The main difference between these two varieties was that Azucena has longer roots compared to Bala. Evidence for the difference in the root length between Azucena and Bala was obtained. After plants were hydroponically grown for 31 days in a glass house, Azucena showed a maximum root length of 41.75 cm and was almost doubled compared to the Bala roots, which were 27.75 cm (Figure 3.1, Appendix I). These results agree with those found by Price *et al.* (1997) in their hydroponics root-screening system of 28 varieties of rice which showed that the Azucena root length was almost doubled compared to the Bala roots. Even though plants grown in hydroponics did not experience the actual situation as if they were grown in soil, the results obtained from the hydroponics screening by Price *et al.* (1997) agreed with the results obtained when plants were grown in the field (Loresto *et al.*, 1983; Price *et al.*, 1997).

Two factors normally contribute to the root length - the rate of cell division and the size of cells after division. In the case of the Azucena and Bala roots, the root-cell length was not related to the maximum root length. This result was obtained from the study by Price *et al.* (1997) who showed that the root-cell length for both varieties of rice was almost identical (54.3 $\pm$ 1.21

μm for the Azucena roots, 54.5 ± 1.21 μm for the Bala roots). They therefore concluded that in these two varieties of rice at least, the major determinant of the rate of root growth was the rate of cell division in the apical meristem of the root. The results from the study by Price *et al.* (1997) may explain why screening for the gene(s) which control root growth in the elongation zone of the Azucena root cDNA library using the subtracted elongation zone of Azucena root cDNA failed. This is because if the elongation of both Azucena and Bala roots was regulated by the same factor (the rate of cell division), it is very likely that the same gene(s) must be involved in controlling the process in these two varieties of rice. Therefore, when the elongation zone of Azucena root cDNA probe was prepared by the subtraction experiment against the cDNAs from the elongation zone of Bala roots, the cDNA probe representing the appropriate gene was not subtracted away from other cDNAs. So, when the subtracted cDNA probe was used to screen the cDNA library, the appropriate clone(s) did not hybridise to the probe because the appropriate cDNA probe(s) was not present. The fact that the Azucena roots were growing faster compared to the Bala roots may be because the gene(s) which control the rate of cell divisions was expressed more in the Azucena roots compared to the Bala roots. Another possible explanation is that the turn over rate of the protein(s) or mRNA(s) product from the expression of the gene(s) that control cell division in the Azucena roots is slower compared to the rate in Bala. This will result in the process of cell division in the Azucena roots being induced for a longer period by the same protein(s) resulting in faster cell division in the Azucena roots compared to Bala.

The DNA fragments seen when the PCR amplification of the subtracted Azucena root cDNA probe was analysed on an agarose gel (Figure 3.4, Appendix I) represents other products of the gene(s) specifically expressed in the elongation zone of Azucena roots rather than the gene(s) which controls cell division. Why the probe was also failed to hybridise to any of the cDNA clones is unknown. The only possible explanation is that the appropriate gene(s) representing the subtracted cDNA probe was expressed to a very low level in the elongation zone of Azucena roots. This will result in the number of mRNA transcripts for this gene being very low in the total mRNA used to

construct the cDNA library. Because of this, the chance of this gene being cloned is low due to competition with other genes that are expressed to very high levels. To overcome these problems, several batches of a cDNA library need to be constructed and the library needs to be screened without amplification.

The isolation of gene(s) that control cell division is still possible. This can be done by differentially screening the elongation zone of the Azucena root cDNA library with cDNA probes prepared from the mature and elongation zones of Azucena root. Plaques that will hybridise to the cDNA probe prepared from the elongation of the root but not to the cDNA probe prepared from the mature zone of the root will then be isolated, sequenced and characterised. However, these experiments were not performed because differential screening techniques quite often end with the isolation of new genes of unknown function.

References

Armenta-Soto J.L., Chang T.T., Loresto G.C., and O'Toole J.C. (1983). Genetic analysis of root characters in rice (*Oryza sativa* L.). *SABRAO J.*, 15: 103-116.

Begg J.E., and Turner N.C. (1976). Crop water deficit. *Adv. Agron.*, 28: 161-217.

Boonjung H., and Fukai S. (1996). Effects of soil water deficit at different growth stages on rice growth and yield under upland conditions. II. Phenology, biomas, production and yield. *Field Crop Res.*, 48: 47-55.

Boyer J.S., and McPherson H.G. (1975). Physiology of water deficits in cereal crops. *Adv. Agron.*, 27: 1-23.

Champoux M.C., Wang G., Sarkarung S., Mackill D.J., O'Toole J.C., Huang N., and McCough S.R. (1995). Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers, *Theor. Appl. Genet.*, 90: 969-981.

Chang T.T., Loresto G.C., O'Toole J.C., and Armenta-Soto J.L. (1982). Strategy and methodology of breeding rice for drought-prone areas. *In* Drought Resistance in Crops with Emphasis on Rice. IRRI, Los Banos, Philippines, pp. 217-244.

Chang T.T., Loresto G.C., and Tagumpay O. (1972). Agronomic and growth characteristics of upland and lowland varieties. *In* Rice Breeding, IRRI, Los Banos, Philippines, pp. 645-661.

Cutler J.M., Shahan K.W., and Steponkus P.L. (1980). Influence of water deficits and osmatic adjustment on leaf elongation in rice. *Crop Science*, 20: 314-318.

David C.C. (1991). The world rice economy: challenges ahead. *In* Rice Biotechnology (Khush G.S., and Teonniessen G.H., eds.). C.A.B. International and IRRI, Los Banos, Philippines, pp. 1-18.

Fukai S., and Cooper M. (1995). Development of drought -resistant cultivars using physio-morphological traits in rice. *Field Crop Res.*, 40: 67-86.

Fukai S., and Inthapan P. (1988). Growth and yield of rice cultivars under sprinkler irrigation in Southeastern Queensland. 3. Water extraction and plant water relations - comparison with maize and grain-sorghum. *Aust. J. Exp. Agric.*, 28: 249-252.

Garrity D.P., and O'Toole J.C. (1994). Screening for drought resistance at the reproductive phase. *Field Crop Res.*, 39: 99-110.

Garrity D.P., Aoldeman L.R., Morris R.A., and Lenka D. (1986). Rainfed lowland rice ecosystem: characterization and distribution. *In* Progress in Rainfed Lowland Rice. IRRI, Los Banos, Phillippines, pp. 3-23.

Hanson A.D., Peacock W.J., Evans L.T., Arntzen C.J., and Khush G.S. (1990). Drought resistance in rice. *Nature*, 345: 26-27.

Hargrove T.R. (1978). The diffusion of genetic materials and the objectives for their use among rice breeding programs in India. *Field Crop Res.*, 1: 197-213.

Hsiao T.C., O'Toole J.C., Yambao E.B., and Turner N.C. (1984). Influence of osmotic adjustment on leaf rolling and tissue death in rice (*Oryza sativa* L.). *Plant Physiol.*, 75: 338-341.

Inthapan P., and Fukai S. (1988). Growth and yield of rice cultivars under sprinkler irrigation in Southeastern Queensland. 2. Comparison with maize and grain-sorghum under wet and dry conditions. *Aus. J. Exp. Agric.*, 28: 243-248.

IRRI (1993). 1993 - 1995 IRRI rice Almanac. Los Banos, Philippines.

Khush G.S. (1984). Terminology for rice growing environments. *In* Terminology for Rice Growing Environments. IRRI, Los Banos, Philippines.

Khush G.S., and Teonniessen G.H. (1991). Rice Biotechnology. C.A.B. International and IRRI, Los Banos, Philippines, pp. vii.

Ko M.S.H., Ko S.B.H., Takahashi N., Nishiguchi K., and Abe K. (1990). Unbiased amplification of a highly complex mixture of DNA fragments by 'lone-linker'-tagged PCR. *Nucl. Acid Res.*, 18: 4293-4294.

Lorens G.F., Bennet J.M., and Loggale L.B. (1987). Differences in drought resistance between two corn hybrids. I. Water relations and root length density. *Agron. J.*, 79: 802-807.

Loresto G.C., Zhang W.X., Chaudhary D., and Chang T.T. (1983). Aeroponic technique for screening the drought avoidance mechanism in rice genotypes by root characters. *Garcia de Orta*, 10: 77-82.

Mackill D.J. (1986). Varietal improvement for rainfed lowland rice in South and Southeast Asia : results of a survey. *In* Progress in Rainfed Lowland Rice. IRRI, Los Banos, Philippines, pp. 115-144.

Mambani B., and Lal R. (1983). Response of upland rice varieties to drought stress. I. Relation between root system development and leaf water potential. *Plant and Soil*, 73: 59-72.

O'Toole J.C., and Chang T.T. (1979). Drought resistance in cereals-rice: a case study. *In* Stress Physiology of Crop Plants. Op. Cit., pp. 373-405.

O'Toole J.C., and Cruz R.T. (1980). Response of water leaf potential, stomatal resistance and leaf rolling to water stress. *Plant Physiol.*, 63: 428-432.

Price A.H., and Tomos A.D. (1997). Genetic dissection of root growth in rice (Oryza sativa L.). II. Mapping quantitative trait loci using molecular markers. *Theor. Appl. Genet.*, 95: 143-152.

Price A.H., Tomos A.D., and Virk D.S. (1997). Genetic dissection of root growth in rice (Oryza sativa L.). I. A hydroponic screen. *Theor. Appl. Genet.*, 95: 132-142.

Puckridge D.W., and O'Toole J.C. (1981). Dry matter and grain production of rice using a line source sprinkler in drought studies. *Field Crop Res.*, 3: 303-319.

Swingdale L.D., and Bidinger F.R. (1981). The human consequences of the drought and crop research priorities for their alleviation. *In* The Physiology and Biochemistry of Drought Resistance in Plants (Paieg L.G., and Aspinal D., eds.). Academic Press, New York, pp. 2-13.

Turner N.C., O'Toole J.C., Cruz R.T., Namuco O.S., and Ahmed S. (1986). Response of seven diverse rice cultivars to water deeficits. I. Stress development, canopy temperature, leaf rolling and growth. *Field Crop Res.*, 13: 257-271.

Widawsky D.A., and O'Toole J.C. (1990). Prioritizing the rice biotechnology research agenda for Eastern India. The Rockfeller Foundation. Gandals Press, New Delhi, pp. 86.

Wright G.C., and Smith R.G.C. (1983). Differences between two grain sorghum genotypes in adaptation to drought stress. II. Root water uptake and water use. *Aust. J. Agri. Res.*, 34: 627-636.

Yoshida S., and Hasegawa S. (1982). The rice root system: its development and function. *In* Drought Resistance in Crops With Emphasis on Rice. Op. Cit, pp. 97-114.