CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF EXTENSINS AND LRR-EXTENSIN IN COTTON

By

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CHARACTERIZATION AND FUNCTIONAL

ANALYSIS OF EXTENSINS AND LRR-EXTENSIN

IN COTTON

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGP	Arabinogalactan proteins
Ala	Alanine
BCIP	5-bromo-4-chloro-3-Indolylphosphate p-toluidine salt
BSA	Bovine serum albumin
CDP-Star	Disodium 3-(4-meth-oxyspiro-{1,2-dioxetane-3, 2'–(5'chloro) tricyclo [3,3,1,1] decan}-4-yl) phenyl phosphate
DEPC	Diethyl-pyrocarbonate
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
GRP	Glycine-rich proteins
GSP	Gene specific primer
HRGP	Hydroxyproline-rich glycoproteins
Hyp	Hydroxyproline
IPTG	Isopropylthio-beta-D-galactoside
Ile	Isoleucine
Leu	Leucine
LRR	Leucine-rich repeat
Lys	Lysine
MALDI	Matrix assisted laser desorption and ionization
MOPS	Morpholinopropanesulphonic acid
NBT	Nitroblue tetrazolium chloride
Ni-NTA	Nickel-Nitrilo triacetic acid
NZY	NZ-amine yeast extract medium
PCR	Polymerase chain reaction
PGIP	Polygalacturonase inhibitor protein
PRK	Pollen receptor-like kinase
Pro	Proline
PRP	Proline-rich proteins
PVDF	Polyvinylidene fluoride
RACE	Rapid amplification of cDNA ends
RGI	Rhamnogalacturonan I
RLK	Receptor-like kinases
Ser	Serine

SH medium	Schenk and Hildebrandt medium
SRK	S-domain class of receptor-like kinase
TBS	Tris bufferred saline
Tyr	Tyrosine
Val	Valine
WAK	Wall associated kinase
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactoside

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

The project began with the aim of isolating extensin cDNA clones from cotton. Previous studies indicated that several extensin peptides in cotton line Acala 44, could be isolated by complete deglycosylation of cotton cell walls by hydrogen fluoride treatment followed by typsin treatment of these walls (Qi, Beherens, West and Mort 1995). Separation of the extensin peptides by reverse-phase HPLC indicated three kinds of repeat motifs (a) Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys, (b) Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys, (c) Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys (Qi, Beherens, West and Mort 1995). The next logical step was to isolate the cDNA clones coding for the proteins containing these peptides. With this purpose in mind, a cDNA library was constructed using the ZAP-cDNA® (Stratagene) construction kit, using RNA from Acala 44 suspension culture cells (Wenjun Huang, Oklahoma State University). This cDNA library was screened by doing plaque lifts and hybridization using degenerate probes coding for Ser-Pro-Pro-Pro-Pro repeats (characteristic of all extensins) and the genomic DNA clone encoding carrot extensin, pDC5A1 (Chen and Varner, 1985). Seven positive clones were isolated and sequenced (Chris Ackerson and Jun Fu, Oklahoma State University). Of the seven sequences found, two were typical extensins, extensin # 2, characterized by two kinds of

repetitive motifs, (a) Ser-Pro-Pro-Pro-Pro-Pro-Pro-Ser-Pro-Pro-Lys-His-Pro-Tyr-Lys-Tyr-Lys and

(b) Ser-Pro-Pro-Pro-Pro-Pro-Pro-Val-Tyr-Lys-Tyr-Lys including the known intramolecular isodityrosine cell wall protein crosslinking motif, Tyr-Lys-Tyr-Lys and extensin # 3, characterized by the repetitive motif (a) Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys, including the isodityrosine cross-linking motif of Tyr-Tyr-Tyr-Lys and (b) Ser-Pro-Pro-Pro-Pro-Val-His-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys including the isodityrosine crosslinking motif of, Tyr-Tyr-Tyr-Lys (Kieliszewski and Lamport, 1994) as shown in Figure 1a. Five of the seven sequences found, (sequences # 4, # 7, # 8, # 13 and # 65631) were not typical extensins because they lacked the cell wall cross-linking motif and though they did have serine and proline rich repeats, they did not have the serine proline repetitive motif as seen in the typical extensins # 2 and # 3. These sequences were called extensin-like sequences and their amino acid sequence is shown (Figure 1b). Three of these sequences, # 4, # 13 and # 8 had 99% identity while comparing their nucleic acid sequence. The longest of these extensin-like sequences, (# 65631) was a chimeric extensin consisting of an N-terminal leucine-rich repeat sequence and a C-terminal extensin-like sequence. This sequence was called leucine-rich repeat extensin (LRR-extensin). The extensins and LRR-extensin sequences were all incomplete at their 5' ends, as judged by the absence of a signal peptide that is present in all extensins. A 5' RACE experiment was done on the incomplete LRR-extensin (# 65631) to determine the 5' end of the sequence. Since the LRR-extensins had not been studied extensively, we decided to study it along with the extensins # 2 and # 3.

The project had two major goals:

1). Characterization of the LRR-extensin (# 65631) by determining the complete cDNA sequence through a 5'RACE experiment, isolating the gene by screening a genomic library and doing southern blot analysis to learn the gene family size.

2). Functional analysis of extensins and LRR-extensin by determining the expression pattern of the genes. The expression pattern of the genes in various organs of cotton was studied by doing northern blots. The expression patterns of the genes during stress by *Xanthomonas* infection and after wounding were analyzed to see if the extensins and LRR-extensins were defense-related. In addition, the LRR domain was expressed in a prokaryotic expression system and used as an antigen to raise polyclonal antibodies, which were used in western blots to estimate the molecular weight of the native LRR-extensin.

Seq # 2 Extensin

SPPPPPPVYKYKSPPPPPPSPPKHPYKYKSPPPPPPSPPKHPY
KYKSPPPPPPSPPKHPYKYKSPPPPPSPPKHPYKYKSPPPPPPSPPKHPYKYKSPPPPPPSPPKHP
YKYKSPPPPSPPKHPYKYKSPPPPPPSPPKHPYKYKSPPPPSPPKHPYKYKSPPPPPPSPPKHPYK
YKSPPPHHPVYKYKSPPPPPPPHYVYASPPPPHHY

Seq # 3 Extensin

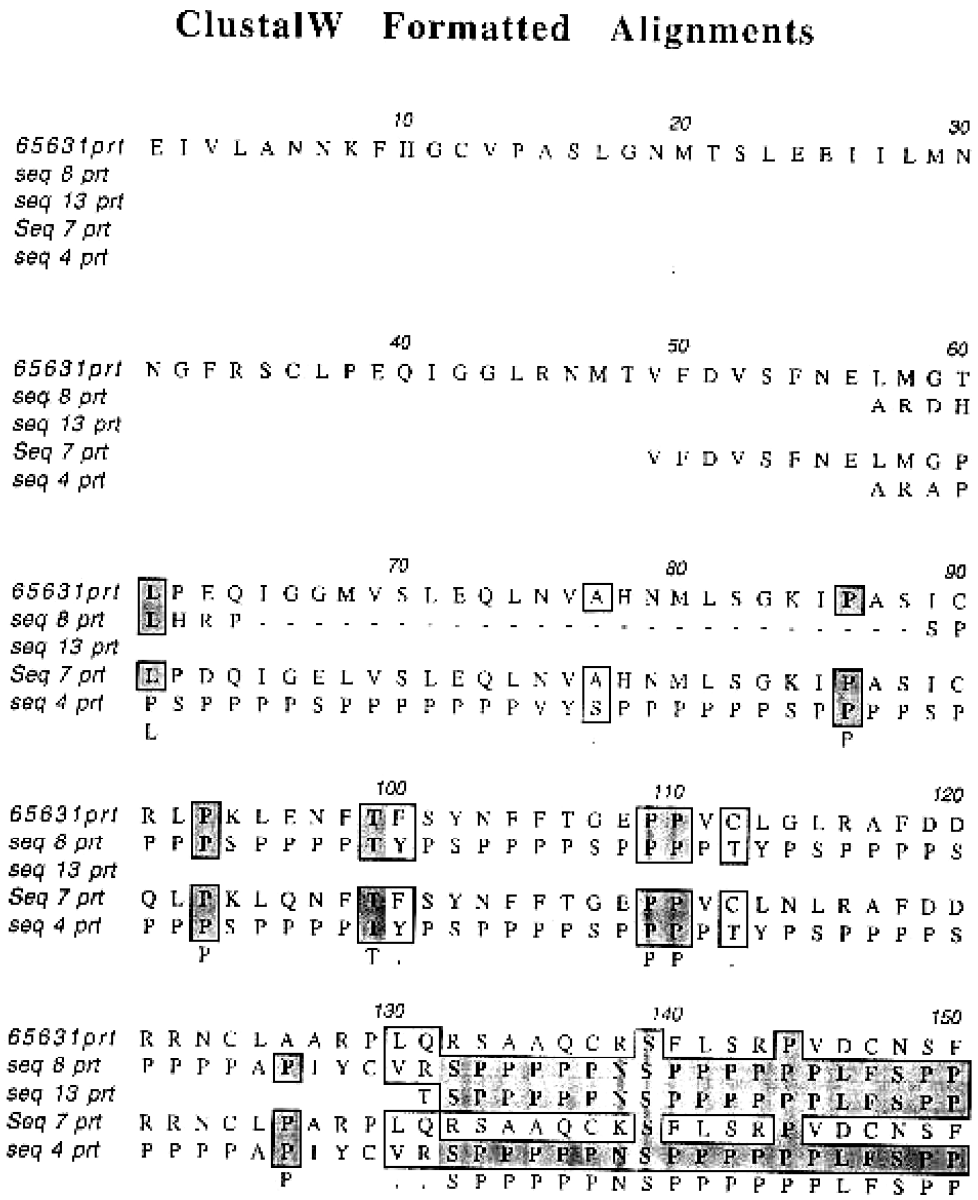
SPPPPSPSPPPPYYYKSPPPPSPSPPPPYYYKSPPPPSPSPPPPYYYK
SPPPPVHSPPPPYYYKSPPPPSPSPPPPYYYKSPPPPSPSPPPPYYYKSPPPPSPSPPPPYYYKSP
PPPSPSPPPPYYYKSPPPPVHSPPPPYYYKSPPPPSPSPPPPYYYHSPPPPVKSPPPPAYIYASPP
PPTHY

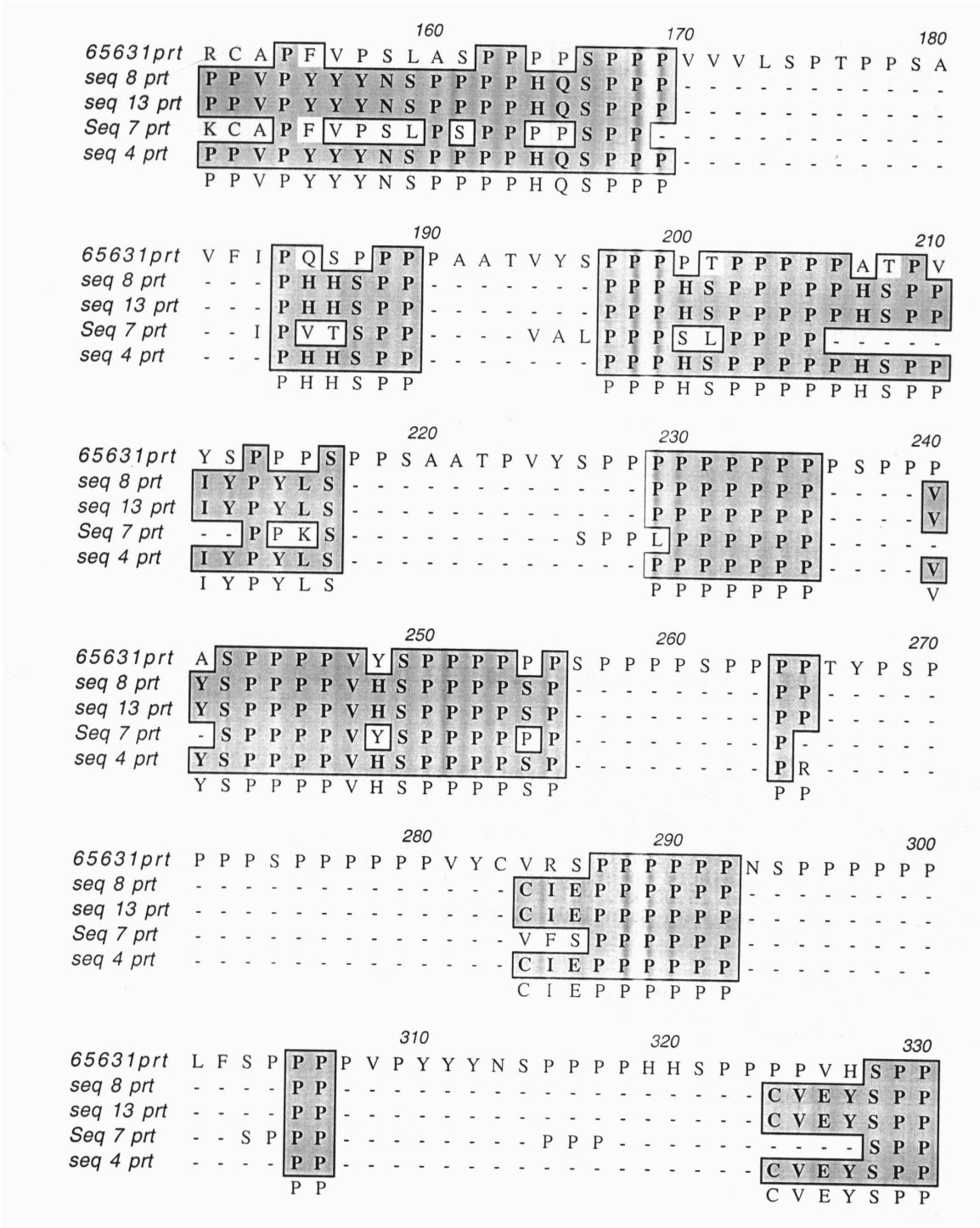
Figure 1a: Extensin sequences, # 2 and # 3 isolated from the cDNA library of cotton suspension cultures.

Extensin # 2 was characterized by the repetitive motifs, (a) Ser-Pro-Pro-Pro-Pro-Pro-Pro-Ser-Pro-Pro-Lys-His-Pro-Tyr-Lys-Tyr-Lys and (b) Ser-Pro-Pro-Pro-Pro-Pro-Pro-Val-Tyr-Lys-Tyr-Lys containing the isodityrosine cell wall cross-linking motif, Tyr-Lys-Tyr-Lys.

Extensin # 3 was characterized by the repeat motif, (a) Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys containing the isodityrosine cross-linking motif Tyr-Tyr-Tyr-Lys and (b) Ser-Pro-Pro-Pro-Pro-Val-His-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys including the isodityrosine crosslinking motif of, Tyr-Tyr-Tyr-Lys.

Figure 1b: ClustalW alignments of five extensin-like sequences isolated from the cDNA library of cotton suspension cultures.

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seq 13 prt P P V P Y Y Y N S P P P P H Q S P P P - - - - - - - - - - -
Seq 7 prt K C A P F V P S L P S P P P P S P P - - - - - - - - - - -
seq 4 prt P P V P Y Y Y N S P P P P H Q S P P P - - - - - - - - - - -
P P V P Y Y Y N S P P P P H Q S P P P
190
200
210
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seq 8 prt - - - P H H S P P - - - - - - - P P P H S P P P P P H S P P
seq 13 prt - - - P H H S P P - - - - - - - P P P H S P P P P P H S P P
Seq 7 prt - - I P V T S P P - - - - V A L P P P S L P P P P - - - - -
seq 4 prt - - - P H H S P P - - - - - - - P P P H S P P P P P H S P P
P H H S P P
P P P H S P P P P P H S P P
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240
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I Y P Y L S
P P P P P P P
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P P
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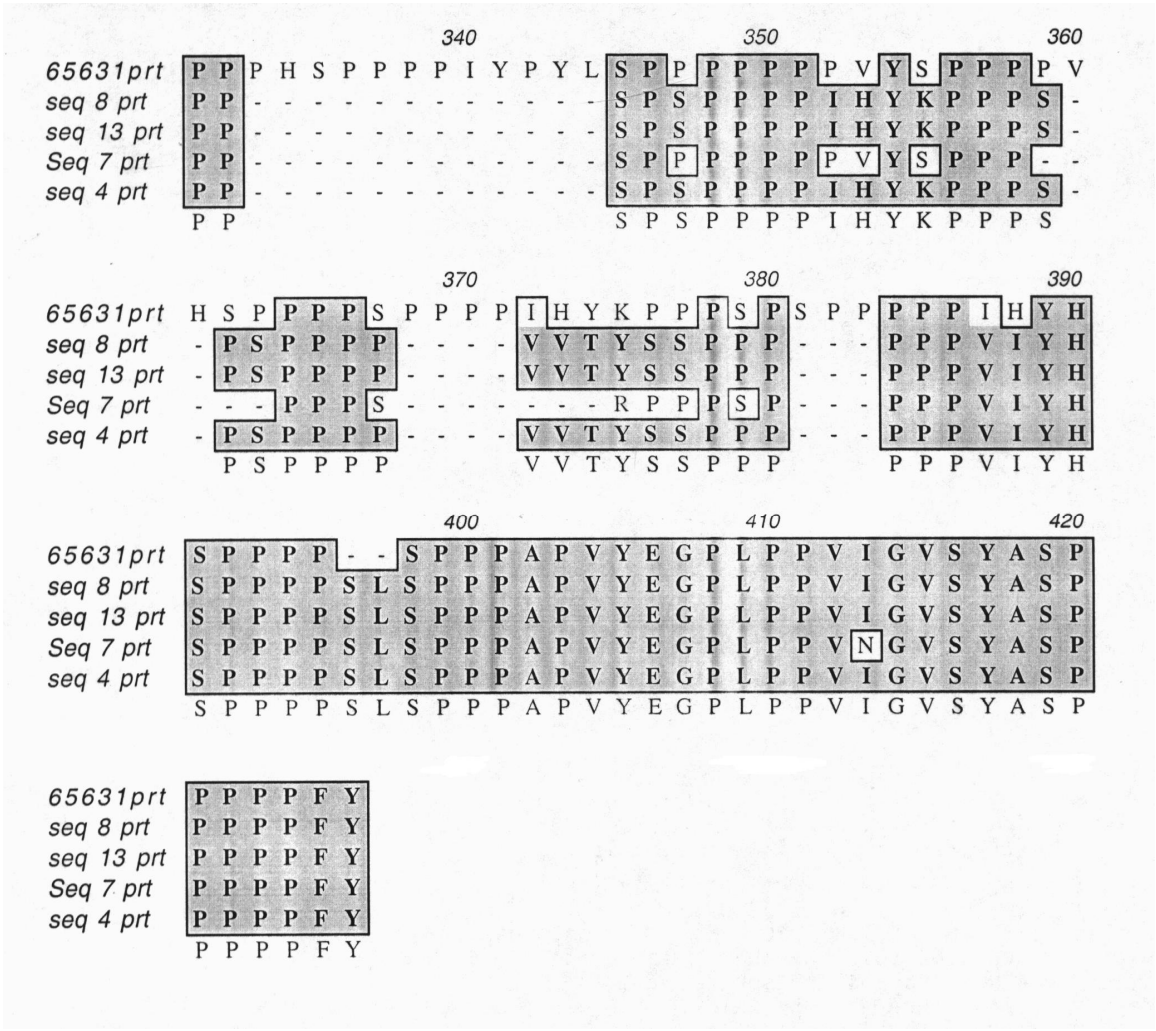
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Seq 7 prt P P P P F Y
seq 4 prt P P P P F Y
P P P P F Y

CHAPTER II

LITERATURE REVIEW

Plant Cell Wall Proteins

Plant cell walls contain a complex mixture of proteins, carbohydrates, water, lignin, cutin, suberin, and certain inorganic compounds. The composition of the plant cell wall changes according to developmental events and abiotic and biotic stresses.

There are four main classes of cell wall structural proteins. These are the hydroxyproline-rich glycoproteins (HRGPs) or extensins, the arabinogalactan proteins (AGPs), the glycine-rich proteins (GRP) and the proline-rich proteins (PRPs) (Showalter, 1993). In addition to the above four classes, there are other proteins present in the cell wall which are mainly enzymes such as polygalacturonases, mannosidases, glucanases, peroxidases, phosphatases, invertases, pectin methylesterases, malate dehydrogenases, arabinosidases, galactosidases, glucuronosidases, xylosidases, ascorbic acid oxidase and proteases (Varner and Lin, 1989). These enzymes have a wide variety of functions that include synthesis of cell wall components, modification of the cell wall components, and degradation of the cell wall components.

EXTENSINS

Extensins are hydroxyproline-rich glycoproteins that are present in the cell walls of higher plants. These proteins are rich mainly in serine and hydroxyproline and some combination of the amino acids lysine, valine, tyrosine and histidine. Extensin genes code for proteins that have a repeating motif of serine followed by at least four hydroxyprolines. The prolines are hydroxylated to hydroxyproline by the enzyme prolyl hydroxylase. The serines in the pentapeptide motif are frequently glycosylated with a single galactose unit, and most of the hydroxyprolines are glycosylated with one to four arabinosyl residues. These are basic proteins due to their high lysine and/or histidine content and have an isoelectric point of ~10. Extensins assume a polyproline II helical structure in solution (i.e. a left handed helix with three residues per turn) (Van Holst and Varner 1984). When viewed under an electron microscope, extensins appears rod-like, approximately 80μm in length (Stafstrom and Staehelin, 1986; Heckman, Terhune and Lamport, 1988).

Extensins can form intramolecular diphenylether linkages called isodityrosine crosslinks between tyrosine residues in the motif Tyr-Tyr-Tyr-Lys and Tyr-Lys-Tyr-Lys (Epstein and Lamport 1984) and form, as yet ill identified intermolecular crosslinks. It has been shown that in cotton suspension culture cells, there exists a covalent linkage between the RG I fraction of the pectin and most of the extensin. This crosslink could be through a 3,6 linked galactan or a phenolic cross-link from a feruloylated sugar in the pectin to an amino acid in the extensin (Qi, Beherens, West and Mort 1995). Tissue print immunoblots of soya bean stems and root show that, extensins are present mainly in

cambium cells and in the cortex cells surrounding the vascular bundle. Extensins have been suggested to contribute to the tensile strength of these cells and help the cells to tolerate tensile stress (Ye and Varner, 1991).

The abundance of extensins in the cell wall of higher plants along with their rodlike appearance suggests a structural role for extensions. Digestion of tomato suspension culture cell wall polysaccharides with anhydrous hydrogen fluoride leaves behind a framework of extensins, thus supporting a structural role for these proteins (Mort and Lamport, 1977).

Extensins may contribute to plant defense by protecting the plant against pathogen attack and mechanical wounding (Showalter, 1993). Immunochemical studies have showed that extensins accumulate in bean cell walls close to where the microbial growth is restricted by the plant (Esquerre-Tugaye, Mazau, Pelissier, Roby, Rumeau and Toppan, 1995). There is evidence to suggest that extensins by virtue of the positively charged lysines bind to and immobilize the negatively charged surfaces of certain plant pathogens and restrict their entry into the plant cell (Mazau, Rumeau and Esquerre-Tugaye, 1987; Mellon and Helgeson, 1982).

Though extensins are said to be structural proteins that may have a role in plant defense, studies in tobacco plants expressing sense or antisense gene constructs can tolerate a large variation in their extensin concentration without visible phenotypic effects (Memelink, Swords, de Kam, Schilperoot, Hoge and Staehelin, 1993). Thus, designing an experiment to prove with certainty the role of extensin will be a challenge.

ARABINOGALACTAN PROTEINS

Arabinogalactan proteins are proteoglycans that are composed of approximately 90% carbohydrate and 10% protein, with the most abundant sugars being arabinose and galactose, though rhamnose, glucuronic acid and other monosaccharides are present in minor amounts. The core protein is rich in hydroxyproline, threonine, alanine, serine and glycine (Clarke, Anderson and Stone, 1979). The sugar groups are O-linked to the amino acids hydroxyproline, threonine and serine in the core protein. These proteins are found in almost all tissues of higher plants. Arabinogalactan proteins are usually soluble and are heterogenous due to the variation in the type of branching of the side chain sugars. A characteristic feature of arbinogalactan proteins is that, they can be precipitated by the β-glucosyl Yariv's reagent (Classen, Witthohn and Blaschek, 2000). Therefore this reagent is used for isolating arabinogalactan proteins and in tissue localization studies.

Many different functions have been attributed to arabinogalactan proteins. Due to their high sugar content resulting in their stickiness, they contribute to the adhesion of the pollen and stigma, they may provide a nutritive role in the style, providing carbohydrate precursors for the growing pollen tube (Lord and Sanders, 1992). A possible role in cellular differentiation has also been proposed for arabinogalactan proteins, as the addition of arabinogalactan proteins from a carrot embryonic cell line to a non-embryonic cell line can induce embryonic potential (Sommer-Knudson, Bacic and Clarke, 1998). Since arabinogalactan proteins are soluble and diffusible components of the extracellular matrix, they may act as intercellular signals between cells and cause modification of the wall composition and ultimately add a new development path.

GLYCINE-RICH PROTEINS

Glycine-rich proteins are structural proteins that are rich in Glycine-X repeat units, where X is usually glycine, but can also be alanine or serine (Condit and Meagher, 1986). Ultrastructural studies in beans have shown that the glycine-rich proteins are localized in the modified primary walls of the protoxylem cells, where they may be helping to repair the walls of these dead cells that are subject to intense stretching (Ryser U and Keller B, 1992).

Glycine-rich proteins may also play an important role in the development of nodules, vascular tissue and flowers and during wound healing and freezing tolerance. (Castonguay, Nadeau and Laberge, 1993; Kuster,Schroeder,Fruhling and Rieping, 1995).

PROLINE-RICH PROTEINS

These proteins have a characteristic repeat motif of Pro-Hyp-Val-Tyr-Lys. These proteins are associated with protoxylem and xylem structures, where they are said to be involved in xylem differentiation or in lignification. The proline-rich proteins participate in different aspects of development, such as nodule and pod differentiation (Loopstra and Sederoff, 1995; Coupe, Taylor, Isaac and Roberts, 1993) and microspore, ovary and embryo development (Jose-Estanyol and Puigdomenech, 1998).

LEUCINE-RICH REPEATS

A Leucine-rich repeat or LRR is a 20-29 amino acid repeat motif that appears to be involved in protein-protein interaction (Kobe and Deisenhofer, 1995). These repeats are present in organisms that range from bacteria to man.

The 24 amino acid consensus sequence of a LRR protein in plant (which is conserved across many taxa) is as follows,

LxxLxxLxxLxLxxNxLxGxIPxx

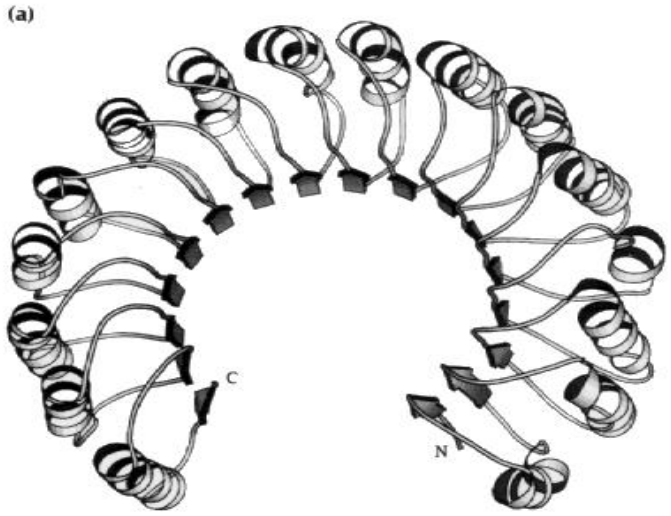
where L is leucine, N is aspargine, G is glycine, I is isoleucine, P is proline and x is any amino acid.

The crystal structure of porcine ribonuclease inhibitor is shown in Figure 2. This LRR protein has 17 LRR repeats, each repeat is 29 amino acids long. Each individual repeat, corresponds to a structural unit comprising of a short β-strand and an α-helix approximately parallel to it. The whole structure, has its 17 repeat units arranged consecutively and in parallel to a common axis, such that the structure adopts a horseshoe shaped structure, with the ligand RNAse binding inside the concave surface of the structure (Kobe and Deisenhofer, 1993).

All known LRR proteins have two things in common, repetitive sequences and involvement in protein-protein interaction. LRR proteins discovered to date in man are involved in cell adhesion, development, signal transduction, DNA repair, transcription, recombination and RNA processing (Buchanan and Gay, 1996).

In plants, the LRR proteins that are characterized so far are mainly involved in plant defense and signal transduction. The four kinds of LRR proteins in plants include,

resistance gene products, receptor-like protein kinases, polygalacturonase inhibitors and LRR-extensins (Jones and Jones, 1997).



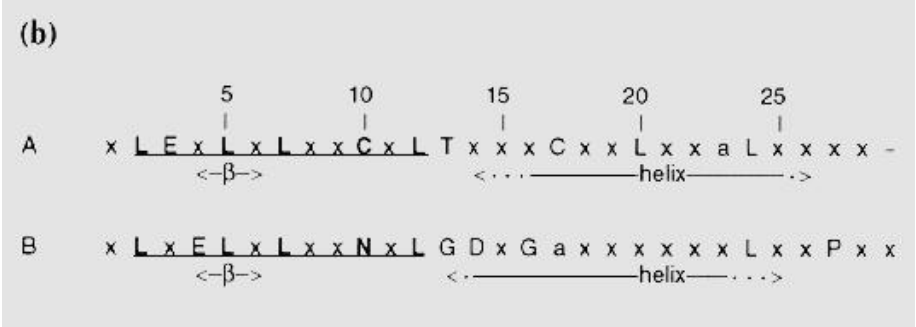


Figure 2: Structure of pig liver ribonuclease inhibitor protein. **(a)** Ribbon diagram of ribonuclease inhibitor protein generated by the program MOLSCRIPT. **(b)** Consensus sequence of two types of repeats (A and B) that alternate in the sequence. Taken from Kobe and Deisenhofer, (1993).

RESISTANCE GENES

The resistance genes are responsible for the recognition of avirulence gene products of the plant pathogens and are responsible for inducing a hypersensitive response. The resistance genes, in addition to having an LRR domain, can also have other domains. All known resistance genes contain leucine-rich repeats except for the *Pto* gene product in tomato that requires an LRR protein *Prf* to function. The resistance genes (Figure 3) fall mainly into three classes.

a). Resistance genes encoding proteins containing extracytoplasmic LRRs with the 24 amino acid consensus: LxxLxxLxxLxLxxNxLxGxIPxx. Examples are the tomato *Cf-9* gene for resistance to the fungus *Cladosporium fulvum* (Jones, Thomas, Hammond-Kosack, Balint-Kurti and Jones, 1994) that contains an extracellular LRR domain and a transmembrane domain to anchor it to the membrane. The rice *Xa-21* gene for resistance to the bacteria *Xanthomonas oryzae* has an extracellular LRR domain connected to a transmembrane domain and an intracellular kinase domain (Song, Wang, Chen, Kim, Pi, Holsten, Gardner, Wang, Zhai, Zhu, Fauquet and Ronald, 1995).

b). Resistance genes encoding cytoplasmic LRRs with 24 amino acid consensus: LxxLxxLxxLxLxx(N/C/T)x(x)LxxIPxx. Examples are the tobacco *N* gene (Whitham, Dinesh-Kumar, Choi, Hehl, Corr and Baker, 1994) for resistance to TMV (tobacco mosaic virus). This protein contains a cytoplasmic LRR domain connected to a domain that shows homology to the *Drosophila* Toll protein and the mammalian interleukin-1 receptor. Another example of a resistance gene encoding a cytoplasmic LRR is the *RPS2* gene in *Arabidopsis* for resistance to the bacterium *P.s*. pv. *tomato* (Bent, Kenkel,

Dahlbeck, Brown, Schmidt, Giraudat, Leung and Staskawicz, 1994). This protein contains the cytoplasmic LRR connected to a nucleotide binding site (NBS) and a leucine- zipper domain.

c) Resistance genes that do not contain the LRR motifs in themselves but require an LRR protein to function. An example is the *Pto* gene in tomato for resistance to the bacterium *P.s*. pv. *tomato* that requires an LRR protein *Prf* to function (Martin, Brommonschenkel, Chunwongse, Frary, Ganal, Spivy, Wu, Earle, and Tanksley, 1993).

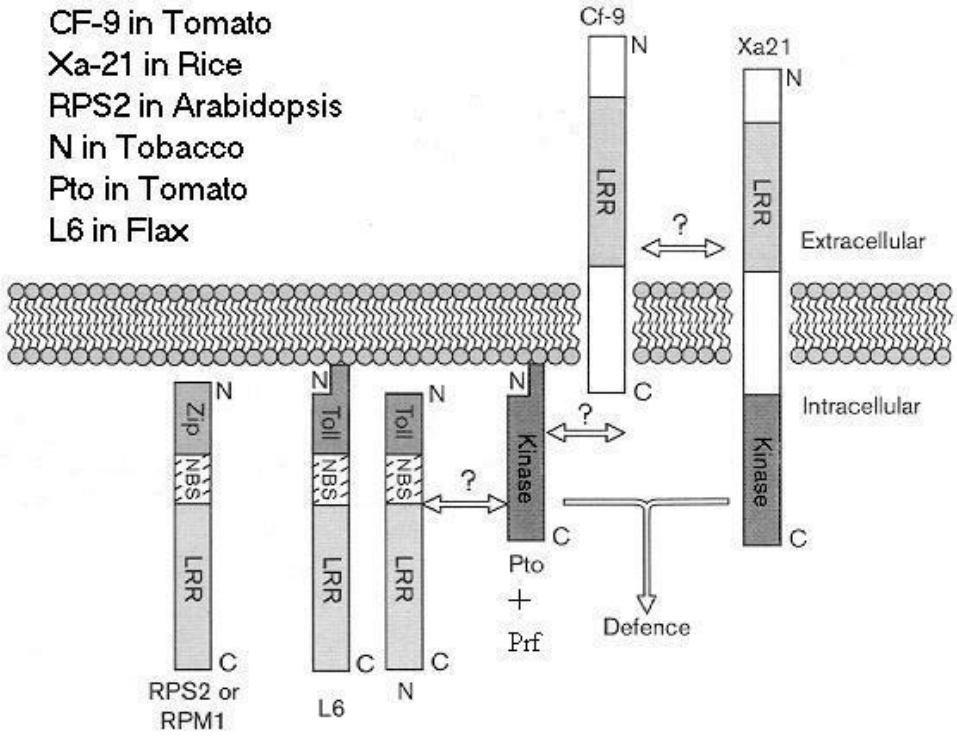


Figure 3: Representation of resistance genes in plants, *Cf-9* and Pto in tomato, *Xa21* in rice, *RPS2* and *RPM1* in *arabidopsis*, *N* in tobacco and *L6* in *Linum usitatissimum*. Taken from Jones and Jones JDG, (1997).

POLYGALACTURONASE INHIBITOR PROTEINS

PGIP or polygalacturonase inhibitor proteins are soluble extracellular glycoproteins that are ionically bound to the extracellular matrix of the plant cells (Salvi, Giarrizzo, De Lorenzo and Cervone, 1990). These proteins are capable of inhibiting fungal endopolygalacturonases by binding to them and slowing down the degradation of pectin, allowing the production of oligogalacturonides 10-15 residues in length, able to induce plant defenses. All the PGIPs isolated to date are composed entirely of 10 LRRs matching the consensus: LxxLxxLxxLxLxxNxLxGxIPxx.

Extraction and bioassay of PGIP activity from bean tissue revealed that the highest activity of PGIP is in the vegetative apex and in flowers, and the lowest activity is in roots, cotyledons, stems, leaves, seeds and embryos. The plant PGIPs do not inhibit bacterial or endogenous endopolygalacturonase enzymes (Salvi, Giarrizo, De Lorenzo, and Cervone, 1990). This leads us to suggest that, since, plants also produce endopolygalacturonases, there may be endogenous PGIPs produced by the plant that are produced against plant endopolygalacturonases, preventing the plant from getting degraded by its very own endopolygalacturonases. Such a protein may also be an LRR protein in the extracellular matrix of the plant cells like the plant PGIPs acting against fungal endopolygalacturonase, but will probably differ from the fungal acting PGIPs in the non-conserved residues in the LRR consensus sequence.

RECEPTOR-LIKE PROTEIN KINASES

Many signals are perceived by transmembrane receptors that activate their intracellular kinase domains by autophosphorylation on their serine or threonine residues. These kinases are called receptor-like protein kinases (RLKs).

The RLKs in plants are divided into three classes based on the structure of the extracellular domain. The kinase domains of these three classes are approximately 40% alike at the amino acid level. The three classes are the S-domain class, the leucine-rich repeat (LRR) class and the epidermal growth factor (EGF)-like repeat class (Plant Receptor Kinase Resource, University of Wisconsin, Madison).

(a). The S-domain class of RLKs (SRKs) are named such, because of their similarity to the S-locus glycoprotein of *Brassica* that functions in the self-incompatibility response of *Brassica*. These proteins have ten conserved cysteine residues in their extracellular domain. Plants that have mutations in the SRK gene are no longer able to distinguish between self and nonself pollen (Goring, Glavin, Schafer and Rothstein, 1993). Therefore, it seems that the SRKs are required to maintain a barrier to self-fertilization.

(b). The leucine-rich repeat (LRR) class of RLKs, have the leucine rich repeat motif in the extracellular domain. A pollen receptor-like kinase (PRK1), present in *Petunia inflata*, belongs to this class. This protein contains five LRRs and is expressed mainly in mature pollen and growing pollen tubes. Expression of an antisense cDNA to the PRK1 resulted in the abortion of half of the pollen. Microspore meiosis proceeded normally, but half of the microspores were arrested at the uninucleate stage. These results show that

PRK1 may be involved in postmeiotic gametophyte development (Skirpan, McCubbin, Ishimizu, Wang, Hu, Dowd, Ma and Kao, 2001).

A gene called ERECTA1 in *Arabidopsis*, is another member of the leucine-rich repeat class of kinase. The gene encodes 20 LRRs. Mutation in ERECTA1 caused compact inflorescense, shortened siliques and leaf petioles. Thus it seems that ERECTA1 is involved in specification of organ shape.

CLAVATA1 is another example of a leucine-rich repeat class of RLK present in *Arabidopsis.* This gene encodes 21 LRRs. Mutation in the CLAVATA1 causes altered floral organ number and fasciated stems. This results from the primary phenotype of very large shoot apical meristems (Clark, Running and Meyerowitz, 1995). A very large number of undifferentiated cells accumulate within the expanded meristem suggesting that the CLAVATA1 gene functions to promote cell differentiation or to restrict cell proliferation.

(c). Epidermal growth factor (EGF) repeat RLKs, is a class repesented by five wall-associated kinases (WAKs) in *Arabidopsis*. These genes code for two epidermal growth factor-like repeats and also collagen, extensin and neurexin motifs. Genetic analysis shows that WAKs play a role in cell expansion and maintainence of cell viability during pathogenesis (Kohorn, 2001).

LRR- EXTENSINS

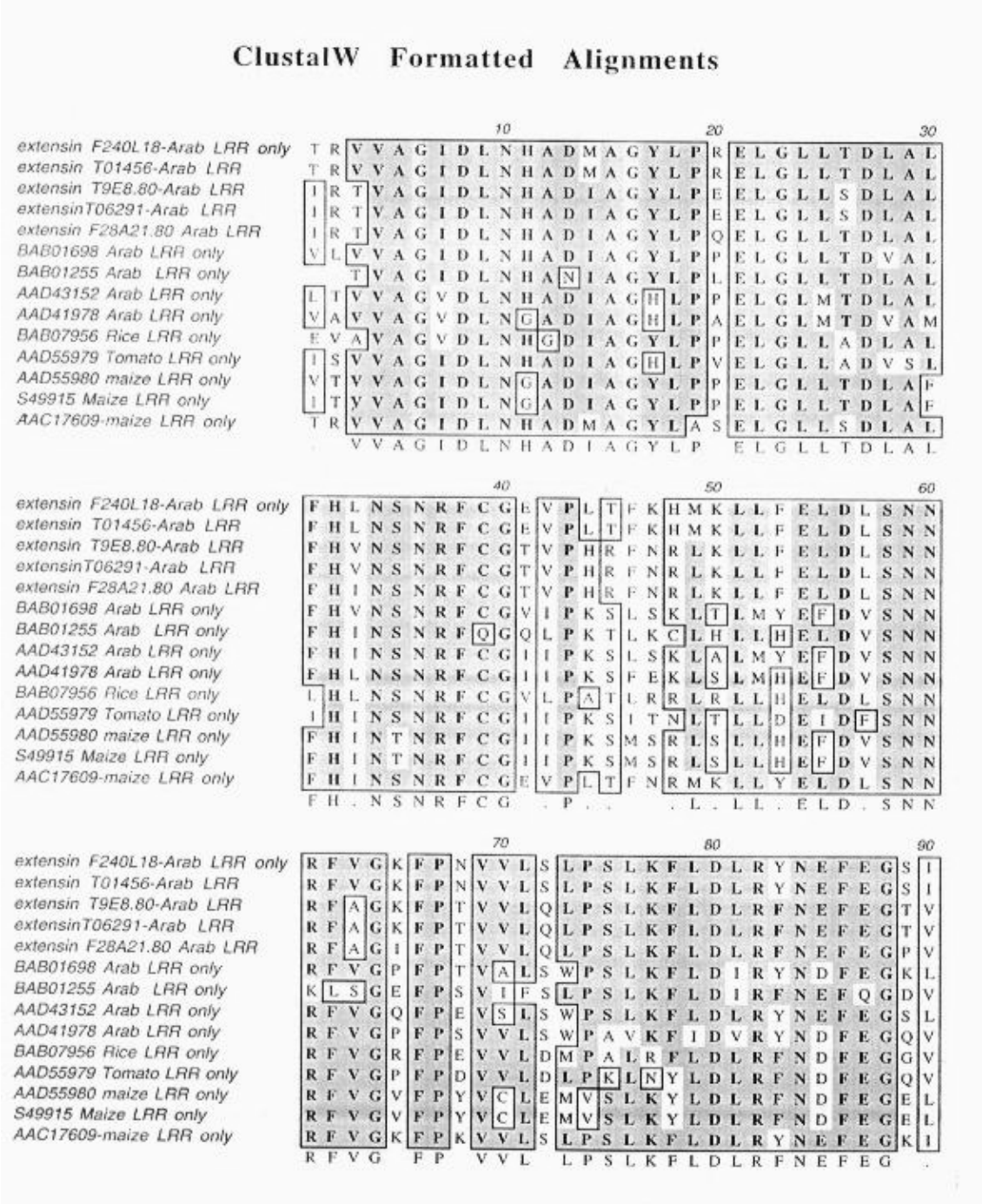
The LRR-extensin gene, codes for chimeric proteins containing two domains, an N-terminal LRR domain and a C-terminal extensin-like domain. To date (12-1-02), there are fourteen LRR-extensin genes in the GenBank database. Nine of these are present in *Arabidopsis,* one in rice, one in tomato and three in maize. Two sequences in *Arabidopsis* (Accession no. CAB79682 and CAA16878), code for the LRR domain only and do not have the extensin-like domain, but have been mistakenly reported as extensin-like proteins.

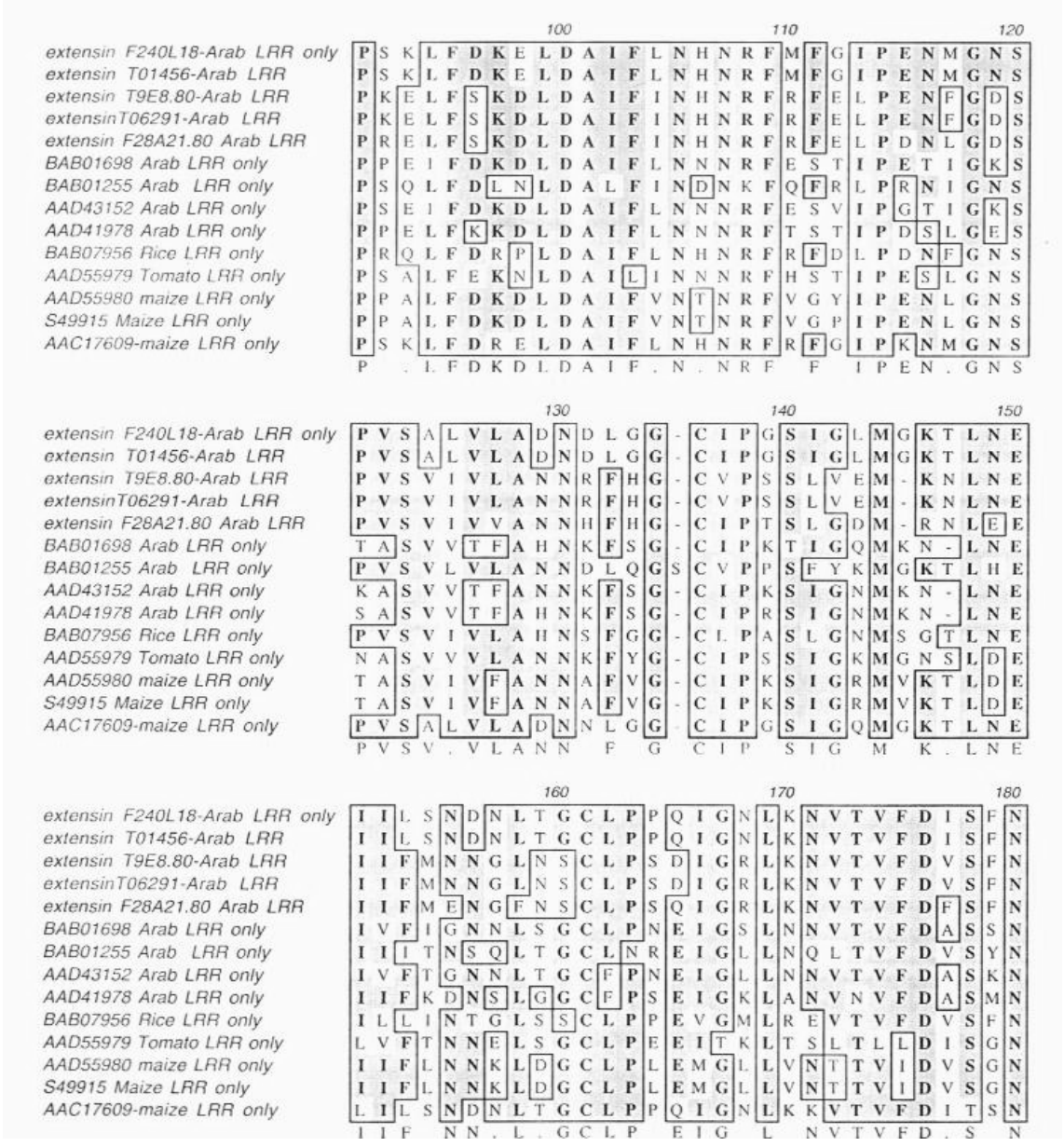
Most of the sequences in the database have come from the genome sequencing programs. Thus far, only two of the sequences in *Arabidopsis* have ESTs (expressed sequence tags). Figure 4 shows the alignment of fourteen LRR-extensins in the database. Only the LRR regions were aligned, because the extensin-like regions were different for each of the sequences.

To date, only three LRR-extensins, one in tomato (Tom-L4), one in maize (Pex1), and one in *Arabidopsis* (LRX1), have been studied. The tomato LRR-extensin was isolated from a tomato genomic library by screening with a carrot extensin sequence. Tom-L4 has ten LRR repeats and is expressed in the green vegetative tissues of plants and northern blot analysis of its leaves indicated that Tom-L4 accumulates in response to wounding (Zhou, Rumeau and Showalter, 1992).

The maize LRR-extensin has been studied in more detail than Tom-L4. This gene was isolated from a pollen cDNA library by differential screening with cDNAs from endosperm. This gene is expressed only in mature pollen and not in other tissues. Unlike Tom-L4, Pex1 is not induced by wounding. Antibodies made to the LRR domain bind to

Figure 4: Alignments of 14 LRR-extensins in the Genbank database.




100
110
120
extensin F240L18-Arab LRR only
extensin T01456-Arab LRR
extensin T9E8.80-Arab LRR
extensinT06291-Arab LRR
extensin F28A21.80 Arab LRR
BAB01698 Arab LRR only
BAB01255 Arab LRR only
AAD43152 Arab LRR only
AAD41978 Arab LRR only
BAB07956 Rice LRR only
AAD55979 Tomato LRR only
AAD55980 maize LRR only
S49915 Maize LRR only
AAC17609-maize LRR only
130
140
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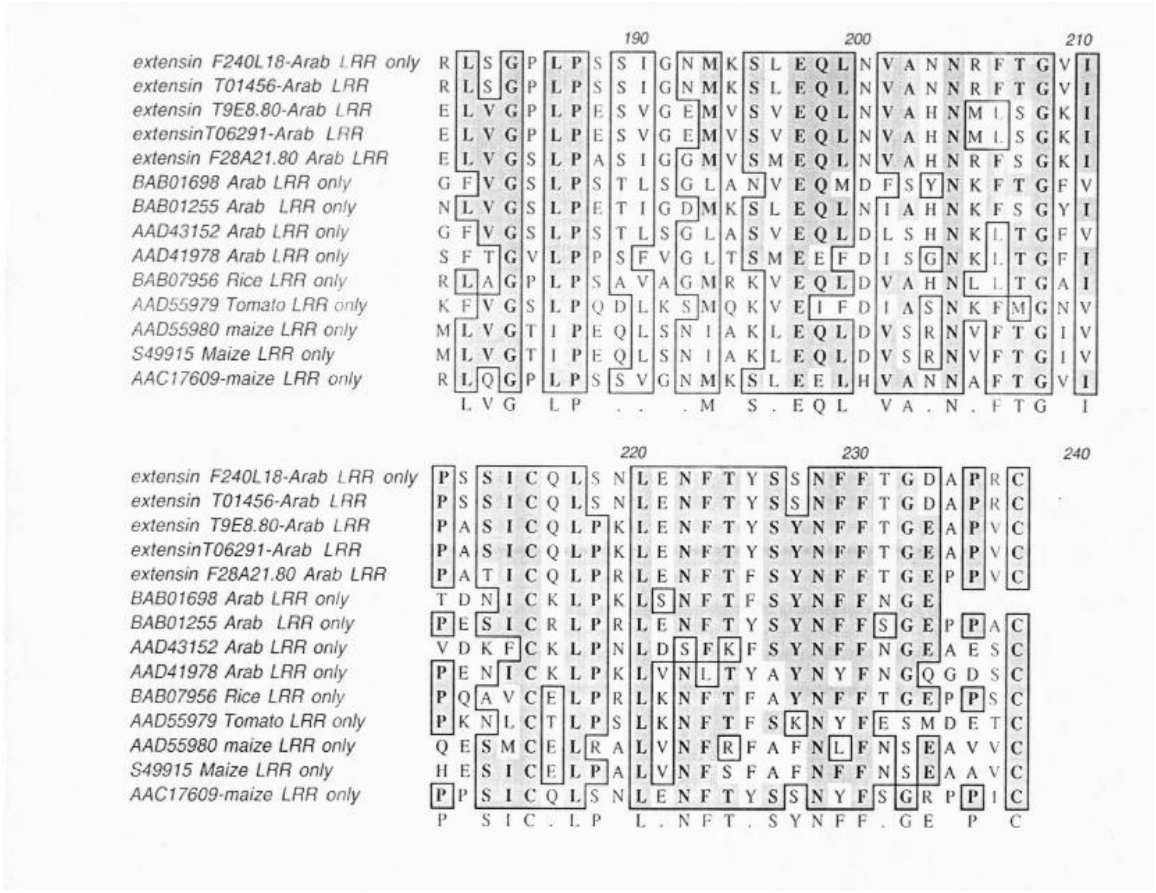
170
180



the intine layer in the pollen wall. The protein is tightly bound to the wall and cannot be liberated by detergents. The authors hypothesized, based on the location of the protein, that it is important in providing support to the mature pollen as it grows inside the style (Rubenstein, Broadwater, Lowrey and Bedinger, 1995). The LRR domain of Tom-L4 and Pex1 show a 56.6% amino acid identity, though their extensin-like domains are very different.

The *Arabidopsis* LRR-extensin LRX1 was isolated from the genomic library using the tomato Tom-L4 sequence (Baumberger, Ringli and Keller, 2001). This gene is

expressed in the cell walls of root hair and not in any other plant organ. Though wounding and infection studies were not done, transposon-tagged LRX1 mutants were identified and these mutants exhibited irregular root hair development. The majority of the root hairs did not elongate fully. Root hair development was arrested soon after initiation resulting in short stumps. Root hairs which proceeded further frequently branched and showed swelling along the main stalk that resulted in root hairs of irregular diameter. LRX1's LRR region, shows a 53% identity to maize Pex1 and a 73% identity to tomato Tom L4, but the extensin-like domains are very different.

Thus the three LRR-extensins are expressed in different plant organs and their responses to wounding are different. This may suggest that they have different functions. However, the intriguing similarity of the LRR domains, suggest that they may be involved in recognizing similar ligands (Jones and Jones, 1997).

CHAPTER III

MATERIALS AND METHODS

Suspension culture cells used in the experiment

Callus cultures from fully expanded Acala 44 cotton leaves were established in 1985 (Janet Rogers, Oklahoma State University) using a modification of the method of Ruyack (Ruyack, Downing, Chang and Mitchell, 1979). The suspension culture cells were cultured by transferring one tube containing 3-4 g of the 3-4 week old callus (in the late log phase) from SH agar medium to 150 ml culture flasks containing 50 ml liquid SH medium (Schenk and Hildebrandt, 1972). Growth curves of the Acala 44 suspension culture cells (Sharlene Ruth Matten, Phd thesis, Oklahoma State University) shows that cells are in the log phase between days 4-10 and reach their stationary growth phase on day 12. Cells on day 9 were the source of RNA for the 5' RACE experiment.

Total RNA isolation from cotton suspension culture cells and plant tissue

Total RNA was isolated by using the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). For the experiment, glassware was baked at 200°C overnight and reagents were treated (with the exception of Tris containing buffers) with 0.1% DEPC

overnight and autoclaved the next day. Tris-containing solutions were made in DEPC treated water. The RNA isolation method involved grinding 1 g of the suspension culture cells or plant material in liquid nitrogen, and extracting the resulting powder in 12 ml of denaturing solution (26 mM sodium citrate pH 4, 0.5% N-lauryl sarcosine, 0.125 M β-mercaptoethanol, 4 M guanidine thiocyanate). The RNA was precipitated from the denaturing solution by adding 1200 µl of 2 M sodium acetate pH 4, followed by the addition of 12 ml of phenol:chloroform:isoamyl alcohol (25:24:1) pH 4.7, chilling on ice for 15 min, and centrifuging at 10,000g for 20 min. The top aqueous phase was collected, an equal volume of isopropanol was added to it, and it was incubated at -20°C for 30 min. The mixture was centrifuged at 10,000g for 10 min and the pellet containing RNA was washed with ice cold 75% ethanol and then dissolved in DEPC treated water.

Estimation of the concentration of RNA sample

The RNA concentration was estimated with a spectrophotometer at 260 nm using the equation (Sambrook, Fritsch, and Maniatis, 1989):

$$\text{RNA concentration in } \mu g/\mu l = \frac{\text{Absorbance at 260nm x dilution factor x 40 } \mu g/ml}{1000\ \mu l/ml}$$

Absorbance of RNA in water of 1.0 corresponds to 40 µg/ml of RNA.

Estimation of the purity of RNA sample

The purity of the RNA sample was estimated by the absorbance ratio of the preparation of RNA: A_{260}/A_{280}.

Very pure RNA will have a ratio of 2.0. In practice, a ratio of 1.7-2.0 is accepted as relatively free of protein contamination. Furthermore, to detect DNA contamination and to check the integrity of the RNA sample, a 1% agarose/MOPS/formaldehyde gel was run. No staining with ethidium bromide in the wells of the gel indicated that the RNA was free of DNA contamination. Two prominent bands representing the 28S and 18S subunits of ribosomal RNA without a smear of smaller pieces indicate there was negligible degradation of the RNA.

1% Agarose/MOPS/Formaldehyde gel (75ml)

Agarose (0.75 g) was melted in 54ml of DEPC treated water and cooled to 65°C. Then, 13.5 ml of formaldehyde and 7.5 ml of 20X MOPS buffer (400 mM 3-N-morpholino) propanesulphonic acid, 160 mM sodium acetate, 20 mM EDTA, pH 7.0) was added, and this mixture was poured into the gel casting tray. RNA was denatured in RNA loading buffer (deionized formamide 62.5% (v/v), formaldehyde 1.14 M, bromophenol blue 200 µg/ml, xylene cyanole 200 µg/ml, MOPS-EDTA-sodium acetate at 1.25X working concentration, ethidium bromide 50 µg/ml) by heating at 65°C for 10 min followed by snap cooling. The denatured RNA was loaded on the gel and run in 1X MOPS buffer at 5V/cm till the dye reached the end of the gel.

cDNA library construction and screening

RNA from 13-day old Acala 44 cotton suspension culture cells was isolated by using RNAgents® Total RNA isolation system (Promega), and mRNA was isolated using Poly(A) Quik® mRNA Isolation Kit (Stratagene). The cDNA library was constructed using the ZAP-cDNA® synthesis kit (Stratagene). The carrot extensin clone pDC5A1 (Chen and Varner, 1985) was random prime labeled using DIG-High Prime (Roche Molecular Biochemicals), and the degenerate probe was labeled with DIG Oligonucleotide Tailing Kit (Roche Molecular Biochemicals). The library was prehybridized using DIG Easy HYB (Roche Molecular Biochemicals), hybridized at 42°C, washed twice at 2X SSC, 0.1% SDS for five min and twice at 0.5X SSC, 0.1% SDS at 60°C for 20 min. Positive clones were identified using the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals) according to the instructions of the Genius™ system user's guide for membrane hybridization (Roche Molecular Biochemicals).

5' RACE Experiment

5' RACE is a technique that allows the isolation and characterization of the 5' ends of cDNAs (Frohman, 1993). For this technique, a 5' RACE kit was used (Gibco BRL). The first strand cDNA was synthesized using total RNA from Acala 44 suspension culture cells by using Superscript™ II reverse transcriptase, and was primed by a gene-specific antisense oligonucleotide primer (GSP-1). This was followed by digesting the RNA with RNAse A and the cDNA was purified with a Glassmax spin cartridge column. Following

purification, the cDNA was tailed at the 3’ end with dC, using the enzyme terminal deoxynucleotidyl transferase. Following this, the dC tailed cDNA was amplified by PCR using an abridged anchor primer that annealed to the dC tail and a second gene specific primer (GSP-2). The primary PCR product was then amplified using a mixture of two primers, a nested gene-specific primer (GSP-3), that annealed 3' to the GSP-2 and an anchor primer that anneals to the sequences in the abridged anchor primer. The procedure is summarized in Figure 5.

The primers used for the experiment were:

GSP 3: 5’-CGGCAAACAGTTCCTCCTATCGTC- 3’

GSP 2: 5’-TGTTCAAGGCTAACCATTCCCCCA- 3’

GSP 1: 5’-CGGCAAAGTACCCATTAACTCAT- 3’

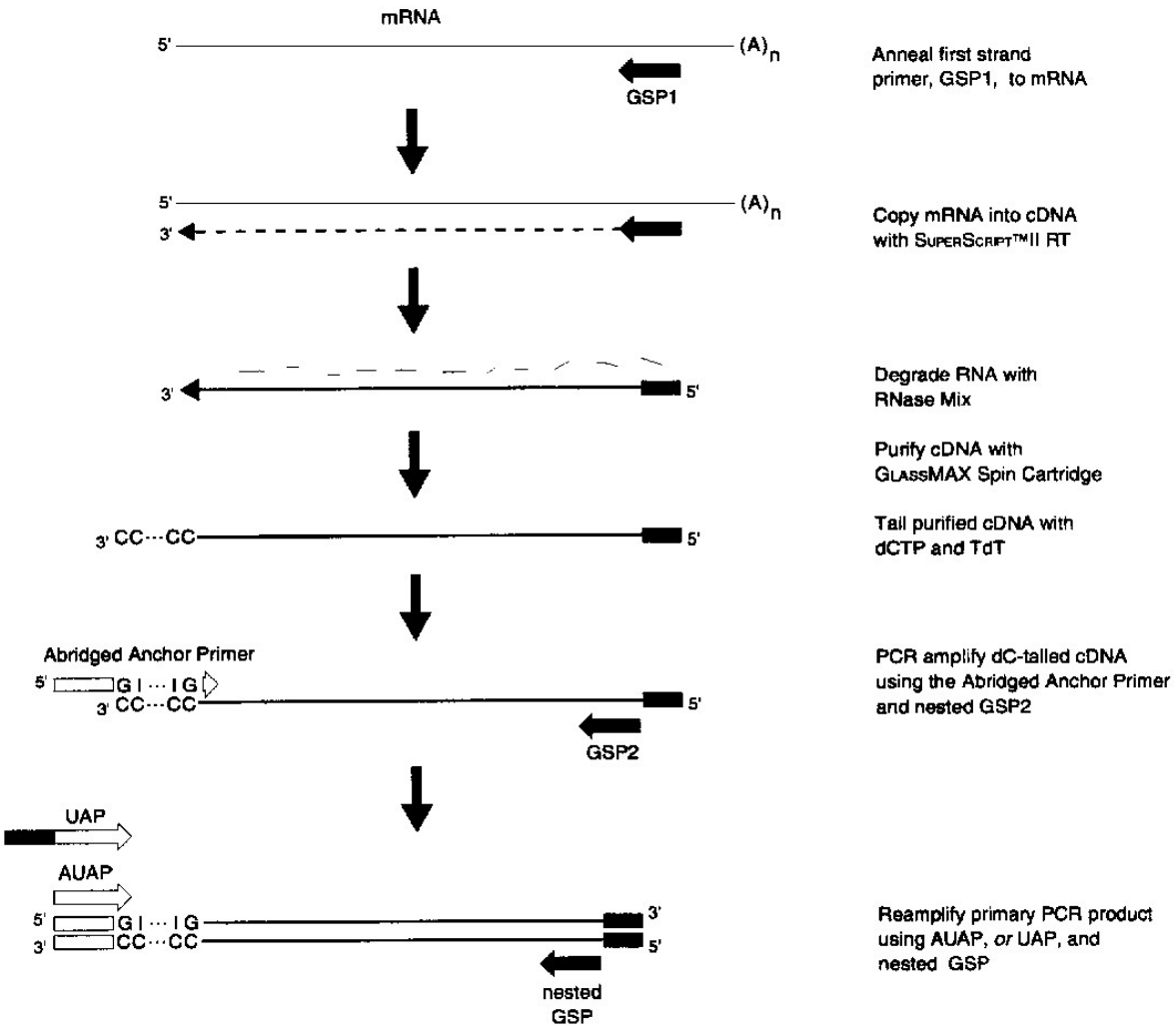


Figure 5: Overview of the 5' RACE Procedure (UAP = Universal Amplification Primer; AUAP = Abridged Universal Amplification Primer). Figure taken from the 5' RACE manual (Gibco BRL).

TA Cloning

TA cloning is a method for directly cloning PCR fragments into a plasmid vector. For the purpose of TA cloning, a TA cloning® kit was purchased (Invitrogen). The cloning is based on the action of *Taq* polymerase, which adds a single deoxyadenosine (A') residue to the 3' ends of PCR fragments. This fragment was ligated to a plasmid vector (pCR™ II) containing a 3' deoxythymidine residue (T'). This plasmid was transformed into the bacterial strain INVaF'. The process of ligation of the PCR fragment into the pCR™ II vector disrupts the gene for the β-galactosidase enzyme and causes the bacteria that are transformed by such a plasmid to be white when grown on LB-ampicillin plates containing X-gal. The white colonies of bacteria were picked up and plasmids were isolated from them by minipreps using the Qiaprep kit (Qiagen). The plasmids were digested with EcoRI (at sites flanking the TA cloning site) and run on 1% agarose gels to confirm the presence of the PCR fragment. The plasmids containing the PCR fragments were sent to the DNA recombinant facility for sequencing (Oklahoma State University).

Synthesis of probes for northern and southern analysis and genomic library screening

The sequences used as probes included the 5'RACE fragment of the LRR-extensin (that contains only LRRs), cloned into the TA cloning kit vector pCR™ II (Invitrogen), the incomplete LRR-extensin cDNA (# 65631), the extensin cDNA # 2, extensin cDNA # 3 (sequences obtained by cDNA library screening by using degenerate probes coding for

extensins and carrot extensin clone) and PCR amplified 3' ends of extensin # 2 and extensin # 3, cloned into the TA cloning vector pCR™ II.

The 5' RACE fragment of LRR-extensin and the 3' ends of extensin # 2 and # 3, were removed from the vector pCR™ II, by EcoR1 digestion. The incomplete LRR-extensin cDNA (# 65631), the incomplete extensin cDNA # 2 and extensin cDNA # 3 were removed from the vector pBluescript, by EcoRI and XhoI digestion. The digests were run on a 1% gel, and the inserts were cut out of the gel and purified using a Qiaquick gel extraction kit (Qiagen). The purified cDNAs were denatured in a boiling water bath for 10 min and snap cooled. Probes were made by random prime labeling, using the DIG-High Prime labeling kit (Roche Molecular Biochemicals) that labels the cDNA with digoxigenin. The DIG-High Prime solution is a single vial that contains random hexamer primer mixture/ Klenow enzyme/ dATP/ dCTP/ dGTP/ dTTP /DIG-11-dUTP. To 30 µl of cDNA (in 10 mM Tris pH 8) containing between 10-3000 ng of cDNA, 8 µl of DIG-High Prime solution was added, and the tube was incubated at 37°C overnight for the labeling to occur. The labeling reaction was stopped, by adding 4 µl of 0.2 M EDTA solution, pH 8. The labeling efficiency was estimated by spotting 1 µl aliquots of the labeled mixture at different dilutions on a nylon membrane and comparing spot intensity between the newly labeled mixture and labeled DNA standards after incubating the nylon membrane in anti-digoxigenin antibody conjugated to alkaline phosphatase and colorimetric detection by NBT and BCIP. Before use, the probes were denatured in a boiling water bath for 10 min, snap cooled, and added to the hybridization solution, DIG Easy Hyb (Roche Molecular Biochemicals) at a concentration of 10 ng of probe/ ml of hybridization buffer. The resulting diluted probes were stored at -20°C and reused 6

times after denaturing the probes in DIG Easy Hyb for 10 min at 68°C before use. Extensin # 2 primers used in PCR to make the 3' unstranslated sequence include,

Forward sequence: AGCCACGGCTTTGACCGTGC

Reverse sequence: AATCCTTACCTGTAATATAA

Extensin # 3 primers used in PCR to make 3' untranslated sequence include,

Forward sequence: CTAGAAAGATCAACCACAA

Reverse sequence: TTTCAGGTTCTAGTTTCTCA

Northern Blot

The RNA samples were subjected to electrophoresis in an agarose/MOPS/formaldehyde gel until the xylene cyanol dye reached the end of the gel (Sambrook, Fritsch and Maniatis, 1989). The gel was photographed and then equilibrated in 20X SSC (3 M NaCl, 300 mM sodium citrate pH 7.0) twice, for 15 min each time. Meanwhile, 200 ml of 20X SSC was poured into a glass tray, and a Whatman 3MM filter paper was wetted with 20X SSC, and wrapped around a polyester support and placed in the tray. The gel after equilibration was placed upside down on this filter paper. Using gloves, a nylon membrane (Roche Molecular Biochemicals) was carefully placed on top of the gel. Two pre-wet Whatman filter papers cut to the size of the gel were carefully placed on the nylon membrane followed by two dry Whatman filter papers. A stack of paper towels were placed on top of the filter papers, and the entire assembly was covered with plastic wrap to prevent evaporation while capillary transfer took place overnight at room temperature. Diagram of transfer is shown in Figure 6.

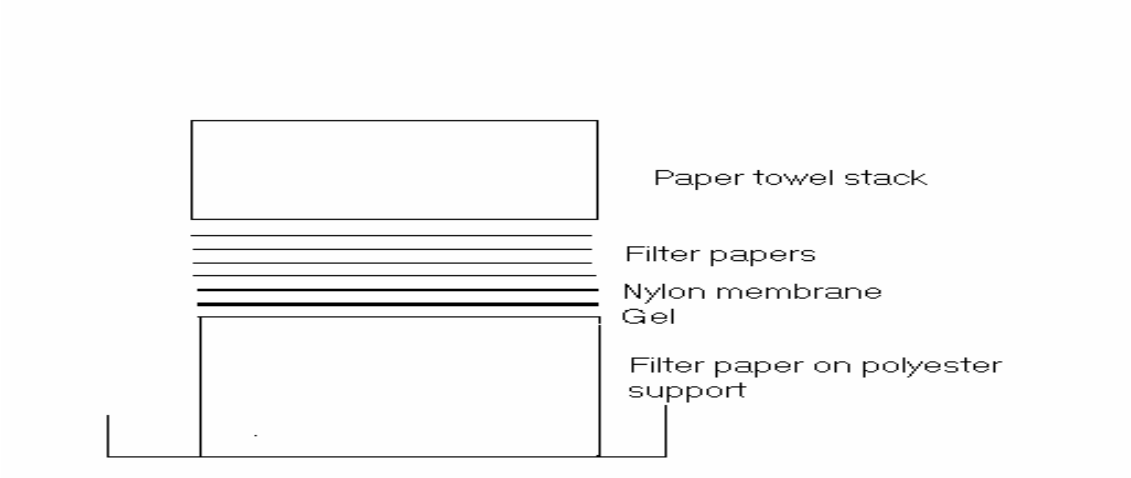


Figure 6: Diagram of capillary transfer for Southern blot and Northern blot

The next day, the nylon membrane was marked for orientation of the RNA samples and then UV crosslinked using the stratalinker (Stratagene) on both sides of the membrane. The membrane was incubated at 42°C for one hour in the hybridization buffer, DIG-easy hyb. After an hour, the membrane was allowed to incubate overnight at 42°C in the probe diluted in the hybridization buffer. The membrane was then washed twice, for 15 min in 2X SSC, 0.1% SDS. This was followed in addition, by stringent washes at 68°C with 0.1X SSC, 0.1% SDS, twice for 15 min. Alternatively, for less stringent washes, we used O.5X SSC, 0.1% SDS. The membranes were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween®20) for 1 min. The membranes were incubated in a blocking solution containing 1% blocking reagent (Roche Molecular Biochemicals) in 100 mM maleic acid and 150 mM NaCl for one hour followed by incubation in 1:20,000 dilution of anti-digoxigenin-alkaline phosphatase conjugated antibody in blocking solution for half an hour. After the incubation period, the membranes were

washed in washing buffer twice, for 15 min each and equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl pH 9.5) for 2 min. CDP-Star reagent (Roche Molecular Biochemicals) was applied to the membrane placed between two plastic sheets and a glass rod was rolled over the sheets to remove air bubbles. The membrane was incubated in CDP-Star reagent for 5 min and then sealed in a plastic bag. For chemiluminiscent detection of signal in the membrane, the membrane was put in an X-ray cassette and exposed to Lumi-Film (Roche Molecular Biochemicals).

Cotton genomic DNA isolation

Acala 44 suspension culture cells were ground in liquid nitrogen and homogenized in extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 500 mM NaCl, 700μL β-mercaptoethanol for every 100 ml extraction buffer). We used 15 ml of buffer for 5-7 g cells. About 1 ml of 20% SDS was added, mixed thoroughly, and the solution was placed at 65°C for 10 min. After 10 min, 5 ml of 5 M potassium acetate was added, mixed and the solution was placed on ice for 20 min. The solution was centrifuged at 5000g at 4°C for 20 min. The supernatant was transferred to a new tube and 10 ml isopropanol was added and incubated at -20°C for 1 hour. The tube was centrifuged at 5000g for 10 minutes at 4°C. The pellet was resuspended in sterile water, and 2-5 μl of RNase A (10 mg/ml) was added and incubated at 37°C for 10 minutes. The DNA was finally precipitated by adding 1/3 volume of 3 M sodium acetate and 1 volume of isopropanol and centrifuging the tube at 3000g for 5 min at 4°C. The DNA pellet was washed with 70% ethanol and finally suspended in 10 mM Tris pH 8.0.

Southern Blot

DNA was subjected to electrophoresis in a 1% gel until the xylene cyanol dye reached the end of the gel. The gel was shaken in 250 mM HCl for 10 min to depurinate the DNA. The gel was then rinsed in water and incubated in denaturing solution (0.5 N NaOH, 1.5 M NaCl), twice for 15 min each time. This treatment denatured the DNA and made it single stranded. We rinsed the gel in water and then incubated the gel twice for 15 min each time in neutralizing solution (0.5 M Tris-HCl, 3 M NaCl pH 7.5). The DNA was then blotted from the gel to the nylon membrane (Roche Molecular Biochemicals) by capillary transfer, as explained in the northern blot protocol.

The membrane was UV crosslinked on both sides using the stratalinker (Stratagene) and incubated for two hours at 42°C in the hybridization buffer, DIG-easy hyb (Roche Molecular Biochemicals), and overnight at 42°C in hybridization solution containing probe. The membrane was washed twice for 15 min in 2X SSC, 0.1% SDS followed by stringent washes, twice for 15 min in 0.1X SSC, 0.1% SDS at 68°C. Alternatively, for less stringent washes, the membranes were washed twice for 15 min in 0.5X SSC, 0.1% SDS. Then, the membrane was equilibrated for 1 min in washing buffer. This was followed by 1hour incubation in blocking buffer and half an hour incubation in 1:20,000 dilution of anti-digoxigenin-alkaline phosphatase antibody in blocking buffer. The membrane was then washed in washing buffer twice for 15 min, incubated in detection buffer for 2 min and CDP-Star was applied to the membrane between two plastic sheets and incubated like this for 5 min and then exposed to Lumi-film for chemiluminescent detection.

Genomic Library Screening

Cotton (*Gossypium hirsutum* L. cv. Acala SJ-2) genomic library in λ FIX II (Stratagene) was given to us by Dr. Thea Wilkins (University of California, Davis). In the primary screen, approximately 600,000 plaques were screened. This was done by plating 50,000 plaques in a single 150mm NZY plate and using 12 plates for the screening procedure. The plates were cooled at 4°C for 30 min. Nylon membranes (Amersham) were placed on the plates and left for1 min to allow for the transfer of the plaques from the NZY plate to the membrane. The membranes were then allowed to soak in trays containing denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 15 min, neutralizing solution (1 M Tris-HCl, 1.5 M NaCl pH 7.5) for 15 min, and 2X SSC (0.3 mM NaCl, 30 mM sodium citrate, pH 7.0) for 10 min.

The membrane was UV crosslinked using the stratalinker (Stratagene) and prehybridized at 42°C with Dig-easy hyb for an hour followed by overnight incubation at 42°C in hybridization solution containing the probe (the probe used was the 5' RACE sequence diluted 10 ng/ml in DIG-easy hyb). The membrane was washed twice for 15 min in 2X SSC, 0.1% SDS and twice in 0.5X SSC, 0.1% SDS. The membranes was equilibrated in washing buffer for 1 min and incubated in blocking buffer for 1hour. This was followed by incubation for half an hour in 1:20,000 dilution of anti-digoxigenin-alkaline phosphatase conjugated antibody. The membranes was washed in washing buffer twice for 15 min, and incubated in detection buffer for 2 min. CDP-Star reagent was applied to the membrane between plastic sheets and the membrane was allowed to incubate for 5

min in this reagent. The membrane was sealed in plastic bags and exposed to Lumi-film for chemiluminescent detection.

The positively hybridizing plaques were picked with a pasteur pipette, dispersed in 1 ml of SM buffer (0.01% gelatin, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) and allowed to stand overnight. These plaques were diluted and plated on XL1-blue MRA bacteria and subjected to secondary and tertiary hybridization to pick up true positives. From these positive plaques, lambda DNA was isolated using the Wizard lambda DNA isolation kit (Promega).

Stripping of membranes for reprobing

For reprobing protocols, the nylon membrane was always kept wet. For southern blots, probes were removed from the membrane by incubating the membrane twice for 10 min each time, in alkaline stripping solution (0.2 N NaOH, 0.1% SDS). The membrane was rinsed thoroughly in 2X SSC and then put into hybridization solution, ready for probing with a different probe. If the second probe was not ready, the membrane was stored in 2X SSC till it was ready to be hybridized to a second probe.

For northern blots, 100 mL of 0.1% SDS was heated till it was ready to boil and poured on the membrane and, the membrane was allowed to incubate in this solution without additional heating for 10 min on a rocking platform.

The membrane was then washed in washing buffer for 10 min and either stored in 2X SSC or put into hybridization solution, ready to be analyzed with a different probe.

In-gel digest for MALDI-MS

The procedure for the experiment was taken from 'Current Protocols in Protein Science', Unit 16.4

Excision of the band from polyacrylamide gel- The LRR protein was run on an 8% polyacrylamide gel. The gel was stained with 0.3% coomassie blue in water and then rinsed twice in water for 10 min/wash. The 45 kD band was then excised from the gel.

Washing of the gel pieces-The gel containing the band was washed with water twice, 15 min/wash and once in water/acetonitrile solution, 1:1 for 15 min. The liquid was then removed and the gel was covered with acetonitrile. When the gels shrank and turned sticky white, the acetonitrile was removed, and the gel was hydrated with 100 mM NH_4HCO_3. After about 5 min, an equal volume of acetonitrile was added and the gel was incubated for 15 min in this solution. Then the liquid was removed and the gel was dried in a speed vac.

Reduction and alkylation- The gel was rehydrated in 10 mM DTT, 100 mM NH_4HCO_3, incubated in this solution for 45 min at 56°C, and then allowed to cool to room temperature. The liquid was then replaced with the same volume of 55 mM iodoacetamide, 100 mM NH_4HCO_3 solution and allowed to incubate for 30 min in this solution in the dark. Then all the liquid was removed and acetonitrile was added to cover the gel and allowed to incubate in this solution for a few minutes. Then all the liquid was removed, 100 mM NH_4HCO_3 was added, and this solution was incubated for 5 min, followed by addition of an equal volume of acetonitrile. This solution was incubated for 15 min at room temperature, following which all the liquid was removed.

In-gel digestion- The gel was completely dried in a speed vac and then rehydrated in digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12.5 ng/µl trypsin) at 4°C. Then enough buffer was added to cover the gel, and the solution was put on ice for 45 min. The supernatant was removed and replaced with the same buffer (without trypsin) and the gel was incubated at 37°C overnight in this buffer.

Extraction of peptides- NH_4HCO_3 (25 mM) was added to cover the gel piece and incubated in a bath sonicator for 15 min. The same volume of acetonitrile was added and incubated for 15 min in a bath sonicator. After 15 min, the supernatant was recovered and the extraction procedure was repeated twice more by adding 5% formic acid and incubating in a bath sonicator for 15 min. The supernatants containing the peptides were pooled, and 10 mM DTT was added to a final concentration of 1 mM DTT. The supernatants were dried in a speed vac to near dryness and resuspended in 10-30 µl of 5% formic acid, 5% methanol.

Zip tip cleanup of the peptide sample for MALDI analysis- A C18 zip tip was equilibrated with 60% methanol, 5% formic acid and then with 5%methanol, 5% formic acid. The peptide sample was allowed to bind to the zip tip by repeatedly drawing and dispensing the sample through the zip tip slowly, about 10-15 times. The zip tip was washed in 5% formic acid, 5% methanol slowly about 3 times. Finally, the peptide sample was eluted from the zip tip with 5% formic acid, 60% methanol about 10-15 times. This eluate was used for MALDI analysis.

Protein extract preparation and Bradford protein assay

Bradford reagent preparation- This reagent was prepared by dissolving 100 mg of Coomassie Blue G-250 in 50 ml of 95% ethanol, adding 100 ml 85% phosphoric acid to the solution, and diluting the resulting solution to 1 liter with water.

SDS-Tris buffer- This buffer was used to make protein extracts from different tissues of cotton. It was prepared by mixing 77.5 ml of 10% SDS, 12.5 ml of 1 M Tris pH, 7.0, and 10 ml of β-mercaptoethanol.

Protein extract preparation-Cotyledons, stems, root and suspension culture cells were ground in liquid nitrogen with SDS-Tris buffer and then heated at 95°C for 5 minutes to denature the samples. The extract was then centrifuged at 12,000g for 1 minute to get rid of insoluble matter.

Bradford assay- The Bradford protein assay is a procedure for determining the protein concentrations in solutions, which depends upon the change in absorbance of Coomassie Blue G-250 upon binding of protein (Bradford, 1976). To prevent interference from the SDS in the protein extracts, we first precipitated the extracts by adding equal amounts of 10% TCA and allowed the solution to stand on ice for 20 min, followed by centrifugation at 12,000g for 10 min. The pellet was washed with ice-cold acetone, air dried and finally dissolved in 0.1 M NaOH. BSA dissolved in 0.1 M NaOH was used as a standard for making standard curves at a concentration range of 200-1500 μg/ml.

About 5 ml of Bradford's reagent was added to 100 μl of the BSA standards and the protein extracts, and mixed by vortexing. The absorbance was read at 595 nm after 5 min,

and protein concentration was estimated by comparison with a curve generated by plotting absorbance versus concentration of BSA standards.

Semi-dry electroblotting

The semi-dry electroblotting apparatus helps in the transfer of proteins present in the gel to the PVDF membrane. Three solutions were made, Anode buffer 1 (0.3 M Tris, 20% methanol), Anode buffer 2 (25 mM Tris, 20% methanol) and Cathode buffer (25 mM Tris, 40 mM aminohexanoic acid, and 20% methanol).

Two pieces of Whatman filter paper were wetted in cathode buffer, one in anode buffer 1 and one in anode buffer 2. The PVDF membrane was first wetted in methanol and then wetted in water. The polyacrylamide gel apparatus was disassembled, leaving the gel on one plate. The wet PVDF membrane was laid on the gel and air bubbles were removed. A filter paper dipped in anode buffer 2 was laid on top of the membrane, and this filter paper was overlaid with a filter paper dipped in anode buffer 1. This assembly was transferred to the bottom carbon electrode of the transblot apparatus, but inverted, so that the gel was on top. Finally, two filter papers dipped in cathode buffer were laid on top of the gel. The transblot apparatus was then assembled and run at 2.5 mA/cm^2 for 30 min.

Western Blotting

An 8% polyacrylamide gel was run with protein samples till the dye reached the end of the gel. The proteins were then transferred from the gel to the PVDF membrane (Biorad) using the transblot apparatus. After transfer, the membrane was washed in TBS (10 mM Tris, 150 mM NaCl pH 7.4) for 1 min at room temperature. The membrane was then put in a blocking solution (5% skim milk in TBS) for 1 hour followed by 1:200 dilution of the ascites fluid in blocking solution (1% skim milk in TBS) and left overnight at 4°C.

(The ascites fluid was obtained from mice, after intraperitoneal injection with appropriate adjuvant and 100 μg of LRR domain of protein, as antigen).

The next day, the membrane was washed in TBS for 5 min at room temperature, twice in TBS, 0.5% Tween 20 for 5 min, and TBS, 5% skim milk for 5 min. The membrane was then allowed to incubate in secondary antibody solution (1:2500 dilution of anti-mouse-alkaline phosphatase antibody in TBS, 1% skim milk) for 2 hours at room temperature. The membrane was washed in TBS for 5 min, twice in TBS, 0.5% Tween 20 for 5 min, and twice in TBS for 5 min. The membrane was finally incubated in warm (37°C) alkaline phosphatase detection buffer (100 mM Tris-HCl, 100 mM NaCl, 100 mM $MgCl_2$) containing 300 μl NBT and 150 μL BCIP. The membrane was washed in water to stop the color reaction and rinsed in methanol to remove background.

Cloning of the LRR domain in the expression vector pQE-30 and transformation into host bacteria M15.

The LRR sequence was amplified from the genomic DNA by PCR. The 5' primer was designed to have an Sph I site, and the 3' primer was designed to have a Pst I site for in-frame cloning of the insert in the expression vector pQE-30 (Qiagen). The PCR product was digested with Sph I and Pst I, and was ligated to the vector, with a molar mass insert: vector ligation ratio of 3:1. The ligation was carried out at 4°C overnight and the ligation plasmid was transformed into the host bacterial strain M15 according to the QIAexpressionist manual (Qiagen) and plated on LB agar plates containing ampicillin (100μg/ml) and kanamycin (25 μg/ml).

5’ Primer: GTGCATGCGATGAGCATTAT

3’ Primer: GACTGCAGAGAAGGAACAAA

Culture growth for preparative purification

About 20 ml of Superbroth containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) was inoculated with a transformed bacterial strain M15, and grown overnight at 37°C shaking at 250 rpm. The next day, a 1 liter culture was inoculated with 1:50 dilution of the overnight grown bacteria and grown at 37°C at 250 rpm until the OD_{600} reached 0.6. At this point, IPTG was added to a final concentration of 1 mM, and the culture was incubated further with shaking for 4 hours. The culture was then centrifuged at 4000g for 20 min and the bacterial cells were frozen at -20°C.

Preparation of cleared lysates under denaturing condition

The frozen bacterial pellet was thawed for 15 min. The cells were resuspended in 40 ml of denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 20 mM β-Mercaptoethanol, 5 mM Imidiazole, pH 8.0), and stirred for 1 hour at room temperature. The lysate was centrifuged at 10,000g for 20 min at room temperature to pellet the cell debri. This supernatant containing the expressed protein was used for binding to the Ni-NTA column (Qiagen).

Binding of the lysate to the Ni-NTA column and elution

About 500 µl of Ni-NTA slurry (Qiagen) was added to 10 ml of lysate and gently mixed by rocking for 1 hour at room temperature. The lysate-resin mix was loaded on an empty column and the flow-through was discarded. The column was washed twice, with 10 ml of wash solution (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 20 mM β-Mercaptoethanol, 20mM Imidiazole, pH 8.0). Finally, the protein was eluted with the elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 20 mM β-Mercaptoethanol, 250 mM Imidiazole, pH 8.0).

Infection studies

Xanthomonas campestris pv. *esicatoria* (*Xcv* 0887-14) was a gift from Dr. Margaret Essenberg (Oklahoma State University), and was grown in 10 ml of nutrient broth (Gibco BRL) at 30°C overnight. The, OD_{600} of the overnight grown culture was 0.14. The bacteria were diluted in nutrient both to an OD_{600} of 0.07 and allowed to grow at 30°C to an OD_{600} of 0.15. The number of cells/ml in the culture medium was estimated by using the equation:

Number of cells/ml = OD_{600} (0.15) x Coleman Jr. factor for *Xcv* (3.4×10^9).

= 5.1×10^8 cells/ml.

The bacteria were finally diluted to 5×10^6cells/ml in saturated $CaCO_3$ and infiltrated into 11-day-old cotyledons of the Ac44 E cotton plants.

11-day-old control cotyledons of the Acala 44E plants were also infiltrated with saturated $CaCO_3$ containing no bacteria.

The experimental plants were infiltrated with 5 ml of 5×10^6 cells/ml of *Xcv*, and the cotyledons were collected from the experimental and control plants 0, 12, 24 and 48 hr after infiltration by plunging the cotyledons in liquid nitrogen and storing the sample at -70°C for RNA extraction.

Wounding studies

For the wounding experiments, cotyledons from 11-day-old Acala 44E cotton plants were sliced into 3mm strips using scalpel blades. Each wounded cotyledon was sealed with parafilm in petri plates containing filter paper moistened with 20 mM Sodium Phosphate and left for 0, 12, 24 and 48 hours before flash freezing in liquid nitrogen for RNA extraction. The controls for the experiments were 11-day-old cotyledons attached to the plants, which were detached at 0, 12, 24 and 48 hours and immediately frozen in liquid nitrogen and stored at -70°C.

CHAPTER IV

RESULTS AND DISCUSSION

5’ RACE results

The incomplete LRR-extensin (# 65631), that was isolated from the cDNA library, was a 1.46 kb nucleic acid fragment that coded for an N-terminal end containing four complete LRR repeats along with one partial repeat, and a C-terminal end containing extensin-like serine/proline rich repeats. 5’ RACE for the incomplete LRR-extensin (# 65631) was done to determine the 5’ end of the incomplete cDNA. The result of the experiment, using primers to the LRR region, showed one prominant band of 1.1 kb (Figure 7). This band was gel extracted, ligated to the TA cloning kit vector, pCR™ II (Invitrogen), and transformed into bacteria INVaF', white colonies were picked from X-gal/Amp plates, and plasmid was isolated from them. The plasmid that showed a 1.1 kb band after restriction digestion with EcoR1, was submitted for sequencing. The sequencing results showed that 183 nucleotide bases in the 5’ RACE sequence were identical to the LRR-extensin (# 65631 sequence). This was because the nested primer was designed to be 183 residues away from the 5’ end of the incomplete cDNA. The experiment showed that the

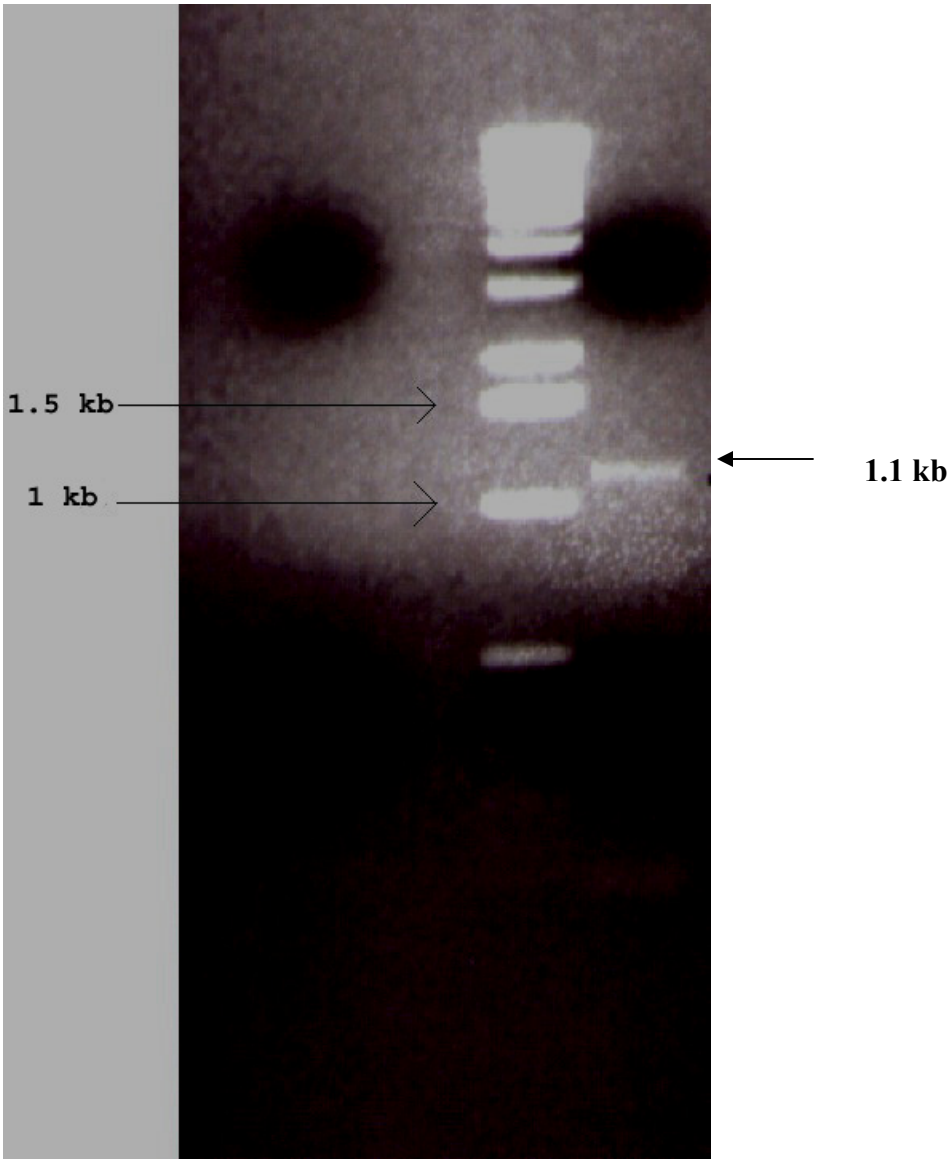


Figure 7: 5' RACE result for the LRR-extensin (# 65631) shows a prominent band of 1.1 kb band on a 1% agarose gel.

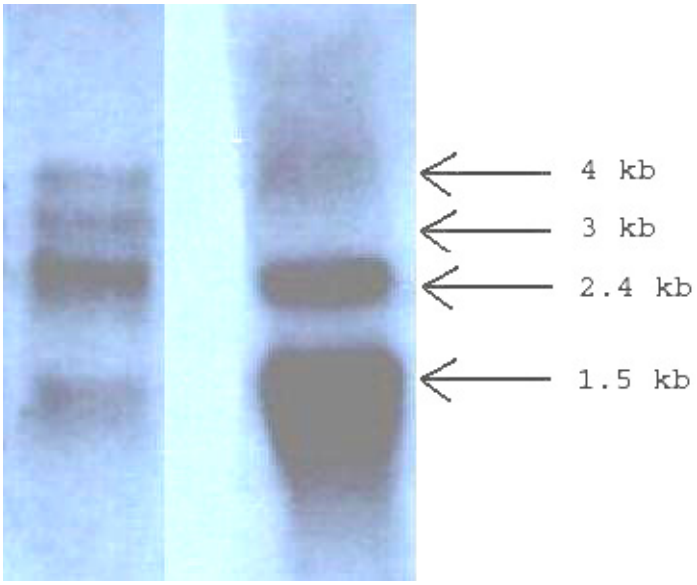
original cDNA was missing 946 nucleotides. The 5' RACE sequence was composed of 7 complete LRR repeats and 1 partial repeat. By incorporation of the 5' RACE sequence in the incomplete LRR-extensin (# 65631), which is by itself a 1.46 kb sequence, a sequence of 2.4 kb was obtained, with a total of 11 LRR repeats.

Northern blot results

Northern blot experiments were done to verify the length of the complete LRR-extensin sequence and also to determine whether, the sequence obtained by the 5' RACE experiment was part of the same cDNA as the LRR-extensin. About 10 μg of total RNA was isolated from 9- day-old Acala 44 suspension culture cells (Chomczynski and Sacchi, 1987) and electrophoresed on a 1% agarose/MOPS/formaldehyde gel, and blotted onto a nylon membrane (Roche Molecular Biochemicals). The blot was UV crosslinked and hybridized with the 5' RACE sequence probe and the incomplete LRR-extensin (# 65631) probe. The results are shown in Figure 8. The results of the northern blot after probing with the two probes revealed that the two probes hybridized to a common transcript of 2.4 kb. This band would correspond to the complete LRR-extensin sequence. In addition, the incomplete LRR-extensin probe, (# 65631) further hybridized to 3 other bands, a 1.5 kb band that is common to both probes, but hybridized more strongly to the 5' RACE probe than to the incomplete LRR-extensin (# 65631) and may correspond to a transcript that is very similar to the LRR domain of LRR-extensin but not similar to the extensin-like domain. This band hybridized to the incomplete LRR-extensin (# 65631) sequence because the incomplete LRR-extensin has a small LRR domain (containing 4 LRR repeats). The *Arabidopsis* database shows protein sequences that contain only the

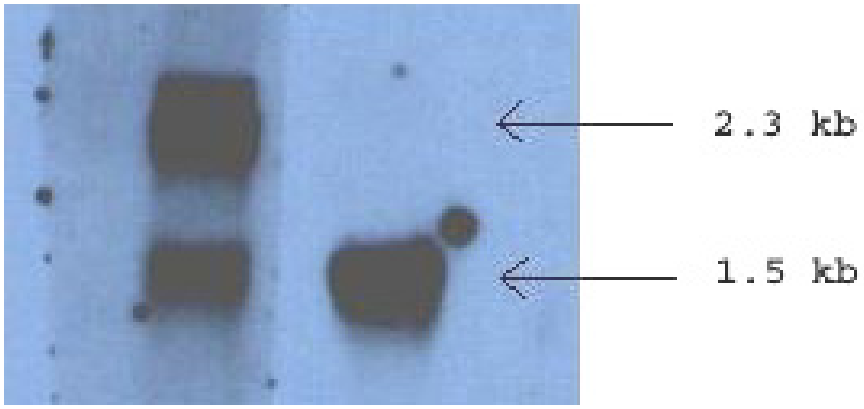
domains similar to the LRR domain of the LRR-extensin, but completely lacking the extensin-like domain (Accession no. CAB79682 and CAA16878). These proteins have mistakingly been identified in the database as LRR-extensin proteins even though they have no extensin domains at all. Therefore, it is possible that in cotton also there would be such sequences that contain domains very similar to the LRR domains of the LRR-extensin but completely lacking the extensin domain. Such a sequence would show a hybridization pattern similar to the 1.5 kb transcript seen in Figure 8. Two other bands, a 3 kb transcript, seen only in the blot hybridized to the incomplete LRR-extensin (# 65631), and a 4 kb transcript, seen in both the blots may correspond to LRR-extensins that have similar LRR domains or totally different LRR domains, but similar extensin domains. The northern blot studies reveal that the extensin domain is less conserved than the LRR domain and that is why one sees more bands hybridizing to the incomplete LRR-extensin (# 65631) than the 5' RACE probe. Northern blots were also done for extensin sequences # 2 and # 3. The results of the experiment are shown in Figure 9. Northern blots were initially probed with extensin # 2 and then stripped and probed with sequence # 3. The results of northern blots probed with extensins # 2 and extensins # 3 show that the extensin # 3 hybridized to 2 transcripts of 1.5 kb and 2.3 kb, which could be two extensins that share a high degree of similarity, or two differently spliced transcripts from the same gene for extensin # 3. The extensin # 2 hybridized to a single transcript of 1.5 kb. Due to the similarity in the sizes of the transcript (1.5 kb), and to make certain that the extensin probes only hybridize to their sequence, and not to the other extensin, new probes were made for extensin # 2 and extensin # 3 by designing primers in the 3' untranslated regions of the extensins and PCR amplifying these regions

of the cDNA and labeling by random prime labeling to generate probes. All the subsequent experiments done on the extensins # 2 and # 3 were done using these probes.



Blot A Blot B

Figure 8: Northern blot results. Blot A probed with incomplete LRR-extensin (# 65631), Blot B probed with 5' RACE derived sequence. The source of RNA was Acala 44 suspension culture cells.



Blot A Blot B

Figure 9: Northern blot results for extensins # 2 and # 3. Blot A probed with extensin # 3. Blot B probed with extensin # 2. The source of RNA was Acala 44 suspension culture cells.

Southern blot results

Southern blots were done to get an idea of the gene family size of the LRR-extensin and extensins # 2 and # 3. Genomic DNA was isolated from suspension culture cells and the DNA was digested with EcoRI, (an enzyme that does not cut within the cDNA of LRR-extensin, extensin # 2 and extensin # 3). For the estimation of the gene family size of LRR-extensin, genomic DNA was additionally digested with Pst I and Kpn I, and was probed with the incomplete LRR-extensin (# 65631) and the 5' RACE derived sequence. The results in Figure 10 show that, after digestion with EcoRI under low stringency hybridization, (0.5X SSC, 0.1% SDS), the blot probed with the incomplete LRR-extensin (# 65631) showed at least 5 bands but under the same conditions, the blot probed with the 5' RACE probe showed at least 3 bands. The genomic DNA after digestion with EcoRI, PstI and KpnI, under high stringency washes (0.1X SSC, 0.1% SDS) of the blot, showed only 2 bands when either probe was used, as seen in Figures 11-a and 11-b. The result indicates that the LRR-extensin may belong to a multigene family but there are 3 closely related members. The 5 bands seen hybridizing to the incomplete LRR-extensin (# 65631) probe may have different LRR domains or not have an LRR domain at all, which is why these bands are not seen in the blots hybridized with the 5' RACE probe at low stringency washes. Their extensin domains may be fairly similar because they are seen in the blots after low stringency washes with the incomplete LRR-extensin (# 65631) probe. Extensin # 2 will not hybridize to the incomplete LRR-extensin (# 65631) or 5' RACE probe because, it shows a low identity (at the nucleic acid level) to either of these probes (17 % identity to # 65631 and 14 % identity to 5' RACE probe). Similarly, extensin # 3

will also not hybridize to incomplete extensin (# 65631) and 5' RACE probe because of low identity of the sequence to both the probes (18 % identity to # 65631 and 14 % identity to 5' RACE probe).

Genomic southern blot results of the extensins reveal that, after genomic DNA digestion with EcoRI, extensin # 2 hybridizes at low stringency (0.5X SSC, 0.1% SDS) to at least 8 bands, but at high stringency (0.1X SSC, 0.1% SDS), binds only to a single band (Figure 12). Extensin # 3 binds to at least 7 bands after low stringency (0.5X SSC, 0.1% SDS) washes, but binds to only one one band after high stringency washes (0.1X SSC, 0.1% SDS) as seen in Figure 13. This means that the isolated extensin clones also belong to a multigene extensin family, but the genes in the family are not very closely related, because only a single member shows up after high stringency washes.

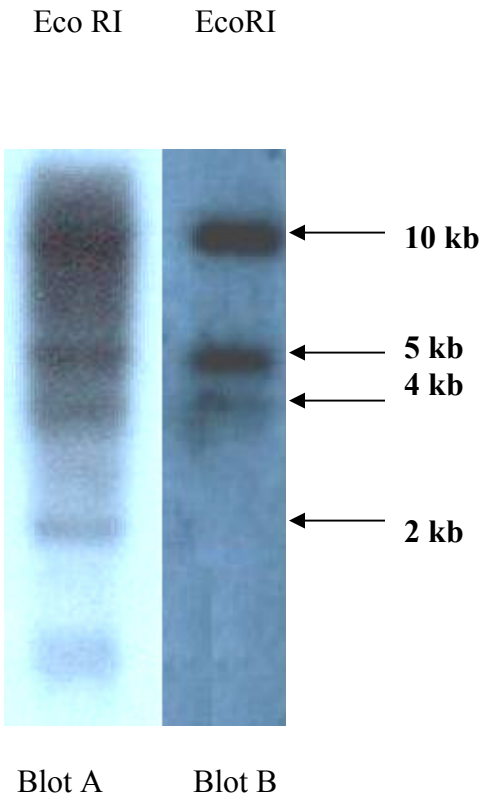


Figure 10: Southern blot of LRR-extensin after low stringency washes. Blot A was hybridized with incomplete LRR-extensin (# 65631). Blot B was hybridized with 5' RACE derived sequence. Genomic DNA was digested with EcoRI, an enzyme that does not cut within the sequence.

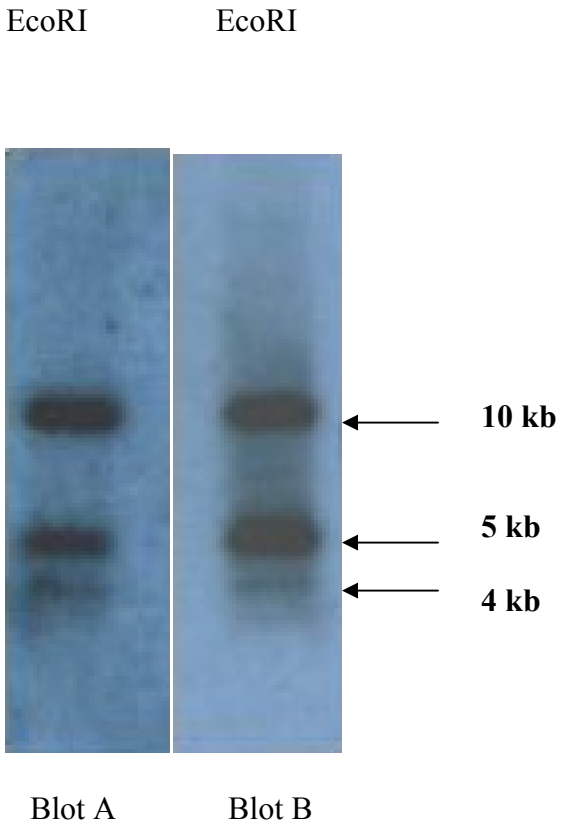


Figure 11-a: Southern blot of LRR-extensin after high stringency washes. Blot A was hybridized with incomplete LRR-extensin (# 65631). Blot B was hybridized with 5' RACE derived sequence. Genomic DNA was digested with EcoRI, an enzyme that does not cut within the sequence.

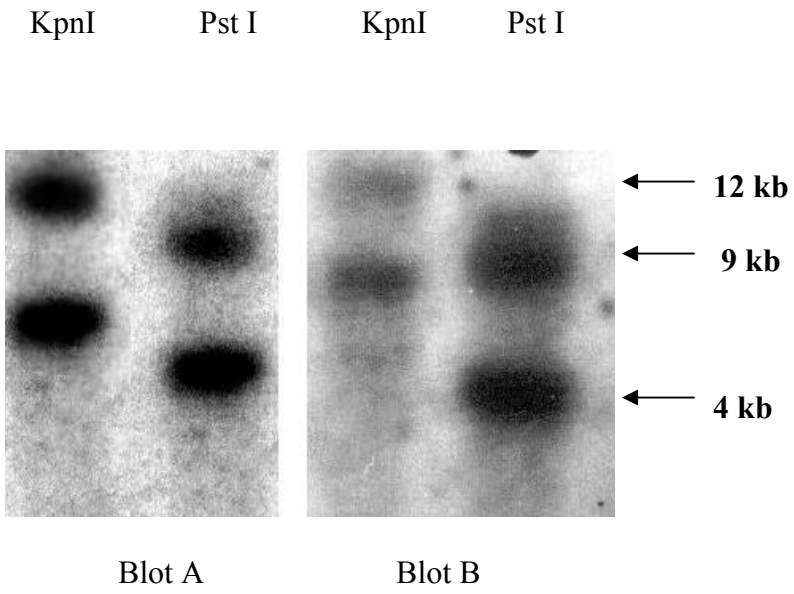


Figure 11-b: Southern blot of LRR-extensin after high stringency washes. Blot A was hybridized with incomplete LRR-extensin (# 65631). Blot B was hybridized with 5' RACE derived sequence. Genomic DNA was digested with KpnI and PstI, enzymes that do not cut within the sequence.

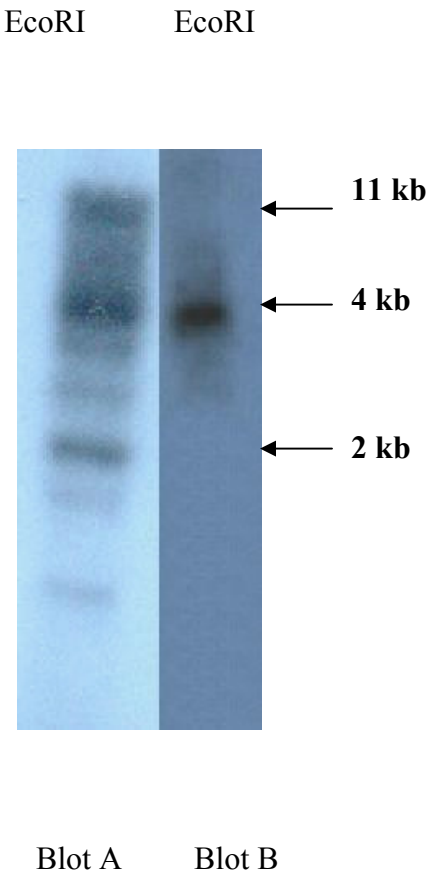


Figure 12: Southern blot of sequence # 2 after high and low stringency washes. Blot A washed under low stringency. Blot B washed under high stringency. Genomic DNA was digested with EcoRI, an enzyme that does not cut within the sequence.

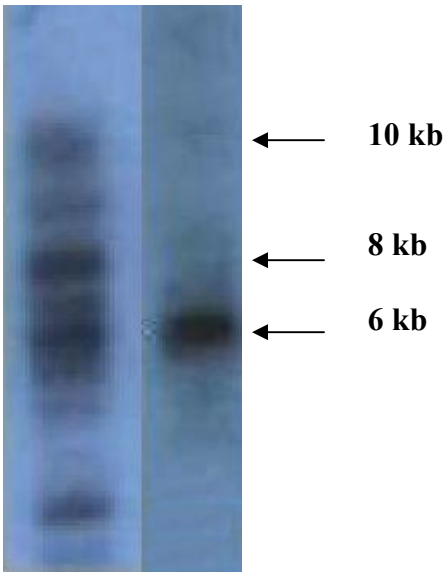


Figure 13: Southern blot of sequence # 3 after low and high stringency washes. Blot A washed under low stringency. Blot B washed under high stringency. Genomic DNA was digested with EcoRI, an enzyme that does not cut within the sequence.

Genomic Library screening

Cotton (*Gossypium hirsutum* L. cv. Acala SJ-2) genomic library in λ FIX II (Stratagene) was given to us by Dr. Thea Wilkins (University of California, Davis). The number of plaques that need to be screened to obtain the LRR-extensin, assuming one copy per haploid genome, was estimated by simple statistics based on the Poisson distribution (Clarke and Carbon, 1976), using the equation shown below.

$$N = \ln (1-P)/ \ln [1-(I/G)]$$

N = number of plaques screened

I = insert size in base pairs

G = size of the genome in base pairs

P = probability of finding a particular sequence in the library

For a 98% chance of finding the gene, assuming the average insert size is 12 kb, for a cotton haploid genome size (1X = 2,118 Mb, although cotton is allotetraploid), 652,000 plaques needed to be screened. For our experiment, about 600,000 plaques were screened with the 5' RACE sequence probe instead of the incomplete LRR-extensin (# 65631) probe because, many positives showed up after hybridization with the incomplete LRR-extensin (# 65631). This result can be easily explained because, we screened the cotton genomic library under low stringency (0.5 X SSC, 0.1% SDS), and under these conditions genes that belong to the extensin-like multigene family are also detected (as seen in Figure 10, where low stringency conditions were applied and 5 bands were seen in the blot). The 5' RACE derived probe showed fewer positives and that is why we

chose this probe to screen the library. Five positive plaques were subjected to primary, secondary and tertiary hybridization and the most strongly hybridizing plaque (as seen by the intensity of the chemiluminescence signal) was picked up and lambda DNA was isolated. The lambda DNA was directly sent for sequencing with the primers used in the 5' RACE experiment and the primers used to sequence the incomplete LRR-extensin (# 65631). The sequencing results revealed that the LRR-extensin is one large open reading frame with no introns because it aligned perfectly with its cDNA sequence. The gene sequence is shown in Figure 14. Figure 15 shows the protein sequence coded by the gene with the domains clearly demarkated. For prediction of splice sites, protein coding exon, translation start site and polyadenylation site, the gene prediction programs, Genscan (MIT, Massachusets, USA), BCM Gene Finder (Baylor College of Medicine, Houston, Texas), NetPlant gene (Center for Biological Sequence Analysis, Technical University of Denmark), NetStart (Center for Biological Sequence Analysis, Technical University of Denmark) and WebGene (Institute of Advanced Biomedical Technologies, Seagrate, Italy) were used.

Figure 14: Gene sequence of LRR-extensin

caatttcggttcaattcatctcaaaataaaaagtgcctattaccaacataatatatgatac

attcttataggatacctataattcttttaacgaatgtttgattttccccttttttaata

taacattaggggtaagggataggataacaccattcaaaccccatgctaaataaatgtata

attaatcactactccatccaaatcaaggcaatgactgattttcatatgatcttaattgtg

aattgaataaaaaagataaaagaattataatgttactttcaatgatatgaagtaattgtg

ttgtccttccgaacctataataatattataagattgtaaaattaaaattatcccaaaatt

gaagtaaaaggactggatatcaaattttaatataatataaggactacagataatattaga

Putative TATA box

ccttaaatctaaataatatatttt**tatatat**aacagctttacaaaaatccttatcttctct

Transcription start site

cccatacacacatactgcatactcgaaaaaaccctaaaagctctctcttttgtctccttc

tctctacattttcttcttcagatctccaaattgaaaccccaaattcgtgttttctgcaca

aaccccattttcaaaaccccagcttttgttcaaaaaaagttcaaaaaaaggtggcatttt

Translation start site

ttaatatcagatgtctcatgtgaa**atg**aagaagacgaacattatcgacctccattgttat
M K K T N I I D L H C Y
ccttctttttgttacctttgtttctttagtttcagttttgtatccttatgttcatgc
P S F C Y L L F L L V S V F V S L C S C
gatgagcattatgtttccagccatagcggacttaccgacaaggaagtatcgtacataaag
D E H Y V S S H S G L T D K E V S Y I K
caacgacagctgctttattacatagatgagttcggtgacagaggggagaacgtctccgtc
Q R Q L L Y Y I D E F G D R G E N V S V
gacccgtcactggtttttgaaaaccagaggttgagagacgcttatatagctttacaagct
D P S L V F E N Q R L R D A Y I A L Q A
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W K K A I L S D P F N L T A D W V G S G
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V C D Y T G V F C A R A L D N K R I R T
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V A G I D L N H G D I A G Y L P E E L G
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L L T D L A L F H I N S N R F C G T V P
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H K F I K L K L M F E L D L S N N R F A
ggtaagttccctgaagtgattcttaagcttcctttacttaaatttttggatttgaggttt

G K F P E V I L K L P L L K F L D L R F
aatgaatttgaaggaactgtgcctaaagagctttttgataaagatttggatgctatttttt
N E F E G T V P K E L F D K D L D A I F
attaatcataaccggtttagatttaatctaccggataattttggtaactcgccggcttct
I N H N R F R F N L P D N F G N S P A S
gttattgttttggctaataacaagtttcacggttgtgtgccggcgagtcttgggaacatg
V I V L A N N K F H G C V P A S L G N M
acgagtcttgaagagataattttaataaacaatgggtttcgatcttgtttgccggagcaa
T S L E E I I L I N N G F R S C L P E Q
attgggggggttgagaaatacgactgtttttgatgttagctttaatgagttaatgggtact
I G G L R N T T V F D V S F N E L M G T
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L P E Q I G G M V S L E Q L N V A H N M
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L S G K I P A S I C R L P K L E N F T F
tcgtataatttcttcaccggagaaccgccggtttgtttggggttacgtgcttttgacgat
S Y N F F T G E P P V C L G L R A F D D
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R R N C L P A R P L Q R S A A Q C R S F
ttgtctagaccggtggattgtaattcatttagatgtgctccttttgttccttctttgctg
L S R P V D C N S F R C A P F V P S L L
tctcctcctccgccttctccgcctccggttgttgtgctgtcaccgccacccccatcgcct
S P P P P S P P P V V V L S P P P P S P
gtttttattccaccatcaccacccccgccgccaccaccgccagtatactctccccgtccc
V F I P P S P P P P P P P P V Y S P R P
ccttcaccatctccaccagtatattcacctccacctccaccgccacctgtgtactctcct
P S P S P P V Y S P P P P P P P V Y S P
cctctgcctccaccatctccaccgccacctgtttattcacccccaccacccccctcacct
P L P P P S P P P P V Y S P P P P P S P
ccaccaccttcaccacccccacaggtatattctccaccaccaccaccatccccacctcca
P P P S P P P Q V Y S P P P P P S P P P
ccatcacccccgccacctacttatccatcaccacctccaccatcttctccaccaccttca
P S P P P P T Y P S P P P P S S P P P S
ccagtatactgtgtgaggtctccacctccttcaccaccaaattcacccccctccaccacct
P V Y C V R S P P P S P P N S P P P P P
cgattgttttcctcacctccaccagttccttactactataactccccaccaccgccacac
R L F S S P P P V P Y Y Y N S P P P P H
cattcaccgccacctcctgtacattctccaccacccccaccacattcacctcctccacca
H S P P P P V H S P P P P P P H S P P P P
atttatccatacttatctccaccaccgccaccacctcctgtttattccccacctcctcca
I Y P Y L S P P P P P P P V Y S P P P P
gttcactcaccaccaccaccgtcacctcctccatgtattgagccacctccaccaccacca
V H S P P P P S P P P C I E P P P P P P
ccatgtgtagagtacacgccatcaccctcacccccaattcattacaaaccacctccatca
P C V E Y T P S P S P P I H Y K P P P S
ccttcacccccacccccaccaatccattatcattcacctccaccaccatcacctccaccg
P S P P P P P P I H Y H S P P P P S P P P
gctcccgtatacgaagggccacttccaccagtaatcggagtatcatacgcttcacctcca
A P V Y E G P L P P V I G V S Y A S P P
ccgccacctttctat**<u>tga</u>**ttctttcaagaatttccctctaaccttgattaaaaaaaaccc
P P P F Y **Stop** **Polyadenylation site**
caaaaaagaggagaaattctctcccataaattatttgatatttggagttgtg**<u>aaaaataa</u>**

<u>aag</u>aagaaaagagtgtagggtgaaaggaaagttgttgcatagggggatgaattcccaaaaa

aaaaaaaaaaa

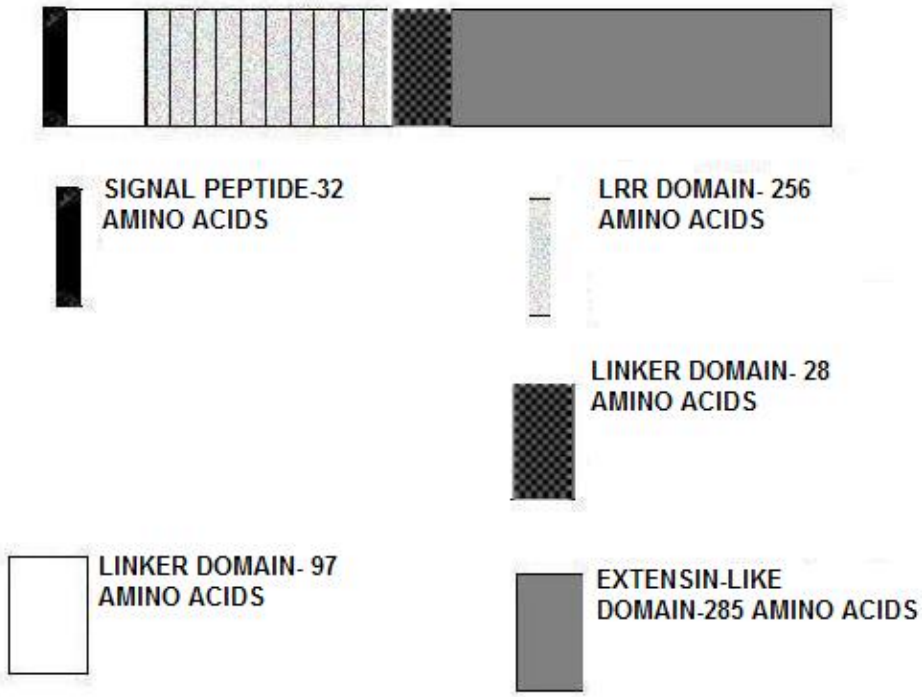


Figure 15-a: Domains of the LRR-extensin

Figure 15-b: Domains of the LRR-extensin

Signal peptide recognition sequence-32 amino acids:

M K K T N I I D L H C Y P S F C Y L L F L L V S V F V S L C S C

Linker domain-97 amino acids:

D E H Y V S S H S G L T D K E V S Y I K Q R Q L L Y Y I D E F G D R G E N V S V D P S L V F E N Q R L R D A Y I A L Q A W K K A I L S D P F N L T A D W V G S G V C D Y T G V F C A R A L D N K R

LRR domain-11 LRR repeats-256 amino acids:

L	x	x	L	x	x	L	x	x	L	x	L	x	x	N	x	L	x	x	G	x	I	P	x	x	
R	I	R	T			V	A	G	I	D	L	N	H	G	D	I	A		G	Y	L	P	E	E	LRR 1
L	G	L	L	T	D	L	A	L	F	H	I	N	S	N	R	F	C		G	T	V	P	H	K	LRR 2
F	I	K	L	K	L	M	F	E	L	D	L	S	N	N	R	F	A		G	K	F	P	E	V	LRR 3
I	L	K	L	P	L	L	K	F	L	D	L	R	F	N	E	F	E		G	T	V	P	K	E	LRR 4
L	F	D	K	D		L	D	A	I	F	I	N	H	N	R	F	R	F		N	L	P	D	N	LRR 5
F	G	N	S	P		A	S	V	I	V	L	A	N	N	K	F	H		G	C	V	P	A	S	LRR 6
L	G	N	M		T	S	L	E	E	I	I	L	I	N	N	G	F	R	S	C	L	P	E	Q	LRR 7
I	G	G	L	R	N	T	T	V	F	D	V	S	F	N	E	L	M		G	T	L	P	E	Q	LRR 8
I	G	G	M	V	S	L	E	Q	L	N	V	A	H	N	M	L	S		G	K	I	P	A	S	LRR 9
I	C	R	L	P	K	L	E	N	F	T	F	S	Y	N	F	F	T		G	E	P	P	V	C	LRR 10
				L	G	L	R	A	F	D	D	R	R	N	C	L	P	A		R	P	L	Q	R	LRR 11

Linker domain-28 amino acids**:**
S A A Q C R S F L S R P V D C N S F R C A P F V P S L L

Extensin-like domain-285 amino acids:

S P P P P S P P P V V V L S P P P P S P V F I P P S P P P P P P P P V Y S P P P P P S P S P P P P P P P V Y S P P L P P P S P P P P P V Y S P P P P P S P P P P P S P P P Q V Y S P P P P P P S P P P P P S P P P P T Y P S P P P P S S P P P S P V Y C V R S P P P S P P N S P P P P P R L F S S P P P V P Y Y Y N S P P P P P H H S P P P P P V H S P P P P P H S P P P P I Y P Y L S P P P P P P P P V Y S P P P P P V H S P P P P P S P P P C I E P P P P P P P C V E Y T P S P S P P I H Y K P P P S P S P P P P P P I H Y H S P P P P P S P P P P A P V Y E G P L P P V I G V S Y A S P P P P P P F Y

Expression studies

To study the expression of LRR-extensin in the different organs of cotton, RNA was extracted from stem, leaves, roots, cotyledons and callus of cotton (Acala 44E plants).

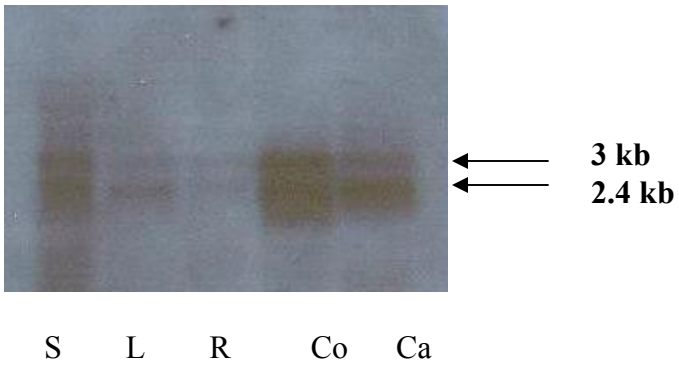
Ten micrograms of total RNA was run in a 1% agarose/MOPS/formaldehyde gel, transferred to a nylon membrane, and hybridized with the incomplete LRR-extensin (# 65631) probe. The results are shown in Figure 16. The blot shows that the LRR-extensin predicted to be a 2.4 kb transcript, is expressed in all organs, but predominantly in stem, cotyledons and callus. In all the organs blots, a band at 3 kb is also visible.

In stem and cotyledons, the 3 kb transcript is expressed to an equal extent as the 2.4 kb LRR-extensin band. In callus and leaf, the 2.4 kb LRR-extensin band is expressed to a greater extent than the 3 kb band. The root shows low level of expression of the 2.4 kb LRR-extensin and the 3 kb band.

Northern blots probed with the 3' untranslated region of extensin # 2 show that this extensin is expressed in all tissues but predominantly in root, stem and callus. Leaf and cotyledons show low-level expression of the 1.5 kb extensin transcript. Figure 17 shows northern blot results when probed with extensin # 2 probe.

Northern blots probed with the 3' untranslated region of extensin # 3 show that this extensin is expressed predominantly in roots, stem and callus (Figure 18). Leaf and cotyledons show low level expression of the 1.5 kb transcript.

(a)



(b)

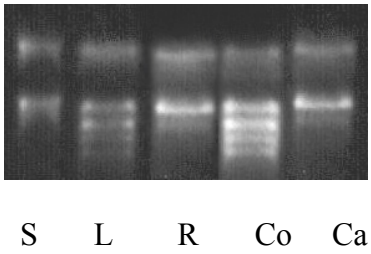
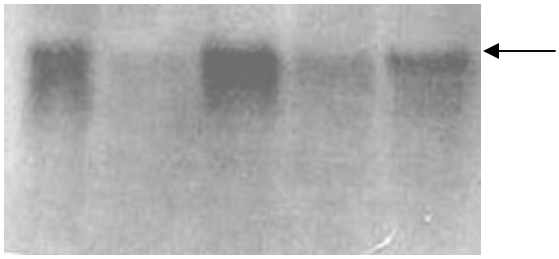


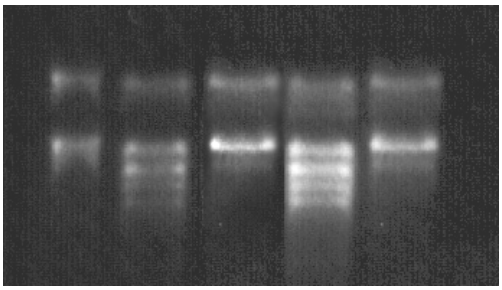
Figure 16: (a) Northern blot of RNA from different organs of cotton probed with incomplete LRR-extensin (# 65631). S, stem; L, leaf; R, root; Co, cotyledons; Ca, callus. **(b)** Ribososomal RNA from stem, leaf, root, cotyledon and callus as control for RNA loading.

(a)



S L R Co Ca

(b)

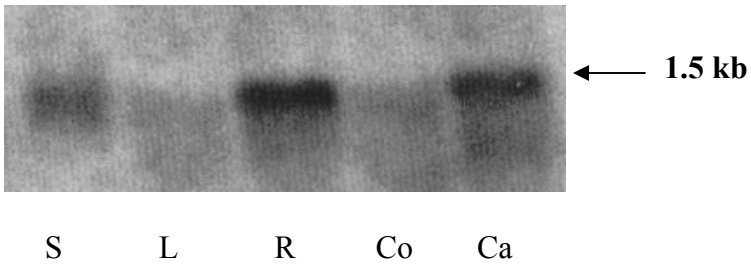


S L R Co Ca

Figure 17: (a) Northern blot of different organs of cotton probed with 3' unstranslated sequence of extensin # 2. S, stem; L, leaf; R, root; Co, cotyledons; Ca, callus.

(b) Ribosomal RNA from stem, leaf, root, cotyledon and callus as control for RNA loading.

(a)



(b)

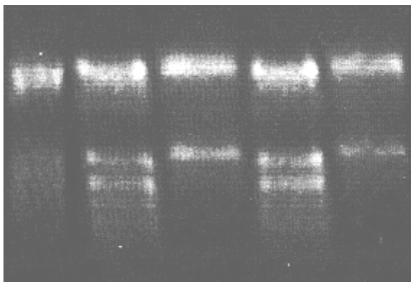


Figure 18: (a) Northern blot of different organs of cotton probed with 3' untranslated sequence of extensin # 3. S, stem; L, leaf; R, root; Co, cotlyledon; Ca, callus.

(b) Ribosomal RNA from apical meristem, stem, leaf and root as control for RNA loading.

Infection Studies

To find out if the LRR-extensin and extensins # 2 and # 3 might be related defense proteins, infection studies were done on Acala 44E plants by infiltrating the plants with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv* 0887-14), a bacterium that induces a hypersensitive reaction in cotton plants. The cotyledons of 11-day-old Acala 44E plants were infiltrated with sterile $CaCO_3$ in control plants and $5x10^6$ cfu/ml of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv* 0887-14) in sterile $CaCO_3$ in infected plants, and RNA was isolated from the cotyledons at 0, 12, 24 and 48 hours after infiltration and northern blot experiments were done. To reduce the variability between cotyledons and between experiments, only the smaller cotyledon (also called the smooth cotyledon) of each plant was used in the study. The experiment was done at noon and all the plants were allowed to absorb the water after infiltration, outside the growth chamber, and returned into the growth chamber at 7 pm. The experiment was done in duplicate, and cotyledons from two plants were pooled and used for each time point.

Figure 19 shows the results of the blots probed with the incomplete LRR-extensin (# 65631) and 5' RACE probe. In control plants and in infected plants, the 2.4 kb transcript that is predicted to code for the LRR-extensin, was the predominant band, with the expression level decreasing at 12 hours, and increasing again at 24 hours. At 48 hours after infiltration, the expression of this gene seems to be suppressed. A 3 kb transcript also shows the same expression pattern as the 2.4 kb LRR-extensin, as seen in the blots hybridized to the incomplete LRR-extensin cDNA # 65631. A 1.5 kb band and two other bands of lower molecular weight also seem to hybridize to the probe to a lesser extent.

This result for the duplicate set of cotyledons for the same time points is also shown (Figure 31 in Appendix A).

This result suggests that, the LRR-extensin gene is probably not an extensin-like defense gene because most extensin genes are induced as a result of the hypersensitive response and show induction after infiltration with *Xcv (*Esquerre-Tugaye, Mazau, Pelissier, Roby, Rumeau and Toppan, 1995).

Figure 20 shows northern blots of RNA isolated at 0, 12, 24 and 48 hours from control plants and, after infiltration with *Xcv* and probed with extensin # 2. The blot shows that there is very low level of expression of the 1.5 kb transcript in controls (except at 12 hours, where it seems to be induced). The reason this gene seems to be induced at this time point may be because the cotyledon that was used in this time point might have been injured and, wounding also can induce extensin # 2 expression. The reason we believe that the 48 hr expression is a result of induction of extensin # 2 due to infiltration with *Xcv*, is because we repeated this experiment three different times and, each time, this gene was induced at 48 hr, but not at 12 hours. The second set of duplicates, for the 12-hour time point for control plants do not show induction at 12 hours (Figure 32 in Appendix A). Therefore this induction can be explained because of injury to the cotyledon while infiltration with $CaCO_3$.

Figure 21 shows northern blots of RNA isolated at 0, 12, 24 and 48 hours from control plants and plants infiltrated with *Xcv* and probed with extensin # 3. The blot shows that there is a low level of constitutive expression of extensin # 3 in controls but it seems to be induced in the 12-hour time point. This is because, the cotyledon at this time point could be injured and wounding can induce extension # 3. The result is similar to the extensin #

2. The second set of duplicates, for the 12-hour time point for control plants do not show induction at 12 hours, consistant with our explanation for this result due to wounding (Figure 33 in Appendix A). However in the cotlyledons infiltrated with *Xcv,* the transcript is induced at 48 hours after infiltration with *Xcv.*

The result for the two extensins is consistent with the proposed role of extensins in defense. Extensins, are thought to be induced as a result of hypersensitive response, and are thought to immobilize the negatively charged surfaces of certain plant pathogens and restrict their entry into the plant cell by binding to their surfaces by their positively charged lysine residues (Mazau, Rumeau and Esquerre-Tugaye, 1987; Mellon and Helgeson, 1982).

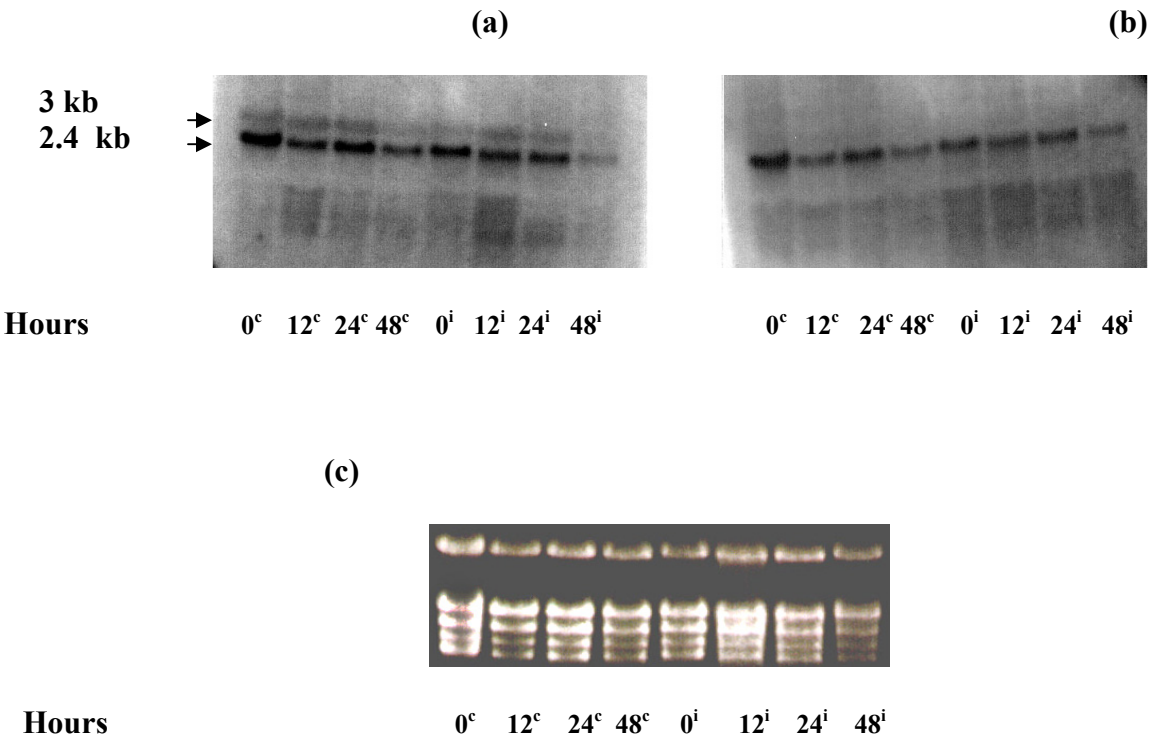


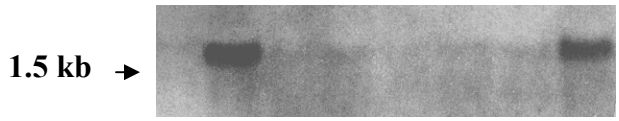
Figure 19: Northern blot of infection studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in controls (0^c 12^c 24^c 48^c), and *Xanthomonas campestris* pv. *vesicatoria* in infected plants (0^i 12^i 24^i 48^i).

(a) Blot probed with incomplete LRR-extensin (# 65631)

(b) Blot probed with 5' RACE probe.

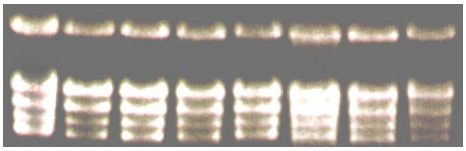
(c) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in control plants, and *Xanthomonas campestris* pv. *vesicatoria* in infected plants, as control for RNA loading.

(a)



Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

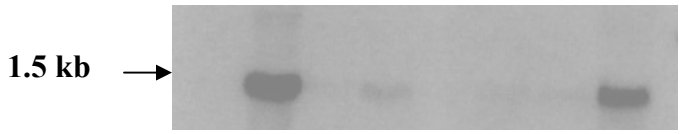
(b)



Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

Figure 20: (a) Northern blot of infection studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in controls (0^c 12^c 24^c 48^c), and *Xanthomonas campestris* pv. *vesicatoria* in infected plants (0^i 12^i 24^i 48^i) and probed with 3' untranslated sequence of extensin # 2. **(b)** Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration of cotton cotyledons as control for RNA loading.

(a)



(b)

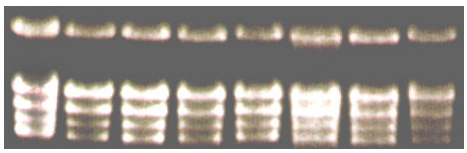


Figure 21: (a) Northern blot of infection studies. RNA was isolated from 11 day old cotyledons at 0, 12, 24 and 48 hours after infiltration by *Xanthomonas campestris* pv. *vesicatoria* and probed with 3' untranslated sequence of extensin # 3. **(b)** Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration of cotton cotyledons as control for RNA loading

Wounding Studies

To find out if the LRR-extensin and extensins # 2 and # 3 are defense genes that are induced by wounding, 11-day-old cotton cotyledons were cut into 3mm strips and incubated in a filter paper moistened with 20mM sodium phosphate buffer for 0, 12, 24 and 48 hours. The controls for the experiments were 11-day-old cotyledons attached to the plants, which were detached at 0, 12, 24 and 48 hours. The cotyledons were then flash frozen in liquid nitrogen, and total RNA was isolated from them. Northern blot results of RNA probed with incomplete LRR-extensin (# 65631) shows that the 2.4 kb LRR-extensin and a 3 kb band were constitutively expressed with slight variations in expression levels. A band at 4 kb is induced at 24 hours after wounding. There is also a 1.5 kb band that shows induction at 24 hours after wounding. In the blots hybridized to the 5'RACE probe, the 2.4 kb LRR-extensin is constitutively expressed, the same 4 kb band and a 1.5 kb band shows signs of induction as in the blot hybridized with incomplete LRR-extensin (# 65631) probe. (Figure 22). The second set of duplicates for this experiment is shown in Figure 34 in Appendix A.

These results, along with the results from the infection studies indicate that the 2.4 kb LRR-extensin is not a defense related gene because, most defense genes are induced as a result of wounding and infiltration with pathogen (Mazau, Rumeau and Esquerre-Tugaye, 1987; Mellon and Helgeson, 1982).

Extensin # 2, which is a 1.5 kb band, is induced at 12 hours after wounding, and expression level remains high at 24 hours and 48 hours. (Figure 23).

There are 2 cross hybridizing bands of 2 kb and 3 kb that are also induced along with the 1.5 kb extensin band. The second set of duplicates for this experiment is shown in Figure 35 in Appendix A.

Extensin # 3, which also shows a 1.5 kb band, is induced along with a band of 2.3 kb and 3 kb at 12 hours after wounding and the expression level remains high at 24 hours and 48 hours (Figure 24). Since the probes for the tissue expression, infection and wounding studies are 3' untranslated sequence probes, the cross hybridizing transcripts may be differential splicing transcripts of the same gene. The second set of duplicates for this experiment is shown in Figure 36 in Appendix A.

The expression patterns exhibited by the extensins # 2 and # 3 are typical of defense genes. These results, along with the results from infection studies indicate that extensins # 2 and # 3 are defense proteins, and their role in wounding is probably to reinforce and strengthen the cell wall after wounding (Mazau, Rumeau and Esquerre-Tugaye, 1987; Mellon and Helgeson, 1982).

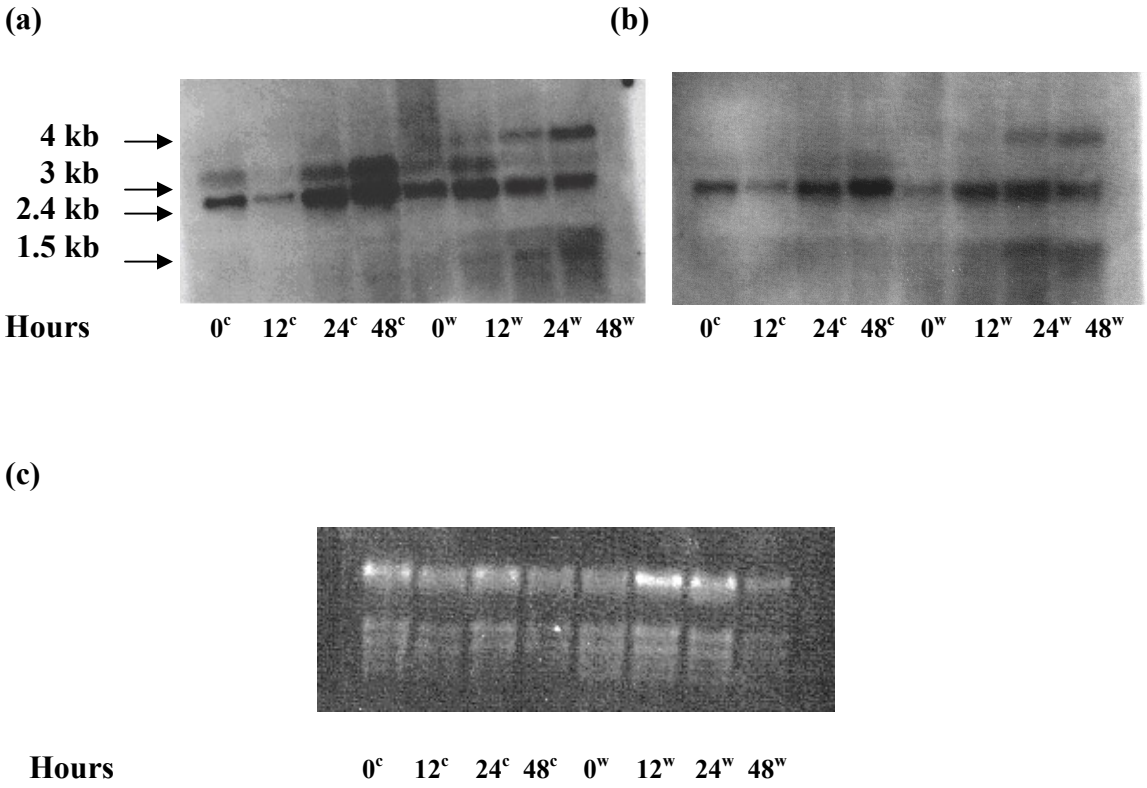


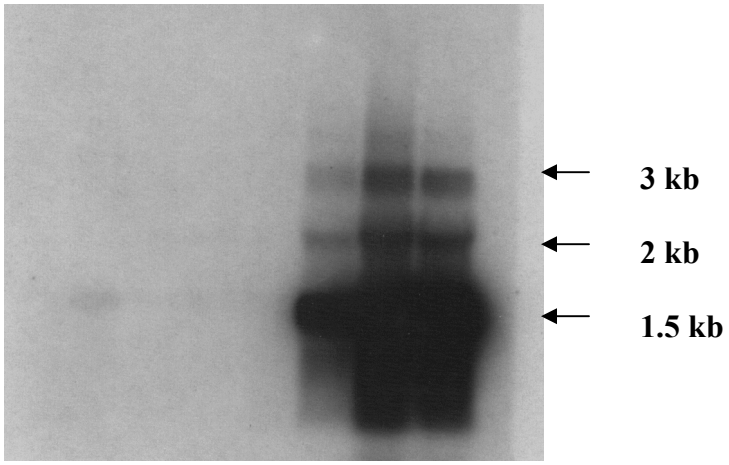
Figure 22: Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w).

(a) Blot probed with incomplete LRR-extensin (# 65631)

(b) Blot probed with 5' RACE probe.

(c) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding, as control for RNA loading.

(a)



(b)

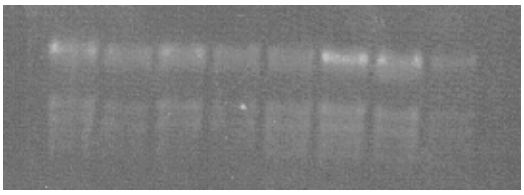
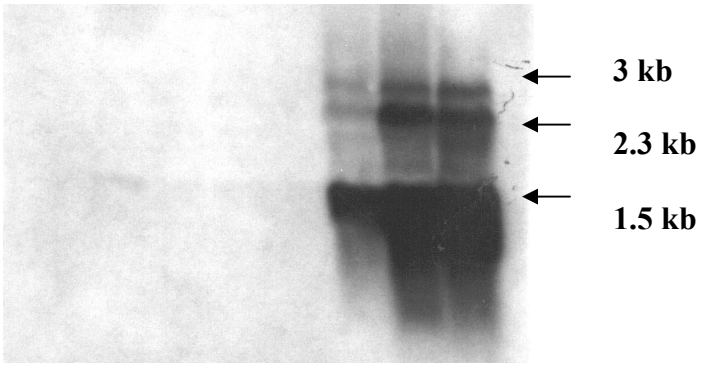


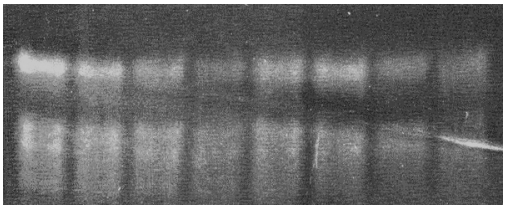
Figure 23: (a) Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w) and probed with 3' untranslated region of extensin # 2. **(b)** Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding, as control for RNA loading.

(a)



Hours 0^c 12^c 24^c 48^c 0^w 12^w 24^w 48^w

(b)



Hours 0^c 12^c 24^c 48^c 0^w 12^w 24^w 48^w

Figure 24: (a) Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w) and probed with the untranslated 3' region of extensin # 3.

(b) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding, as control for RNA loading.

Expression Studies of the LRR region in E.coli

To express the LRR domain in bacteria, primers were made in the linker domains preceding and succeeding the LRR-domain and the entire LRR domain was cloned in frame to the prokaryotic plasmid vector pQE-30, after amplification from genomic DNA by PCR. Figure 25 shows the result of PCR amplifying the 1.14 kb fragment coding for the LRR-domain. The PCR fragment was run on a 1% gel, gel extracted, digested with Sph I and Pst I (the 5' and 3' primers contained these sites) and ligated to the plasmid vector pQE-30. The ligated vector was finally transformed into host bacteria M15 and plated on LB/Amp/Kan plates. A single colony was picked and grown overnight at 37°C in Superbroth and then diluted 1:50 in 1 liter Superbroth and allowed to grow at 37°C till the OD_{600} reached 0.6. At this point, 1 ml of the culture was taken and bacteria were pelleted and resuspended in 5X SDS-PAGE sample buffer. This was the uninduced control (UC). To the rest of the culture, IPTG was added to a final concentration of 1mM and bacteria were allowed to grow for 4 hours. After 4 hours, a 1 ml culture was taken and bacteria were pelleted from the culture and resuspended in 5X SDS-PAGE sample buffer. This was the induced control (IC). These samples were later run on a 12% acrylamide gel to find out if the LRR domain protein (LD) was expressed. A prominent band at 45 kD (the expected size of the LRR domain if it was expressed) showed that the LRR domain was indeed expressed by the bacteria. The rest of the culture was centrifuged to pellet the bacteria, and the LRR protein was purified from the bacteria under denaturing conditions by binding to the Nickel-NTA column. Figure 26 shows a polyacrylamide gel with the uninduced control, induced control and the LRR domain

protein after purification under denaturing conditions on a Nickel-Nitrilotriacetic acid (Ni-NTA) column. The LRR domain protein after purification was run on a 12% gel and sent to the Hybridoma Center (Oklahoma State University) for gel extraction of the 45 kD band and injection into mice for polyclonal antibody synthesis. To test if the LRR domain was translated correctly from the LRR coding sequence, the LRR domain protein was gel extracted, reduced, alkylated, and digested with trypsin and the fragments were analyzed by MALDI-MS. If the LRR domain was translated correctly, we would expect that MALDI-MS would identify fragments of the predicted molecular sizes. The predicted masses of the LRR domain peptides after digestion with trypsin was generated by the program PeptideMass (ExPASy, Swiss Institute of Bioinformatics). The MALDI-MS data shown in Figure 27 identified many fragments with molecular weights expected from the LRR protein after alkylation and trypsin digestion. This proves that the LRR protein was translated correctly from the LRR coding sequence. To show that the polyclonal antibody recognized the LRR as antigen, a western blot experiment was done, by incubating the LRR antigen with the polyclonal antibody. Figure 28 shows the western blot results of the LRR antigen after incubation with the polyclonal antibody made from the Hybridoma Center, against the LRR antigen. This result indicated that the polyclonal antibody did recognize the LRR antigen. Since we were interested in finding out the size of the native LRR-extensin in the plant, total protein was extracted from stem, leaf, root and callus culture cells of cotton (Acala 44) and a western blot experiment was done using the LRR polyclonal antibody as the primary antibody. Figure 29 shows the western blot results of an 8% polyacrylamide gel run with total protein extracted from leaf, stem, root and callus culture cells and incubated with anti-LRR antibody. A protein band of ~

300 kD was detected in the callus culture cells. A very high molecular weight band that barely entered into the stacking gel was detected in the stem, leaf and root extracts. By accident we discovered that if the protein extracts from the callus culture were left in the -70ºC freezer for a week, the 300 kD band disappeared and a band was detected in the stacking gel. This result leads us to believe that the LRR-extensin in the stem, leaf and root extracts is a high molecular weight protein that barely enters into the stacking gel because, it is either heavily glycosylated, (more than in the callus cultures), or it is cross-linked to other cell wall polysaccharides or to itself. The callus LRR-extensin is perhaps less heavily glycosylated because of its faster growth rate than the plant organs. Storing the callus protein extracts in the -70ºC freezer for a week somehow caused the LRR-extensin to cross-link to other cell wall polysaccharides or to itself, and caused the LRR-extensin in callus to become much bigger and not fully enter into the stacking gel, just as we see for the stem, leaf and root samples.

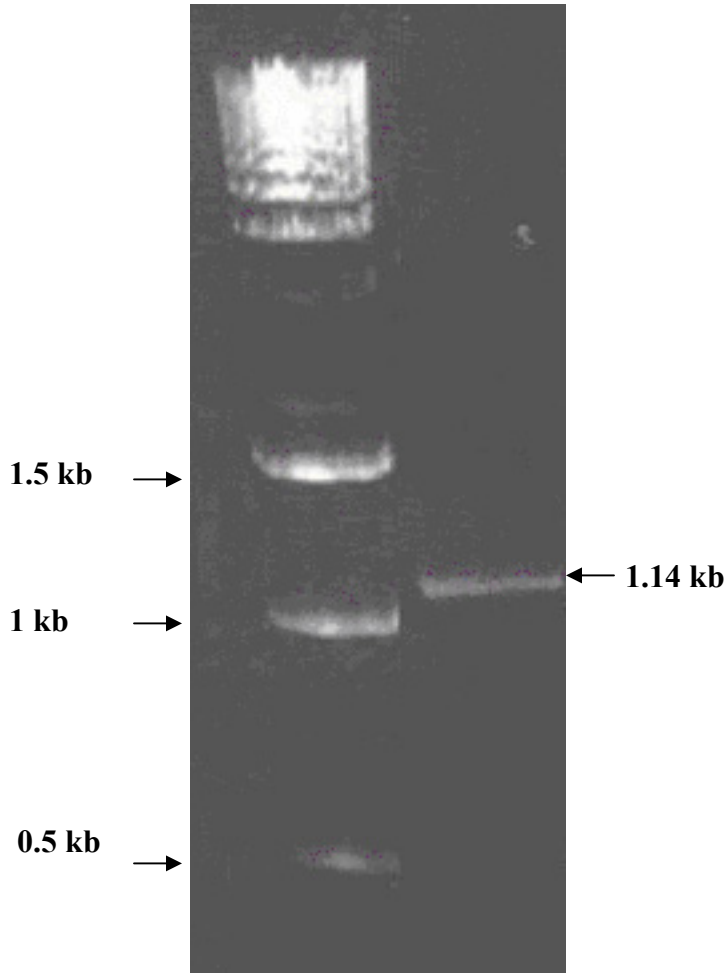


Figure 25: PCR results of amplifying a 1.14 kb fragment coding for the LRR domain from the genomic DNA.

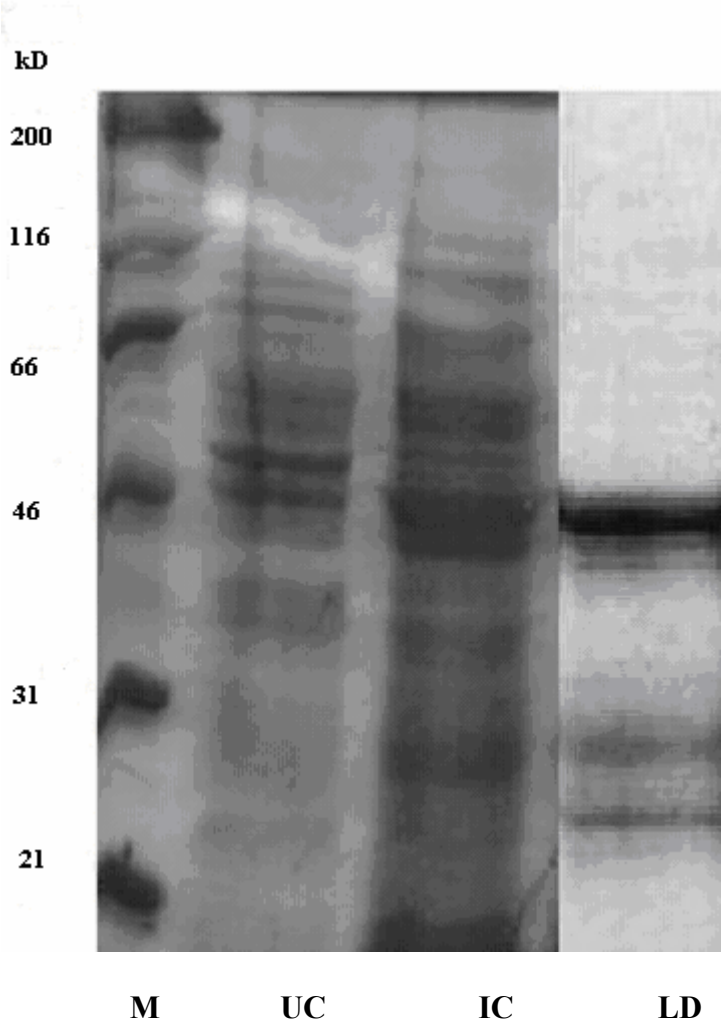


Figure 26: A 12% polyacrylamide gel. M, broad range molecular weight marker; UC, uninduced control; IC, induced control; LD, LRR domain protein. Coomassie blue staining.

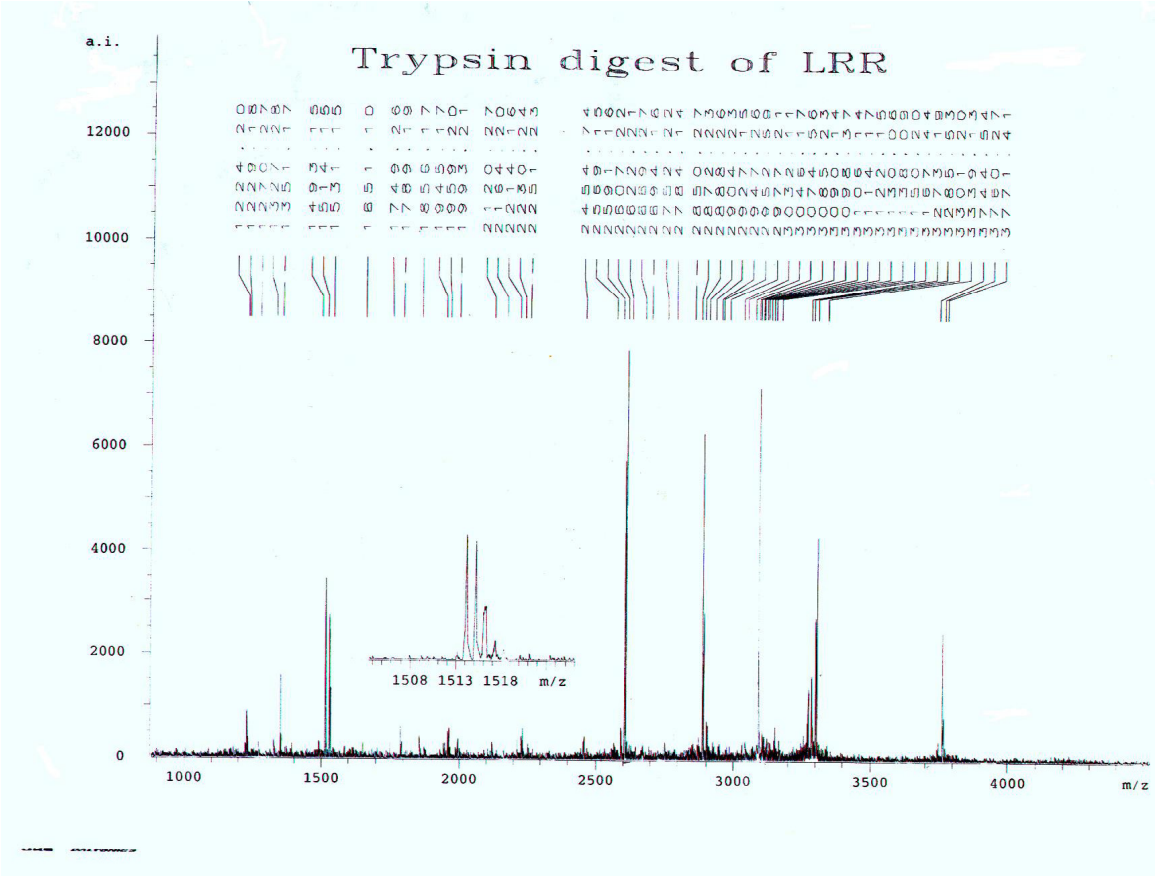


Figure 27-a: MALDI/MS analysis of the LRR peptide after trypsin digestion.

Figure 27-b: MALDI/MS analysis of the LRR peptide: Theoretical mass was predicted by PeptideMass program from ExPASy. Experimental mass was predicted by MALDI-MS.

Experimental mass	Theoretical mass	Peptide sequence
1224.20	1224.663	NCLPARPLQR
1229.18	1229.63	SCLPEQIGGLR
1327.28	1327.675	DLDAIFINHNR
1531.15	1531.743	QLLYYIDEFGDR
1351.15	1351.667	LMFELDLSNNR
1789.19	1789.871	GENVSVDPSLVFENQR
1959.2	1959.992	ELFDKDLDAIFINHNR
2230.24	2231.145	FNLPDNFGNSPASVIVLANN K
2607.22	2608.254	LENFTFSYNFFTGEPPVCLG LR
2850.27	2850.516	FCGTVPHKFIKLKLMFELDL SNNR
2888.26	2889.439	FHGCVPASLGNMTSLEEIIL INNGFR
2947.26	2946.486	LPKLENFTFSYNFFTGEPPV CLGLR
2977.21	2977.386	AILSDPFNLTADWVGSGVCD YTGVFCAR
3090.14	3091.429	AILSDPFNLTADWVGSGVCD YTGVFCAR
3106.14	3105.481	KAILSDPFNLTADWVGSGVC DYTGVFCAR
3114.17	3115.502	SAAQCRSFLSRPVDCNSFRC APFVPSAAK
3301.2	3302.596	QLLYYIDEFGDRGENVSVDP SLVFENQR
3744.54	3743.908	IPASICRLPKLENFTFSYNF FTGEPPVCLGLR
3760.27	3760.81	KAILSDPFNLTADWVGSGVCDYTGVFCARALDNK

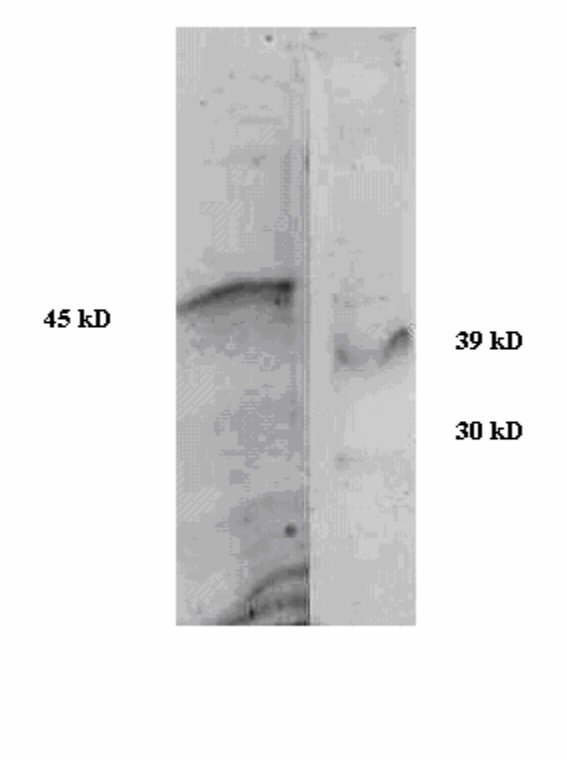


Figure 28: Western blot showing that the polyclonal antibody made in the Hybridoma center reacted with the LRR protein expressed in pQE-30. The lower molecular weight proteins in the LRR prt lane may be the degradation product of the larger molecular weight protein.

LRR prt, LRR domain protein; KM, Kaleidoscope markers.

(a)

Figure 29: (a) Western blot results of the total protein from L, leaf; S, stem; R, root and Ca, callus culture cells after incubation with anti-LRR antibody shows a 300 kD protein band in the callus culture cells.

DISCUSSION

Cotton extensins

Extensins are the most well studied cell wall proteins. In cotton, two extensin cDNAs were discovered, which we call extensin # 2 and extensin # 3. Extensin # 2, has a 1.5 kb mRNA sequence as seen in northern blots, but, the cDNA sequence we got is only 1 kb long and missing the 5' ends. This incomplete cDNA has an open reading frame of 696 nucleotides that codes for a protein containing 232 amino acids and is missing the initiating methionine. This sequence has two kinds of repeats, SPPPPPPSPPKHPYKYK and SPPPPPPVYKYK, where the motif YKYK signifies the intramolecular isodityrosine cross-link within extensins. This extensin fragment is missing a signal peptide sequence characteristic of all extensins. Extensin # 2 is expressed in all tissues but predominantly in root, stem and callus. Leaf and cotyledons show a low level of expression of the 1.5 kb extensin transcript. Extensin # 2 is induced in cotton cotyledons at 48 hours after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria* and is also induced in cotton cotyledons at 12 hours after wounding of the cotton cotyledons. This kind of expression as a result of wounding and infection suggests that extensin # 2 contributes to plant defense (Showalter, 1993).

Extensin # 3 is a 1.5 kb sequence as shown by northern blots, but the sequence we isolated is 700 bp long. It has an open reading frame of 606 nucleotides and codes for 202 amino acids. It has one kind of repeat of the type SPPPPSPSPPPPYYYK, where the motif YYYK signifies the intramolecular isodityrosine cross-link within extensins. It is missing the signal peptide sequence that is characteristic of all extensins. This extensin is expressed predominantly in roots, stem and callus with leaf and cotyledons showing low-level expression of the transcript. Extensin # 3 is also induced in cotton cotyledons 48 hours after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria,* and like extensin # 2, it is also induced at 12 hours after wounding the cotton cotyledons.

Though extensins # 2 and # 3 have different repeat sequences and cross-linking motifs, northern blot results suggest that both have a transcript size of about 1.5 kb. They are expressed in similar organs and their response due to wounding and infection is the same. From this, we can conclude that they have similar roles, which are to protect the plant against pathogen attack and wounding by either binding to the surface of the bacteria and restricting their entry into the plant or by strengthening the cell wall during wounding (Mazau, Rumeau, Esquerre-Tugaye, 1987).

Cotton LRR-extensin

The LRR-extensin is a 2.46 kb transcript with an open reading frame of 2091 bp, encoding a protein of 697 amino acids. The protein primary structure consists of a 32 amino acid signal sequence, a 97 amino acid domain with no homology to any known functional motif, an LRR domain of 256 amino acids, a 28 amino acid domain with no

homology to known motifs and an extensin domain of 285 amino acids. The LRR domain contains 11 repeats of 21-24 residues that match the plant extracytoplasmic LRR consensus sequence of LxxLxxLxxLxLxxNxLxGxIPxx (Jones and Jones, 1997). When the LRR regions were aligned, cotton LRR-extensin shows 55% identity to *Arabidopsis* LRX1, 63% identity to tomato TomL4 and 52% identity to maize Pex 1. Comparison of the cotton LRR-extensin to ESTs from developing cotton fiber available from Brookhaven National Laboratory, Upton, NY revealed that, there is a 100% identity with an EST named BNLGHi5349 (GenBank Acc AI729848) from 6-day old immature cotton fiber (6-days post anthesis) and 97% identity with an EST named GA_Ea0024C08f (GenBank Acc BG 444367) from a 7-10 days post anthesis library from the Clemson University Genomics Institute, SC. At this stage in the development of cotton fibers, the fiber is going through the elongation phase (Jasdanwala, Singh and Chinoy, 1977), which is responsible for the expansion of the fiber and synthesis of the primary cell wall. In our experiments, the cotton LRR-extensin is expressed predominantly in stem, cotyledons, callus and leaves and to a lesser extent in roots. The cotton LRR-extensin was not induced within 48 hr after wounding. In experiments after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria,* there was no induction of the LRR-extensin. However, the expression of this gene seems to follow a circadian rhythm and decrease at 48 hours after infiltration with the bacteria. This indicates that, unlike the extensins, # 2 and # 3, the LRR-extensin may not play a role in plant defense.

Western blots with anti-LRR polyclonal antibodies recognized a protein of 300 kD, though the protein sequence predicts a mass of 76 kD. The difference in molecular mass may be due to extensive glycosylation of the serine-hydroxyproline repeat sequences.

To understand the role of the cotton LRR-extensin, it is important to to review the functions of other plant LRR-proteins and undestand their role in the plant kingdom.

LRR-extensins form a sub-family of plant LRR-proteins

Besides LRR-extensins, plants have three other kinds of LRR-proteins, the resistance gene products that bind to the avirulence gene products in plant pathogens and are responsible for initiation and transduction of defense gene activation signal (Gabriel and Rolfe, 1990; Keen, 1992), the polygalacturonase inhibitor proteins that are proposed to regulate the activity of endopolygalacturonases of fungi by binding to the enzyme leading to the production of oligogalacturonic acid of defined lengths that are then supposed to transmitting the information through the plasma membrane to induce defense gene activation and prevent fungal infection (De Lorenzo, Cervone, Bellincampi, Caprari, Clark, Desiderio, Devoto, Forrest, Leckie, Nuss and Salvi, 1994), receptor-like protein kinases like ERECTA1, which is involved in specification of organ shape and CLAVATA1, which is involved in cell differentiation and to restrict cell proliferation. Thus, all the LRR-proteins mentioned above are involved in either defense related signal transduction (resistance gene products and polygalacturonase inhibitor proteins) or signal transduction during developmental processes (CLAVATA1 and ERECTA1).

LRR-extensins in the plants

To date (12-1-02), there are fourteen LRR-extensin genes in the GenBank database. Nine of these are present in *Arabidopsis,* one in rice, one in tomato and three in maize.

Alignment of the 14 LRR-extensins to the cotton LRR-extensin is shown in Figure 30. The LRR-extensins studied to date are expressed in various plant organs. Maize Pex 1, is specifically expressed in pollen (Rubinstein, Broadwater, Lowrey and Bedinger, 1995), the *Arabidopsis* LRX1 is expressed only in roots (Baumberger, Ringli and Keller, 2001), and the tomato TomL4 is expressed in green vegetative tissue (Zhou, Rumeau and Showalter, 1992). The wounding expression patterns of the LRR-extensin genes are also very different in maize and tomato, even though the two LRR-extensins show a 56.6% amino acid sequence identity in the LRR domains. In maize, the Pex1 gene is not induced in leaf 24 hours after wounding the leaf, whereas in tomato, the L4 gene is highly induced in stems 24 hr after wounding. Immunolocalization experiments have shown that at least two of the LRR-extensin proteins are localized to the cell wall (Rubinstein, Broadwater, Lowrey and Bedinger, 1995; Baumberger, Ringli and Keller, 2001) and cannot be extracted from the cell wall with high salt, chaoptropic agents, SDS, or reducing agents, which suggests that they are covalently cross linked to the cell wall. Western blot results with polyclonal antibodies raised against the LRR domain have shown cross-reaction with a protein of higher molecular weight than predicted for the LRR-extensin. The polyclonal antibody raised against the LRR domain of Pex1 reacts with a protein of 300 kD although the predicted molecular mass for Pex1 is 118 kD (Rubinstein, Marquez, Suarez-Cervera and Bedinger, 1995). Similarly, in *Arabidopsis*, polyclonal antibody raised against the LRR domain cross reacts with a 160 kD protein though the predicted molecular mass of LRX1 protein is 85 kD (Baumberger, Ringli and Keller, 2001). To account for the difference in molecular weights, it is assumed that the protein is heavily glycosylated in the extensin domain due to the presence of the serine-

hydroxyproline repeats. Functional analysis of the LRR-extensin gene was done for LRX1, by identifying transposon tagged mutants of LRX1 in *Arabidopsis*. The mutants showed irregular root hair development suggesting a role for this gene in root hair morphogenesis.

Since *Arabidopsis* is the model plant system, identification of transposon mutants of the other remaining eight *Arabidopsis* LRR-extensins will help us functionally characterize the LRR-extensins present in organs other than root. Furthermore, identifying the interacting partners by yeast two-hybrid analysis or immunoprecipitation with the anti-LRR antibodies in Acala 44 suspension culture cells will further help us undestand the role of these LRR-extensins.

Possible functions of the LRR-extensin

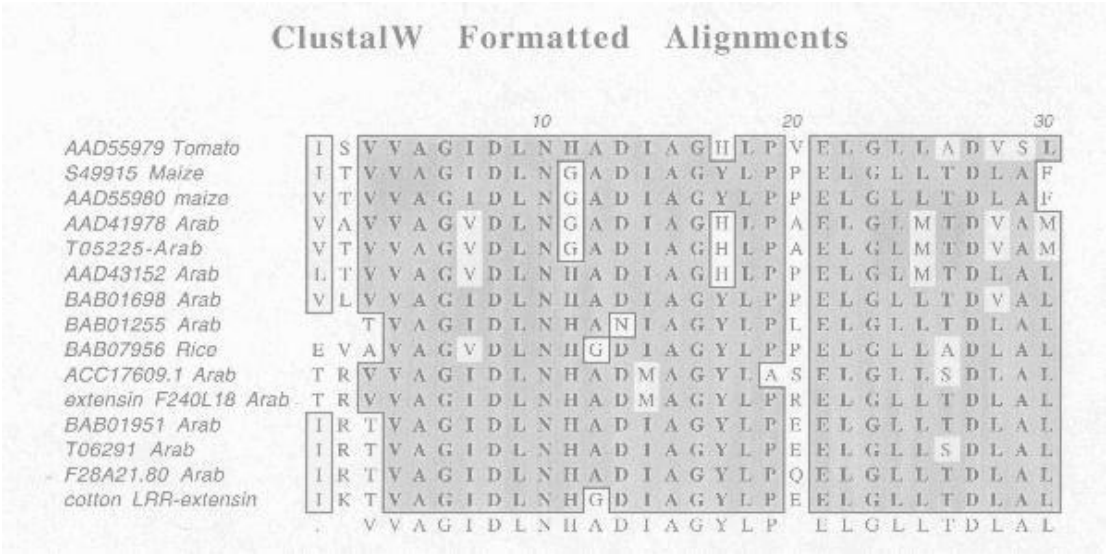
Isolation of transposon-tagged LRX1 mutants in *En*-1 mutagenized *Arabidopsis* plants (Baumberger, Ringli and Keller, 2001) showed that these plants exhibited an irregular root hair development. Most of the root hairs did not elongate completely and were arrested soon after initiation, resulting in short stumps. The root hairs that did elongate frequently branched and showed swelling along the main axis, resulting in spherical structures several fold the normal diameter of a root hair. However, the number of root hairs and the initiation site were the same as in the normal root hair. Using antibodies, the protein was localized in the cell wall of the root hair proper throughout all stages of root hair development. From these observations, LRX1 is proposed to be involved in root hair morphogenesis and elongation by controlling polarized growth or cell wall formation

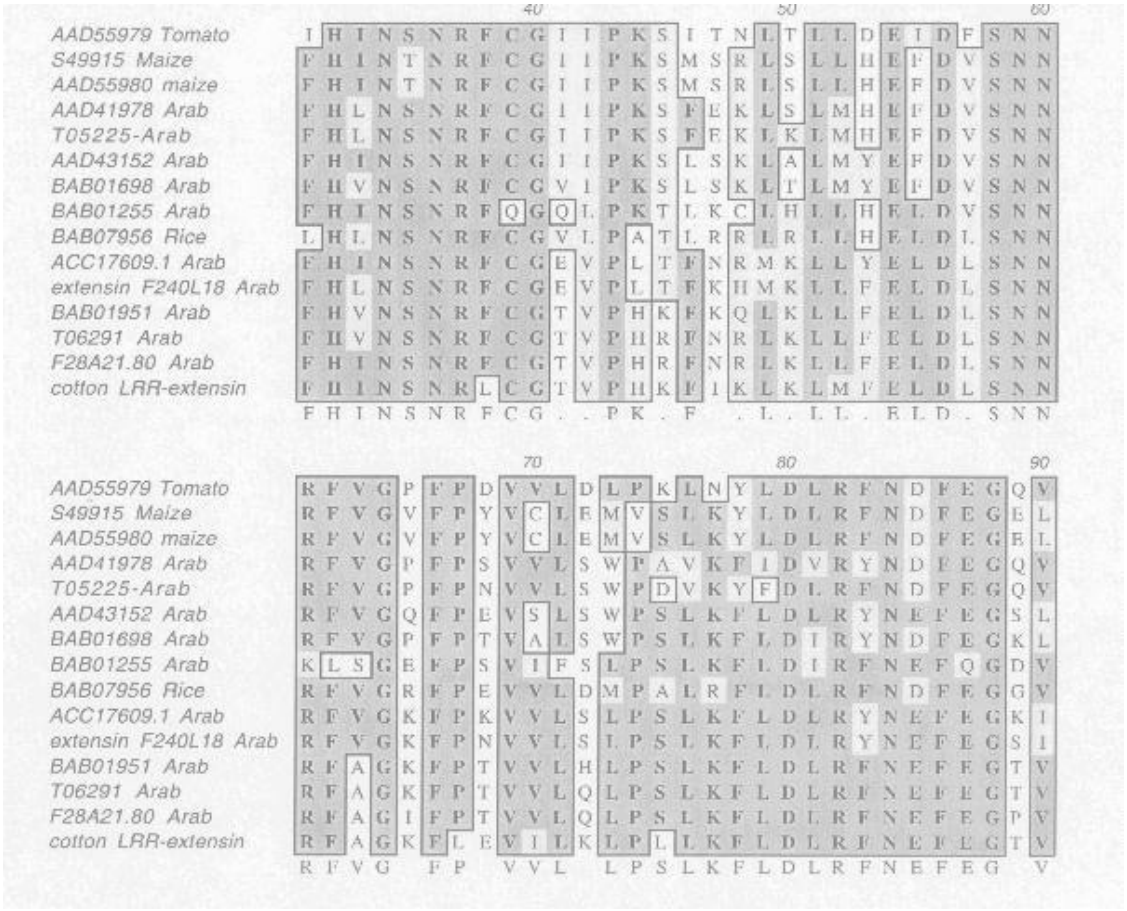
and assembly. The LRX1 mutant phenotype might be explained by defective cell expansion, resulting from a spatially deregulated exocytosis or an altered deposition of new cell wall material. In this context, the LRX1 may be involved in stabilizing root hair polarization. Since this root hair polarization and growth orientation depends on the microtubule and Ca^{2+} gradient at the tip of the root hair, root hair defects in the LRX1 mutants may point to microtubules and membrane Ca^{2+} channels as direct or indirect targets for LRX1 action. It has already been found that drugs that disrupt or stabilize microtubules in elongating root hairs can cause loss of growth directionality and induce branching (Bibikova, Blancaflor, Gilroy, 1999). Therefore, formation of root hairs that are unbranched, straight, and oriented correctly require an intact microtubule assembly. It is assumed that for proper cell wall synthesis, microtubules form guard-rail like barriers that constrain the movement of cellulose synthase enzyme and thereby direct the correct orientation of the cellulose microfibrils in the cell wall (Giddings, Staehelin, 1991). Identification of other root hair mutants, RHD3 (Galway, Lane, Schiefelbein, 1999), which is morphologically close to LRX1 mutants, is associated with irregular thickness of cell wall, and KOJAK, that codes for a cellulase synthase-like enzyme and shows root hair abortion (Favery, Ryan, Foreman, Linstead, Boudonck, Steer, Shaw and Dolan, 2001), shows that defective cell wall synthesis or cell wall alteration can result in defective root hair synthesis.

Since the cotton-LRR extensin and *Arabidopsis* LRX1 are quite similar in the LRR domain (55% identity and 69% similarity), and, one can assume that the cotton fiber EST named BNLGHi5349 (that is expressed 6 days post anthesis when the fiber is undergoing the elongation process, which is the stage responsible for the expansion of the fiber and

synthesis of the primary cell wall) is our cotton LRR-extensin, since it has 100% identity to our cotton LRR-extensin, one can propose the hypothesis that the cotton LRR-extensin is involved in coordinating growth or cell wall formation and assembly.

Figure 31: Clustaw alignment of 14 LRR-extensins in the Genbank database with cotton LRR-extensin.

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AAD55980 maize
AAD41978 Arab
T05225-Arab
AAD43152 Arab
BAB01698 Arab
BAB01255 Arab
BAB07956 Rice
ACC17609.1 Arab
extensin F240L18 Arab
BAB01951 Arab
T06291 Arab
F28A21.80 Arab
cotton LRR-extensin
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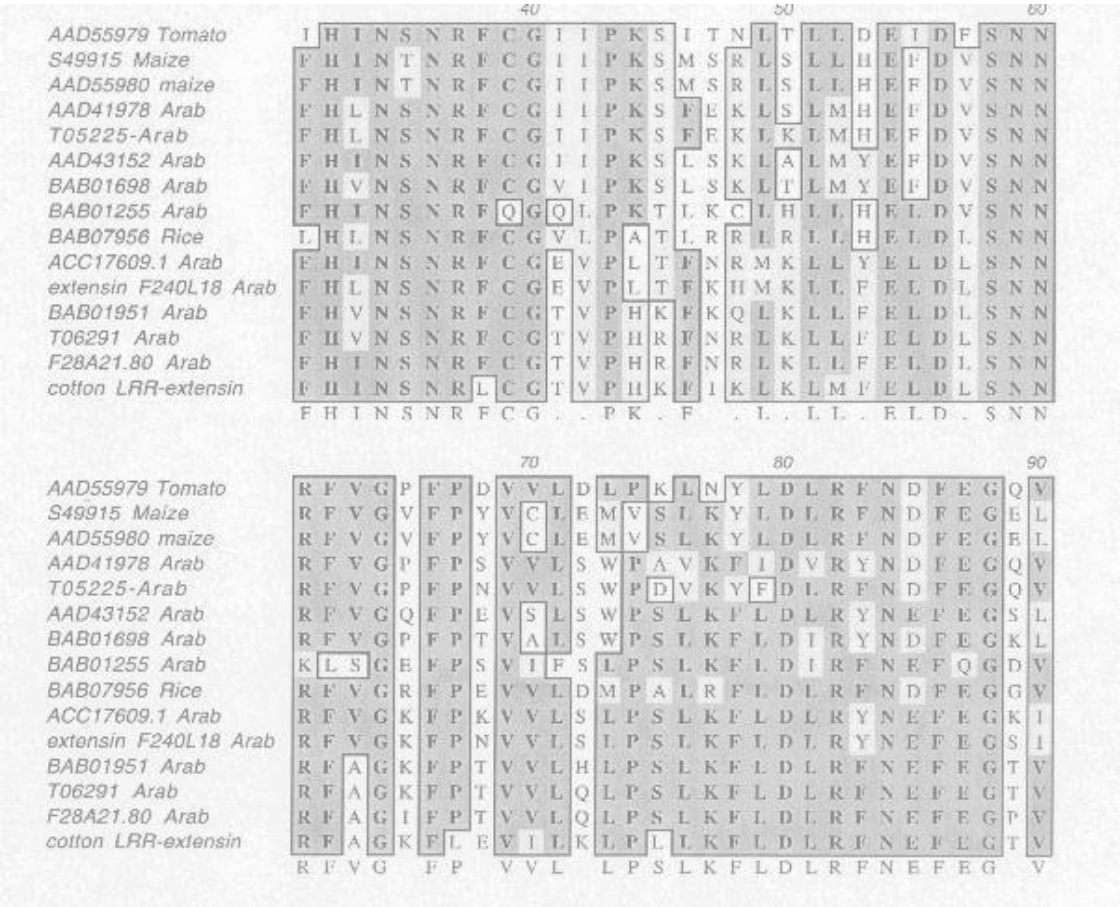
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BAB07956 Rice
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extensin F240L18 Arab
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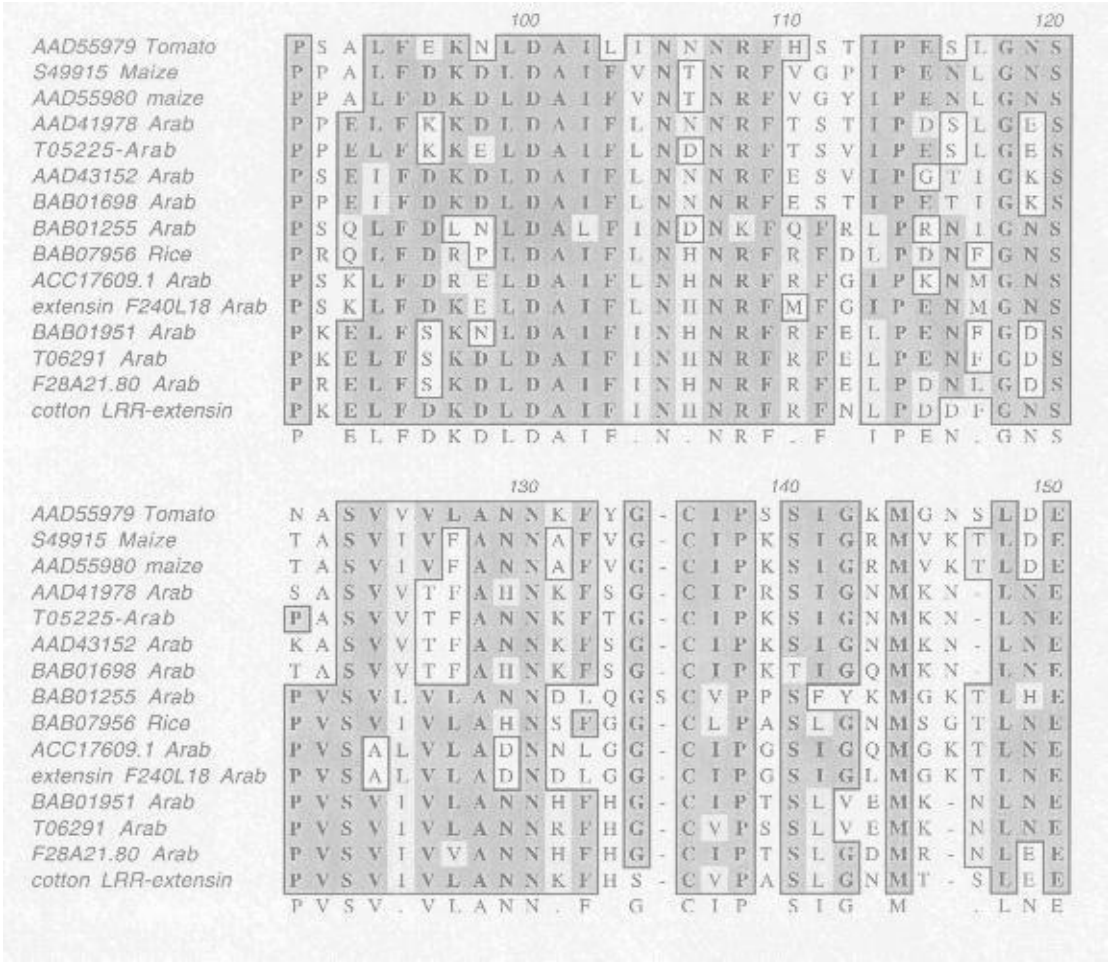
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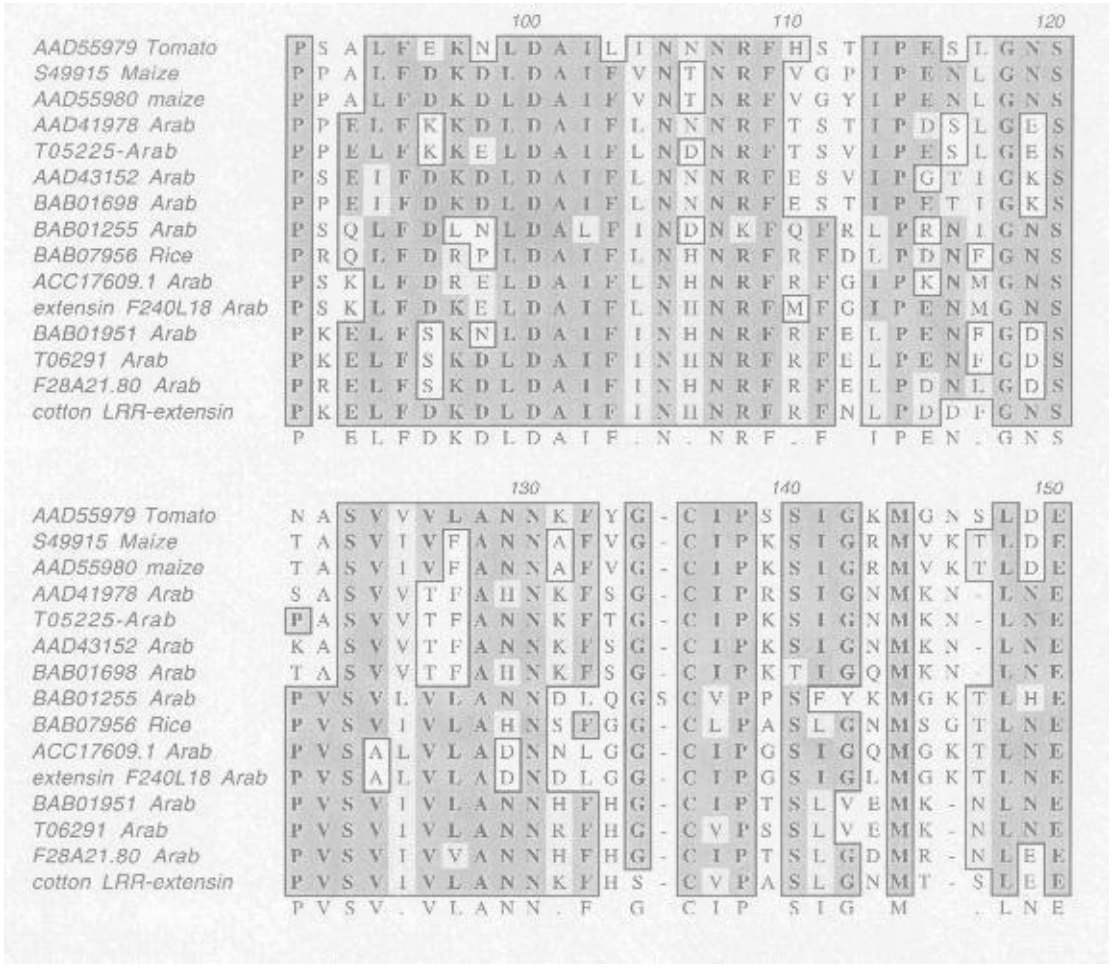
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extensin F240L18 Arab
BAB01951 Arab
T06291 Arab
F28A21.80 Arab
cotton LRR-extensin
P E L F D K D L D A I F . N . N R F . F I P E N . G N S
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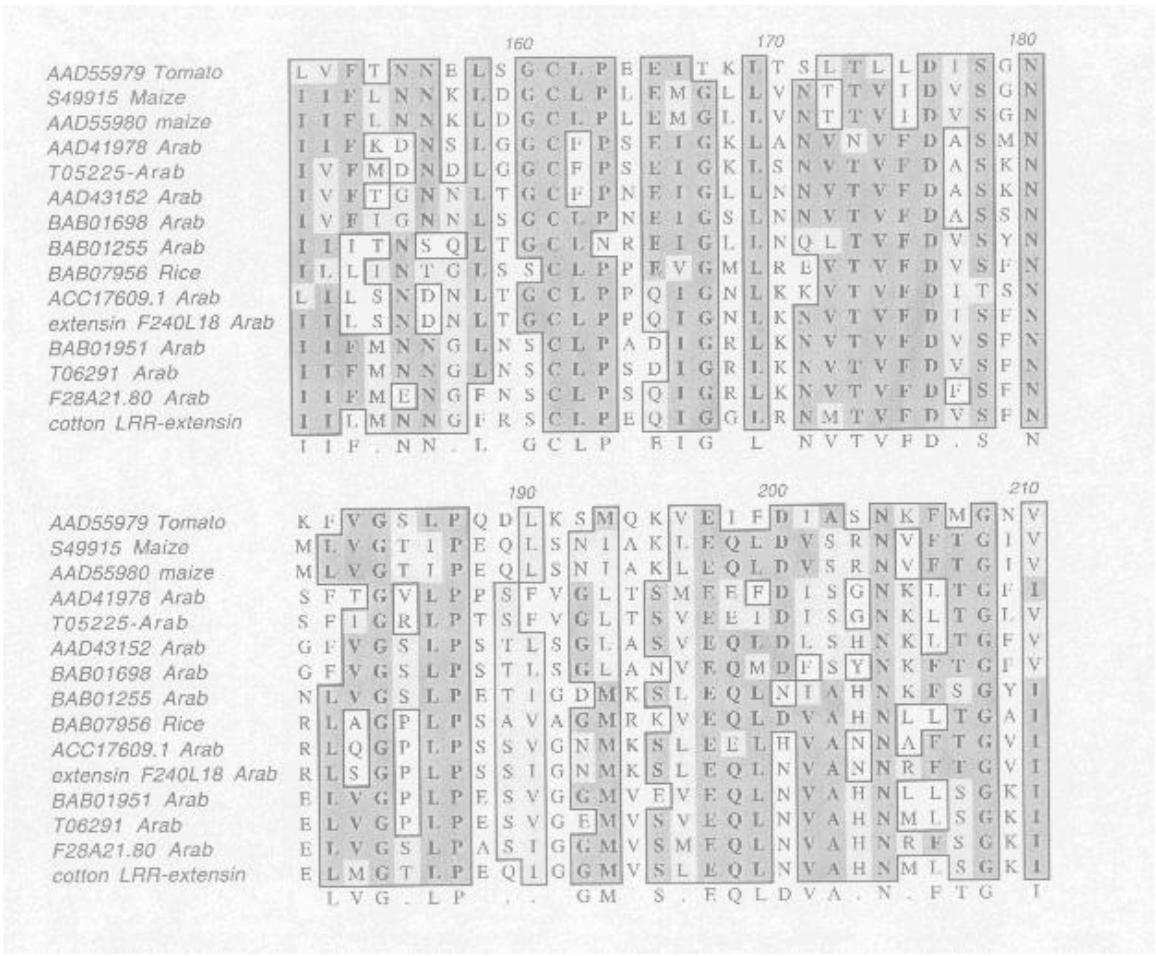
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ACC17609.1 Arab
extensin F240L18 Arab
BAB01951 Arab
T06291 Arab
F28A21.80 Arab
cotton LRR-extensin
190
200
210

CHAPTER V

SUMMARY AND CONCLUSION

The project began with the aim of isolating extensin cDNA clones from cotton. For this purpose, a cDNA library was constructed (Wenjun Huang, Oklahoma State University), and probed with 2 kinds of probes, a carrot genomic extensin clone pDC5A1 (Chen and Varner, 1985) and a degenerate probe coding for $Ser(Pro)_4$ repeats, which are characteristic of all typical extensins. Seven cDNAs were isolated. Two of the cDNAs encoded typical extensins and were called extensins # 2 and # 3.

The extensin # 2 cDNA was a 1 kb long incomplete cDNA sequence, lacking the initiation methionine and signal peptide found in typical extensins. This incomplete cDNA has an open reading frame of 696 nucleotides and codes for a protein fragment containing 232 amino acids, containing two kinds of repeats, Ser-Pro-Pro-Pro-Pro-Pro-Pro-Ser-Pro-Pro-Lys-His-Pro-Tyr-Lys-Tyr-Lys and Ser-Pro-Pro-Pro-Pro-Pro-Pro-Val-Tyr-Lys-Tyr-Lys, which include the isodityrosine cross-linking motif of Tyr-Lys-Tyr-Lys.

The extensin # 3 cDNA was a 700 bp long incomplete cDNA sequence, lacking the initiation methionine and signal peptide found in typical extensins. This incomplete cDNA has an open reading frame of 606 nucleotides and codes for a protein fragment

containing 202 amino acids, containing a repeat motif of Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys which include the isodityrosine cross-linking motif of Tyr-Tyr-Tyr-Lys. Northern blot results indicate that the full length of the cDNAs of extensin # 2 and # 3 is around 1.5 kb. Southern blot results indicate that extensin # 2 and # 3 are both single copy genes, as seen by their hybridization pattern under stringent conditions. Under low stringency, however, both the extensins hybridize to multiple bands.

Tissue expression analysis by Northern blot shows that extensin # 2 is expressed in all tissues but predominantly in root, stem and callus. Infection studies of cotton cotyledons after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria* shows that extensin # 2 is highly induced in cotton cotyledons at 48 hours after infiltration with the bacteria. Wounding studies on cotton cotyledons shows that, extensin # 2 is highly induced at 12 hours after wounding and remains high at 24 hours and 48 hours after wounding. In addition to the 1.5 kb extensin band, there are 2 more bands of 2 kb and 3 kb that hybridize to the extensin # 2 probe. Since only the 3' untranslated region was used in the synthesis of the extensin # 2 probe, these additional bands may be differentially splicing transcripts of the extensin # 2 transcript.

Extensin # 3 is expressed predominantly in roots, stem and callus. Leaf and cotyledons show low-level expression of this transcript. Infection studies of cotton cotyledons after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria* shows that extensin # 3 is highly induced at 48 hours after infiltration of the bacteria. Wounding studies on cotton cotyledon shows that extensin # 3 is highly induced at 12 hours after wounding, but the expression remains high at 24 and 48 hours after wounding. In addition to the 1.5

kb extensin # 3 band, there is a band at 2.3 kb and 3 kb, which could correspond to a differently spliced transcripts of extensin # 3 because the extensin # 3 probe was made from the 3' untranslated region of this gene.

The expression pattern shown by extensin # 2 and # 3 are typical of genes involved with defense related functions. It is hypothesized that, extensins protect the plant against pathogen attack and wounding by binding to the surface of the bacteria and restricting their entry into the plant and/or strengthening the cell wall during wounding (Mazau, Rumeau, Esquerre-Tugaye, 1987).

Besides the two extensins mentioned above, five more sequences with homology to extensions were found after screening the cDNA library. These five sequences, (sequences # 4, # 7, # 8, # 13 and # 65631) were not typical extensions, because they lacked the cell wall cross-linking motif and they did not have multiple tandem repeats as seen in the typical extensins # 2 and # 3. These sequences were called extensin-like sequences (Figure 1b). Three of these sequences, # 4, # 13 and # 8, were identical (99% identity). The longest of these extensin-like sequences, (# 65631) was a chimera, consisting of an N-terminal leucine-rich repeat sequence and a C-terminal extensin-like sequence. This sequence was called leucine-rich repeat extensin (LRR-extensin).

The LRR-extensin (# 65631) is a 1.46 kb incomplete cDNA sequence lacking the initiation methionine and signal peptide. The sequence has an N-terminal end containing four complete LRR repeats and one partial repeat, and a C-terminal end containing extensin-like serine/proline rich repeats. A 5' RACE experiment was done on this sequence to obtain the 5' end of the sequence. The 5' RACE sequence was composed of seven complete LRR repeats and one partial repeat. Putting the 5' RACE sequence and

the incomplete cDNA sequence (# 65631) together, we obtained a 2.46 kb complete cDNA sequence. Northern blot analysis with the 5' RACE sequence and the incomplete cDNA sequence (# 65631) shows that both these sequences hybridized to a transcript of about 2.4 kb. In addition, the incomplete LRR-extensin (# 65631) hybridized to a transcript of 3 kb that did not hybridize to the 5' RACE fragment, and both probes hybridized to a transcript of 1.5 kb. This can be explained by the reasoning that the 3 kb transcript may contain sequences similar to the extensin-like sequence in the incomplete LRR-extensin (# 65631), and the 1.5 kb sequence is similar to the LRR repeats that are present both in the incomplete LRR-extensin (# 65631) and the 5' RACE sequence.

Evidence that the complete sequence of the LRR-extensin that was deduced from the combination of the # 65631 clone and the 5' RACE clone is correct was obtained by isolating the genomic clone for LRR-extensin and sequencing it. The genomic clone of LRR-extensin did not have any introns because the sequence of the gene aligned perfectly with the complete cDNA sequence of LRR-extensin. The complete sequence of the LRR-extensin is 2.46 kb, with an open reading frame of 2091 bp, encoding a protein of 697 amino acids. The protein primary structure consists of a 32 amino acid signal sequence, a 97 amino acid domain with no homology to any known functional motif, an LRR domain of 256 amino acids, a 28 amino acid domain with no homology to known motifs and an extensin-like domain of 285 amino acids. The LRR domain contains 11 repeats of 21-24 residues that match the plant extracytoplasmic LRR consensus sequence of LxxLxxLxxLxLxxNxLxGxIPxx (Jones and Jones, 1997). Southern blot analysis after digestion of genomic DNA with EcoR1, Pst 1 and Kpn 1, and hybridization under stringent conditions with either the incomplete LRR-extensin (# 65631) or 5' RACE

sequence probe, showed at least two bands, whereas, hybridization under low stringency with the incomplete LRR-extensin (# 65631) showed at least five bands. This means that there are multiple sequences in cotton that show homology to the incomplete LRR-extensin (# 65631). But since the five bands were not seen in the blots hybridized under low stringency with the 5'RACE sequence, they may have domains similar to the extensin-like domains of the incomplete LRR-extensin (# 65631) and have different LRR repeats or not have these LRR- repeats at all.

Cotton LRR-extensin is expressed predominantly in stem, cotyledons, callus and leaves and to a lesser extent in roots. Infection studies of cotton cotyledons after infiltration with the bacteria *Xanthomonas campestris* pv. *vesicatoria* show cotton LRR-extensin is not induced as a result of infection. In fact, the gene seems to follow a circadian rhythm, with the expression level decreasing at 48 hours after inoculation with the bacteria. Wounding studies on cotton cotyledon showed that, the LRR-extensin is not induced after wounding even after 48 hours. This expression pattern is not like a gene involved in defense related function, because most genes that have a role in defense of the plant are induced as a result of wounding or infection. Therefore, we conclude that the cotton LRR-extensin may not have a defense related function.

The LRR domain was expressed in a prokaryotic expression vector and polyclonal antibodies to this domain were made (Hybridoma Center, Oklahoma State University). In Western blot experiments, a protein band of ~ 300 kD was detected in the callus culture cells. A very high molecular weight band that barely enters into the stacking gel was detected in the stem, leaf and root extracts. If the protein extracts from the callus culture cells were left in the -70ºC freezer for a week, the 300 kD band disappeared and a band

was detected in the stacking gel, in western blots. This result leads us to suggest that, the LRR-extensin in the stem, leaf and root extracts are very high moleculae weight proteins and do not completely enter into the stacking gel, because, either they are heavily glycosylated (more than the callus cultures), or they are cross-linked to other cell wall polysaccharides or proteins. The callus LRR-extensin may be less heavily glycosylated because, callus grows faster than the plant organs and hence the glycoproteins in callus may not be as completely glycosylated as their counterparts in stem, leaf and root. Storing the callus protein extracts in the -70ºC freezer for a week apparently caused the LRR-extensin to cross-link to other cell wall polysaccharides or to proteins, and cause the LRR-extensin in callus to become much bigger and not fully enter into the stacking gel, as we saw for the stem, leaf and root samples.

Comparison of the cotton LRR-extensin to ESTs from developing cotton fiber available from Brookhaven National Laboratory, Upton, NY, showed a 100% identity with an EST named BNLGHi5349 (GenBank Acc AI729848) from 6-day old immature cotton fiber (6-days post anthesis) and a 97% identity with an EST named GA_Ea0024C08f (GenBank Acc BG 444367) from 7-10 days post anthesis library from the Clemson University Genomics Institute, SC. At this stage in the development of cotton fibers, the fiber is going through the elongation phase (Jasdanwala, Singh and Chinoy, 1977), which is responsible for the elongation of the fiber involving synthesis of the primary cell wall. The only functional analysis study involving LRR-extensin was done with *Arabidopsis* LRR-extensin (LRX1). Isolation of transposon-tagged LRX1 mutants in *En*-1 mutagenized *Arabidopsis* plants (Baumberger, Ringli and Keller, 2001) showed that these plants exhibited an irregular root hair development. Most of the root hairs did not

elongate completely and were arrested soon after initiation, resulting in short stumps. From these observations, the authors hypothesized that LRX1 is involved in root hair morphogenesis and elongation by controlling polarized growth or cell wall formation and assembly. For proper root hair, cell wall synthesis and growth polarization, an intact microtubule assembly is very important. This is because the microtubules form guard-rail like barriers that constraint the movement of cellulose synthase enzyme and thereby allow the correct orientation of the cellulose microfibrils in the cell wall (Giddings, Staehelin, 1991). Identification of other root hair mutants like RHD3 (Galway, Lane, Schiefelbein, 1999), and KOJAK, that show a phenotype similar to LRX1, (Favery, Ryan, Foreman, Linstead, Boudonck, Steer, Shaw and Dolan, 2001), prove that defective cell wall synthesis or cell wall alteration can result in rupture of the root and defective root hair synthesis.

The fact that the cotton-LRR extensin and *Arabidopsis* LRX1 are quite similar in the LRR domain (55% identity and 69% similarity), and assuming that the cotton fiber EST BNLGHi5349 (that is expressed 6-days post anthesis when the fiber is undergoing the elongation process, a stage which is responsible for the expansion of the fiber and synthesis of the primary cell wall) is our cotton LRR-extensin, neatly fits in into the hypothesis that cotton LRR-extensin may be involved in coordinating growth or cell wall formation and assembly.

APPENDIX A

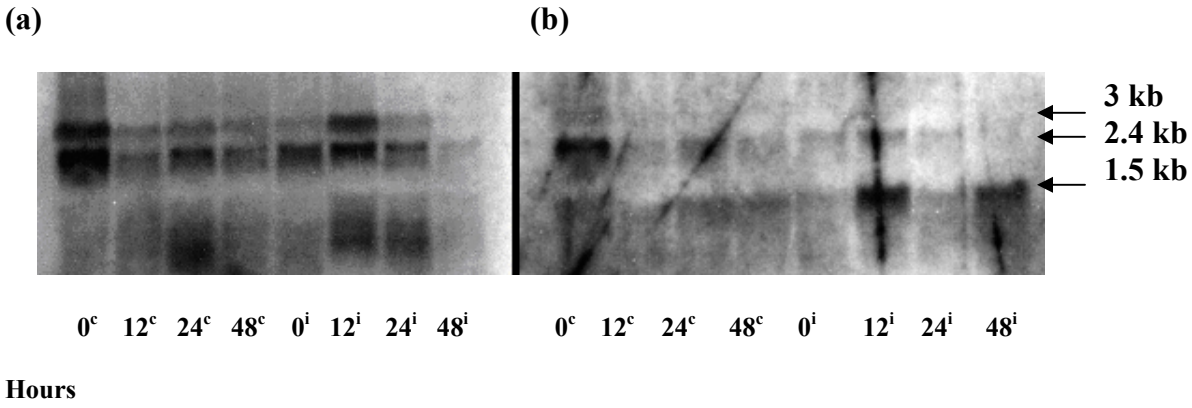


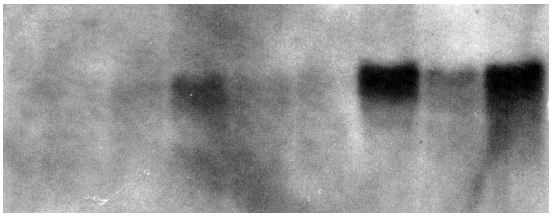
Figure 31: Northern blot of infection studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in controls (0^c 12^c 24^c 48^c), and *Xanthomonas campestris* pv. *vesicatoria* in infected plants (0^i 12^i 24^i 48^i).

(a) Blot probed with incomplete LRR-extensin (# 65631)

(b) Blot probed with 5' RACE probe of LRR-extensin.

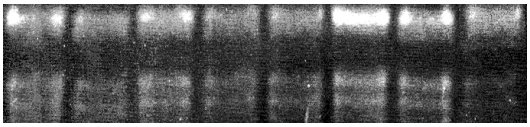
(c) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in control plants, and *Xanthomonas campestris* pv. *vesicatoria* in infected plants, as control for RNA loading.

(a)



Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

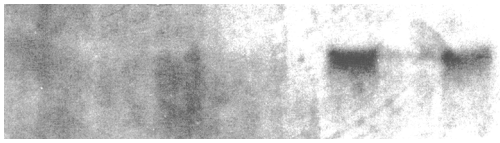
(b)



Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

Figure 32: (a) Northern blot of infection studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours after infiltration with $CaCO_3$ in controls (0^c 12^c 24^c 48^c), and *Xanthomonas campestris* pv. *vesicatori*a in infected plants (0^i 12^i 24^i 48^i) and probed with the 3' untranslated end of extensin # 2. **(b)** Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration of cotton cotyledons as control for RNA loading.

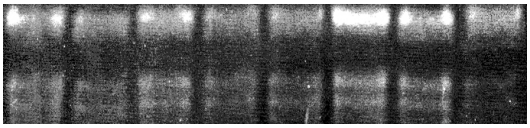
(a)



1.5 kb

Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

(b)



Hours 0^c 12^c 24^c 48^c 0^i 12^i 24^i 48^i

Figure 33: (a) Northern blot of infection studies. RNA was isolated from 11 day old cotyledons at 0, 12, 24 and 48 hours after infiltration by *Xanthomonas campestris* pv. *vesicatoria* and probed with the 3' untranslated end of extensin # 3. **(b)** Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours after infiltration of cotton cotyledons as control for RNA loading.

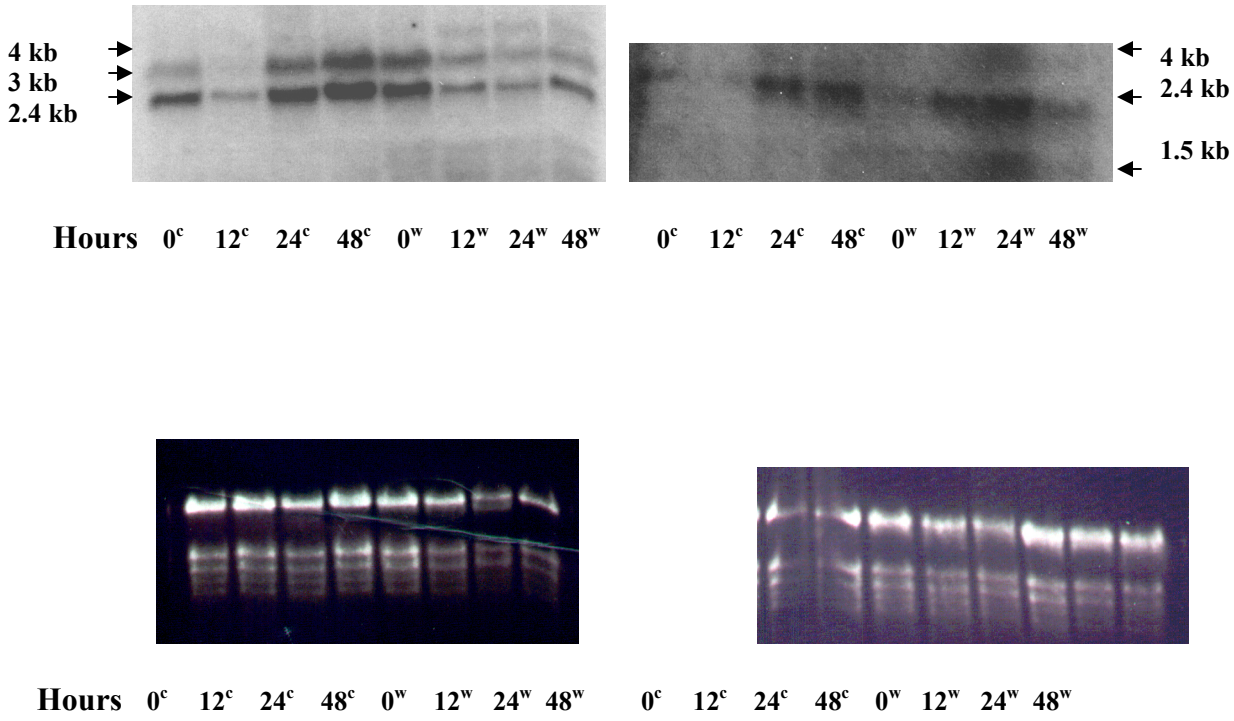


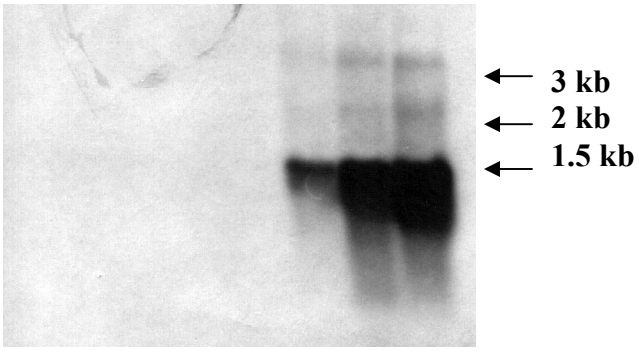
Figure 34: Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w).

(a) Blot probed with incomplete LRR-extensin (# 65631).

(b) Blot probed with 5' RACE probe of LRR-extensin.

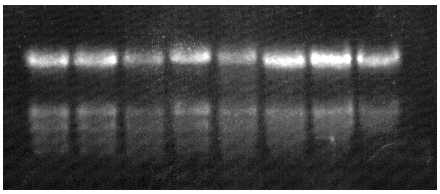
(c) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding.

(a)



Hours 0^c 12^c 24^c 48^c 0^w 12^w 24^w 48^w

(b)

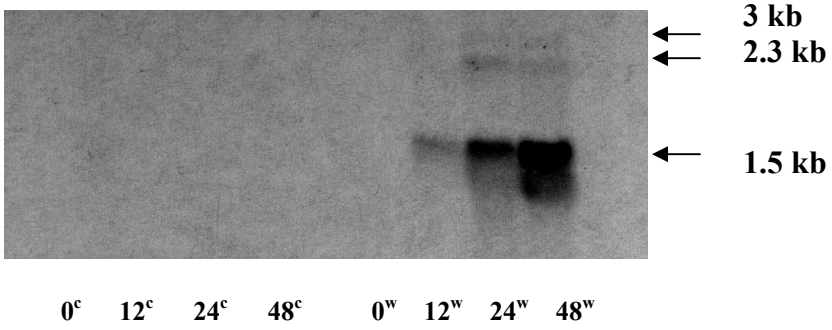


Hours 0^c 12^c 24^c 48^c 0^w 12^w 24^w 48^w

Figure 35: (a) Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w) and probed with 3' untranslated end of extensin # 2.

(b) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding, as control for RNA loading.

(a)



(b)

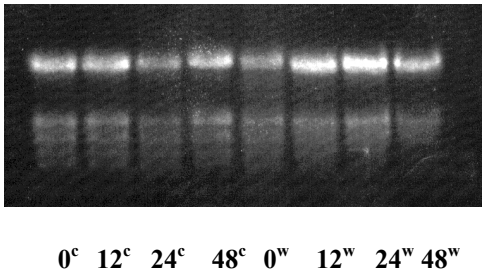


Figure 36: (a) Northern blot of wounding studies. RNA was isolated from 11-day-old cotyledons at 0, 12, 24 and 48 hours without wounding (0^c 12^c 24^c 48^c), and after wounding (0^w 12^w 24^w 48^w) and probed with the 3' untranslated end of extensin # 3.

(c) Photograph of ribosomal RNAs from 0, 12, 24 and 48 hours with and without wounding, as control for RNA loading.

Figure 37: Extensin # 2 gene sequence

tctccacccccaccacctccagtttacaagtataagtctccaccacctccacctccgtca
 S P P P P P P V Y K Y K S P P P P P P S
ccacccaagcacccttacaagtacaagtccccaccacctccaccaccatcaccaccaaag
 P P K H P Y K Y K S P P P P P P S P P K
catccttacaaatacaagtccccaccacctccaccaccatcaccaccaaagcatccttac
 H P Y K Y K S P P P P P P S P P K H P Y
aaatacaagtctccaccaccaccaccatcaccacctaagcatccctacaagtacaagtcc
 K Y K S P P P P P S P P K H P Y K Y K S
ccaccaccaccaccaccatccccaccaaagcatccctacaagtacaagtccccccccacca
 P P P P P P S P P K H P Y K Y K S P P P
ccaccaccgtcaccaccgaagcacccttacaagtacaagtccccacccccaccgtcacca
 P P P S P P K H P Y K Y K S P P P P S P
cccaagcacccctacaagtacaagtcacctccaccaccaccaccatcacccccaagcat
 P K H P Y K Y K S P P P P P P S P P K H
ccttacaagtacaagtccccacccccaccgtcaccacccaagcacccctacaagtacaag
 P Y K Y K S P P P P S P P K H P Y K Y K
tcacccccaccacccccaccatcacccccccaagcacccttacaagtacaagtctccacca
 S P P P P P P S P P K H P Y K Y K S P P
cctcatcatccagtttacaaatacaagtctcctccaccaccaccaccccattatgtctac
 P H H P V Y K Y K S P P P P P P H Y V Y
gcttcacccccctcctcctcaccactac**taa**gccacggctttgaccgtgctccaatccaag
 A S P P P P H H Y **Stop**
tcgtttgcaggaaaagataatgctgtgatgcaaataaagctacaaaaagatagtttgaaa

aggatatatctagagagataaagcaaaagattgggagcctagactagtctccgcgtttga

actgaagaacaataatgatgtagacaaattgcatttcaatgctgtattattcagtggttt

cgctcgtatttatttatttccaattctgcttaataataaaaatgattagtatatataatc

ctgtatgtgttttccaaccattttgcatgagtttattccactaataaaatataatgtcca
      Polyadenylation site
ttcct<u>aaaaaaaaaaaaaaaaaaaaaaaaaaa</u>

Figure 38: Extensin # 3 gene sequence

tctccatcaccaccaccaccatattactacaaatctccnncctcccccatcgccatctcct
 S P S P P P P Y Y Y K S X P P P S P S P
cctcctccctactactacaaatctccaccacctccatctccatcaccaccaccaccatac
 P P P Y Y Y K S P P P P S P S P P P P Y
tactacaaatctccacctcccccatcaccatctccacctcctccctactactacaaatct
 Y Y K S P P P P S P S P P P P Y Y Y K S
ccaccaccaccagtccactctccaccaccaccctactattacaagtccccacctccccca
 P P P P V H S P P P P Y Y Y K S P P P P
tctccatcaccaccaccaccatactactacaaatctcctcctcccccatcgccatctcct
 S P S P P P P Y Y Y K S P P P P S P S P
cctcctccttactactacaaatctccaccacctccatctccatcaccaccaccaccatac
 P P P Y Y Y K S P P P P S P S P P P P Y
tactacaaatctccacctcccccatcaccatctccacctcctccctactactacaaatct
 Y Y K S P P P P S P S P P P P Y Y Y K S
ccaccaccaccagtccactctccaccaccaccctactattacaagtccccacctcctcca
 P P P P V H S P P P P Y Y Y K S P P P P
tccccttcacccccctcccccatactactatcactcacctcccccaccagtgaaatcacct
 S P S P P P P Y Y Y H S P P P P V K S P
ccacctccagcctatatttacgcttctcctccaccacctactcactat**tga**gtctagaaa
 P P P A Y I Y A S P P P P T H Y **Stop**
gatcaaccacaaaatcgcaacgttcatgtaagtaaaattttagtcaaacctatagttaac

taataataatctaacaattaatttaatattctaaatttttttgtttaaattaaaatttcag
          Polyadenylation site
gttctagtttctc**aaaaaaaaaaaaaaaaaa**

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VITAE

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Candidate for the Degree of

Doctor of Philosophy

Thesis: CHARACTERIZATION AND FUNCTIONAL ANALYSIS

OF EXTENSIN AND LRR-EXTENSIN IN COTTON

Major Field: Biochemistry

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Title of Study: CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF EXTENSINS AND LRR-EXTENSINS IN COTTON.

Pages in Study:135 Candidate for Degree of Doctor of Philosophy

Major Field: Biochemistry

Scope and Method of Study: This research involved the characterization and functional analysis of two extensins (extensins # 2 and # 3), and an LRR-extensin (# 65631). 5' RACE experiment was done to determine the 5' ends of the LRR-extensin, and the genomic clone was isolated after screening the genomic library of cotton with extensin specific probes. Tissue expression analysis was done by northern blots to find out the expression pattern of the extensins and LRR-extensin in the different tissues of cotton. Infection and wounding studies were done on cotton cotyledons to see if the extensins and LRR-extensins were defense-related genes. The LRR domain of the LRR extensin was expressed in a prokaryotic expression vector, and polyclonal antibodies were synthesized to the LRR domain. Western blot analysis was done using these antibodies to detect the molecular weight of the LRR-extensin in plant tissues.

Findings and Conclusions: The LRR-extensin is a 2.46 kb transcript as demonstrated by RACE, Northern blot and genomic library screening. It is expressed predominantly in stem, cotyledons, callus, and leaves. Infection studies show that the expression of this gene is not induced at 48 hours after infiltration with *Xanthomonas campestris* pv. *vesicatoria.* Cotton LRR-extensin is not induced in cotyledons 48 hours after wounding. Western blot results with anti-LRR polyclonal antibodies, recognized a protein of 300 kD in callus cultures. Clones for Extensin # 2 and # 3 are 1 kb and 700 bp long, respectively, and both are missing their 5' ends. They are mainly expressed in root, stem and callus. Infection studies show that both the extensins are induced at 48 hours after infection, and wounding studies show that both the genes are expressed at 12 hours after wounding the cotyledons. From these results, one can conclude that the extensins are defense related genes and the LRR-extensin is not involved in plant defense.