ISOLATION, CHARACTERIZATION AND TRANSFORMATION STUDIES WITH LECTIN GENES FROM COWPEA (*Vigna unguiculata* L.Walp.)

Thesis

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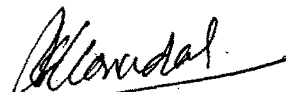

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ISOLATION, CHARACTERIZATION AND TRANSFORMATION STUDIES WITH LECTIN GENES FROM COWPEA (*Vigna unguiculata* L.Walp.)

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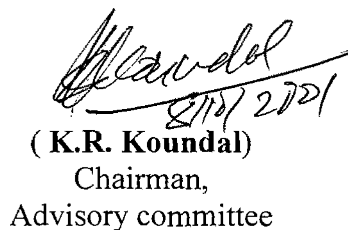
CERTIFICATE

This is to certify that the thesis entitled "**Isolation,Characterization and Transformation Studies With Lectin Genes from Cowpea (*Vigna unguiculata* L.Walp.)**", submitted to the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in Molecular Biology and Biotechnology, by **Mr. Subhojit Datta** embodies the results of *bonafide* work carried out by him under my supervision and guidance. No part of the thesis has been submitted by him for any other degree or diploma.

I further certify that such help or information, as have been availed of in this investigation, is duly acknowledged.



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LIST OF ABBREVIATIONS

A	:	Absorbance
APS	:	Amonium per sulfate
bp	:	Base pair(s)
cDNA	:	Complementary DNA
cpm	:	Count per minute
dATP	:	Deoxyribo adenosine tri phosphate
dCTP	:	Deoxyribo cytidine tri phosphate
dGTP	:	Deoxyribo guanosine tri phosphate
dTTP	:	Deoxyribo thymidine tri phosphate
DMF	:	Dimethyl formamide
DNase	:	Deoxy ribonuclease
dNTPs	:	Deoxy ribonucleotide triphosphates
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
h	:	Hour(s)
IAA	:	Indole acetic acid
IPTG	:	Isopropyl β-D-thio galactoside
Kb	:	Kilo base pair(s)
Kd	:	Kilo dalton
LA	:	Luria agar
LB	:	Luria broth
mCi	:	Milli curie
min	:	Minute(s)
mM	:	Milli molar

μ	:	Micro
MOPS	:	[3-(N-morpholino) propane sulphonic acid]
PEG	:	Poly ethylene glycol
pfu	:	plaque forming units
POPOP	:	1,4-Bis-2- (5-phenyloxazol-2-yl)-benzene
PPO	:	2,5-Diphenyloxazole
rpm	:	rotations per minute
RNase	:	Ribonuclease
S	:	Sedimentation coefficient in Svedberg units
SDS	:	Sodium dodecyl sulphate
sec	:	Second(s)
SSC	:	Saline sodium citrate
STE	:	Saline Tris EDTA
TAE	:	Tris-Acetate EDTA
TBE	:	Tris-Borate EDTA
TE	:	Tris-EDTA
TEMED	:	N,N,N',N'-tetramethylethylene diamine
v/v	:	Volume by volume
w/v	:	Weight by volume
Xgal	:	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
YEMA	:	Yeast extract mannitol agar

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1. INTRODUCTION

Revolution in plant breeding, supplemented by wide scale mechanization gave an impetus to world agriculture by breaking the yield plateau through introduction of high yielding varieties. With the introduction of hybrids and high yielding varieties, monoculture of certain crops or crop varieties has favored drastic increase in insect population that fed upon these crops. Every year about 35% of our harvest is lost due to harmful crop pests and diseases, with loss due to insects alone measuring an alarming 15% (Gatehouse *et al.*, 1992). Thus crop protection is an integral part of any agricultural practices that aims to make the revolution in crop sciences sustainable in long run.

In the recent past for protecting our crops the most widely adopted approach has been indiscriminate use of synthetic and semi synthetic chemical pesticides. But this approach has several hazardous problems-the most important one being the long-term damage to environment and health. Beside this, the wide spread use of pesticides has resulted in rapid resistance build-up by insect-pests to a number of such compounds. The endotoxin of soil bacterium *Bacillus thuringiensis* has been able to give protection against many important crop pests. But as more and more numbers of field trials are performed, the incidences of resistance build up in insects are also growing. All these factors taken together have necessitated changes in crop protection technologies to move towards true sustainability.

In this context a 'copy nature' strategy of insect control using insecticidal plant genes is expected to be sustainable and environment friendly. Pathogenesis related proteins (PRPs) are the class of secondary metabolites giving protection against pathogens. Sometimes, primary molecules in addition to their main function serve a role as defense chemical. Endogenous plant defensive proteins, interfering with digestion and thus affecting the nutritional status of the insect is a strategy widely employed by plants to defend themselves against pests. Over expression of several protease inhibitors from constitutive promoters has been shown to confer protection in transgenic plants against Lepidopteran and Coleopteran insect pests. Many major insect pests of crops suck the sap

directly from the content of phloem and parenchymatous tissues, and thus their digestive system is directly exposed to the content of the feed. A different approach must be taken with sucking insect pests, as they do not rely on proteolysis and till date no *Bt* toxin effective against Homoptern pests have been reported. Lectins are currently receiving most interest as insecticidal agents against these sap sucking Homopteran plant pests which includes aphids, leaf hoppers and plant hoppers.

Lectins are a heterogeneous group of carbohydrate binding proteins that bind to the sugar moieties of glycoproteins, glycolipids and polysaccharides with high affinity. Plant lectins form a unique class of proteins because of their highly specific carbohydrate binding activity, which distinguishes them from other classes of defense proteins. They are found in many different species and in different organs and tissues. Because of their binding specificity, lectins have the capability to serve as recognition molecule within a cell, between cells and between organisms. Plant lectins are secretory proteins, which are synthesized during seed development along with other storage proteins and can accumulate up to 15% or even higher of total protein. However the level of accumulation of lectins in storage tissues is far in excess of that required for any role in cell interaction or role as storage proteins. Based on this a role of lectins in plant defense has been proposed. Although lectins have been implicated in plant defense for a very long time, but remained a low profile candidate perhaps because of the relative difficulty in their genetic engineering.

With the advent of recombinant DNA technology and plant genetic engineering it has now been possible to isolate candidate genes and put them into host crops. Genetic engineering offers several advantages over conventional plant breeding by reducing the time of variety development and widening the gene pool. Instead of single resistance factor in breeding approach, through this technique, several genes can be transferred to the same host extending the resistance spectrum. Transgenic plant technology has allowed production of resistant crops by introducing entirely novel resistance genes into crop species. After some initial skepticism, genetic engineering of crops for insect resistance has now been adopted both by the agricultural industry and by government

agencies with some enthusiasm. Insect resistance has now been one of the major success stories of application of plant genetic engineering to agriculture, and genetically modified insect tolerant corn, potato and cotton plants expressing *Bt* endotoxin genes are now a commercial reality.

Cloning, structural characterization of and transformation with lectin genes is virtually prerequisite for the genetic manipulation of these genes for insect resistance. Therefore present investigation with cowpea lectin gene was undertaken with the following objectives-

1. To clone and characterize cowpea lectin gene
2. To transform *Brassica juncea* with isolated cowpea lectin gene, and
3. To characterize developed transgenics using molecular techniques.

In the present investigation, cDNA clones of lectin have been isolated from cDNA library constructed in λZAPII vector. Isolated lectin gene has been sequenced and fully characterized. *Brassica juncea* cultivar RLM198 has been transformed by *Agrobacterium* method with isolated cowpea lectin gene, cloned in a binary vector. Regenerated transgenic lines have been analyzed by molecular techniques.

2. REVIEW OF LITERATURE

2.1 Plant Lectins

The term 'lectin' was coined by William C. Boyd in 1954 for their property of selectivity (Latin '*legere*', to pick up or choose). In 1888 H. Stillmark of Russia gave the first description of what we now know as lectin. While investigating the toxic effects on blood of extracts of the castor bean (*Ricinus communis*), Stillmark observed that the red blood cells were being agglutinated by a protein and gave it the name 'ricin'. Shortly afterward 'arbin' was extracted from *Arbus precatorius*. Because they were first isolated from plants, lectins came to be known as 'phytohaemaglutinin'. Now it has become clear that lectins are present not only in plants but also in some bacteria, vertebrates and invertebrates. However, lectins are most widely distributed in plants, reported in almost 1000 plant species.

Plant lectins are a very heterogeneous group of proteins from the point of view of their occurrence within the plant kingdom and distribution over tissues (Etzler 1986; Rüdiger 1988). They show high degree of heterogeneity with respect to biochemical and physiological role, because of their widely different carbohydrate binding specificities. (Goldstein and Hayes,1978). Besides the fact that they all exhibit carbohydrate binding activity, most of the plant lectins behave as typical storage proteins (Peumans and Van Damme, 1995 a).

Although, the term lectin initially referred to the ability of some carbohydrate binding proteins to selectively agglutinate erythrocytes from particular human blood group (Peumans and Van Dame, 1995 b) they exhibit most unusual chemical and biological properties. Molecular cloning of lectins and lectin related products has led to new insights necessitating an update of earlier definition of lectin as carbohydrate binding proteins. First, some plant enzymes are fusion proteins composed of a carbohydrate binding and a catalytic domain, which are separated by a hinge region (Collinge *et al.*, 1993). Similarly type II ribosome inactivating proteins (RIP) such as ricin and arbin are

fusion products of a toxic A chain and a carbohydrate binding B chain (Barbieri *et al.*, 1993). Second, several carbohydrate binding proteins posses only one binding site and therefore, are not capable of precipitating glycoconjugates (Van Damme *et al.*, 1994). Third, several legume species contain proteins that are clearly related to lectins but are devoid of carbohydrate binding activities e.g. α-amylase inhibitor of *Phaseolus vulgaris* (Mirkov *et al.*, 1994). Keeping these facts in mind, Peumans and Van Damme (1995 b) have defined plant lectins as 'any storage proteins that posses atleast one noncatalytic domain, that binds reversibly to a specific mono or oligosaccharide'. This new definition includes a broad range of proteins that behave quite differently in agglutination or glycoconjugates precipitation properties.

Based on their overall structure, three major types of lectins are distinguished, namely 'merolectins', 'hololectins' and 'chimerolectins'. Merolectins are proteins built exclusively of a single carbohydrate-binding domain. They are small single polypeptide proteins and because of their monovalent nature, they fail to precipitate glycoconjugates or agglutinate cells e.g. hevein (Van Parijs *et al.*, 1991). Hololectins are built of a carbohydrate-binding domain, but contain two or more such domains that are identical or very homologous. This group comprises all lectins that have multiple binding sites and hence, are capable of agglutinating cells or precipitating glycoconjugates. Obviously, the majority of all known plant lectins are hololectins, because they behave as haemaglutinin. Chimerolectins are fusion proteins possessing a carbohydrate-binding domain tandemly arrayed with an unrelated domain, which has a well-defined catalytic activity that acts independently of the carbohydrate-binding domain. Depending on the number of sugar binding sites, chimerolectins behave as merolectins or hololectins (Peumans *et al.* 1995 a).

The legumes are particularly rich in lectin and may account for as much as 50% of the soluble protein content as in *Phaseolus* seeds. Legume lectins for decades have been a paradigm in the area of protein- carbohydrate interaction. The legume lectin monomer is structurally well conserved. It consists of two large β pleated sheets that form a scaffold on which the carbohydrate-binding region is grafted. Most known legume lectins contain a structure termed as 'canonical legume lectin dimer'. Carbohydrate

binding of legume lectins depends on the simultaneous presence of both the calcium and a transition metal ion. Most legume lectins in addition to their sugar binding sites, contain one or more binding sites for hydrophobic ligands e.g. adenine and adenine related plant hormones.

2.2 Plant Lectins as Plant Storage Proteins

Usually a plant storage protein is defined as a protein which is found in large amounts in the cells of reserve tissues and whose function is to serve as source of nitrogen and/or sulphur. Like lectins, plant storage proteins are highly heterogeneous but biochemically they all have an amino acid composition characterized by a high content of glutamine/glutamic acid, asparagine/aspartic acid, serine and glycine and a low content of the sulphur amino acids methionine, cysteine and lysine (Shotwell and Larkins, 1989). Many seeds particularly those of legumes contain relatively large concentrations of lectins. For example Con A constitutes 2-3% of the protein from Jackbean (*Canavalia ensiformis*) and soybean agglutinin (SBA) accounts for 1-15% of the soyabean protein (Glycine max) (Peumans and Van Damme, 1995 b). A general survey for the occurrence of lectins in different plant tissues indicates that the majority of these proteins are found in typical storage tissues. For example, most seed lectins are located in the parenchyma of the seed storage organs such as the cotyledons e.g. legume lectins (Van Driessche, 1988) and the endosperm e.g. *Ricinus communis* lectins (Lord, 1985). Lectins and storage proteins are not only found in close association with each other but are also developmentally regulated in a markedly similar way. For instance, legume seed lectins are synthesized during seed development together with major storage proteins and are degraded together with them during seed germination (Van Driessche, 1988). Since they meet all the criteria imposed by the definition of plant storage proteins, most plant lectins are regarded as genuine storage proteins, which distinguishes themselves from the classical types of storage proteins by their carbohydrate binding properties. The other natural physiological roles assigned to lectins include biological nitrogen fixation, plant tissue growth, cell - cell recognition and adhesion density dependent inhibition of growth and gamete recognition (Bohlool & Schmidt, 1978; Begbie and King 1985; Etzler, 1986).

2.3 Plant Lectins as plant defense

The presence of lectin at relatively high concentrations in legume seeds has been associated with a possible role in insect and disease resistance. Theoretical approach to the physiological role of plant lectins demonstrates that these proteins have some role in plant defense (Diaz *et al.*, 1989; Bohlool and Schmidt, 1978, Peumans *et al.*, 1995 a). Most lectins are stable over a wide pH range, able to withstand heat and resistant to animal and insect proteases. In these respects, they strongly resemble other defense-related proteins such as some Pathogenesis-Related Proteins (PRPs), proteases, chitinases and glycanases (Peumans and Van Damme, 1995 a). Due to the broad interest in the possible involvement of plant lectins in various defense mechanisms, intensive efforts have been undertaken during the last three decades to demonstrate deleterious effects of numerous lectin on different kind of plant pathogens and predators. The possibility that they play a protective role in plants was first demonstrated by Janzen *et al.* (1976) using *Phaseolus vulgaris* agglutinin (PHA) which killed larvae of bruchid beetle, *Callosobrochus maculatus*. When the purified lectin was incorporated into artificial diet of insects at different concentrations, there was no insect survival at 5% level, and even at 0.1% the lectin was found to cause a significant reduction in the number of larvae developing into adulthood. Gatehouse *et al.* (1984) subsequently confirmed the toxicity of these purified lectins towards developing larvae of the bruchid beetle. Ironically, this first indication for a protective role of lectins against insects was based on a false positive result, since the effects were due to contaminating α-amylase inhibitor (Huesing *et al.*, 1991). However, this observation appears to be specific to *P. vulgaris* and is not the case with lectin preparations from other species. Winged bean (*Phosphocarpus tetragonolobus*) lectin has also been shown to be involved in seed resistance to non pest species (Gatehouse *et al.*, 1991). Besides demonstrating protective roles of lectins within seeds, several studies have also been carried out involving the screening of purified lectin against insect pests in an attempt to identify insecticidal proteins (Murdock *et al.*, 1990) and hence to isolate the genes encoding them for subsequent plant transformation. Recently wheat germ agglutinin (WGA) was found to have an inhibitory effect on development of the major pests of maize - the European corn borer (*Ostrinia nubilalis*)

and the Southern corn root worm (*Diabrotica undecimpunctata*) (Czapla and Lang 1990). Chrispeels and Raikhel (1991) have reported a chitin binding domain in wheat germ agglutinin (WGA) and homologous lectins from barley and rice. All the above mentioned lectins have been shown to have mammalian toxicity (Pusztai *et al.*, 1979; Higuchi *et al.*, 1984). This, therefore, limits their potential use in the production of transgenic plants, particularly in crop plants. The lectin isolated from pea (*Pisum sativum*) on the other hand has been shown to be innocuous to mammals as it is readily broken down in the gut (Begbie and King 1985). However, it was shown to be insecticidal when the purified protein was incorporated into artificial seeds. Purified snowdrop and garlic lectins have also been shown to be insecticidal but do not exhibit mamamlian toxicity (Pusztai *et al.*, 1992). Snowdrop lectin (GNA) and wheat germ agglutinin (WGA) particularly found to be toxic to rice brown plant hopper, *Nilaparvata lugens*, a sap-sucking insect, when incorporated into artificial diet @ 1% (Powell *et al.* 1993). Similar results were obtained with the green leafhopper (Powell *et al.*, 1993). Soybean lectin has been shown to be toxic to larvae of Lepidopteran, *Manduca sexta* when tested in bioassay (Shukle and Murdock, 1983). Transgenic plants expressing different lectin genes have been produced and were shown to have enhanced level of protection against insects.

Due to the absence of glycans on plant viruses, plant lectins fail to inhibit viral infection and replication. However, type II RIPs has been reported to inhibit systemic spread of some plant viruses (Balzanini *et al.*, 1992). The insecticidal plant lectins indirectly prevent and/or reduce the spread of insect transmitted viral diseases.

Bacterial cell wall precludes any interaction between glycoconjugates on their membrane and carbohydrate binding proteins. Therefore, plant lectins can not disturb cell wall structure or intracellular processes of invading bacteria. Potato lectin was found to immobilize avirulent strains of *Pseudomonus solanacearum* in the cell wall (Sequeria and Graham, 1977). Another indirect defense mechanism is the blocking of the movements of normally motile bacteria at the air-water interface by the thorn apple (*Datura stromonium*) seed lectin (Broekaert and Peumans, 1986).

By virtue of their specificity, chitin-binding lectins have a role in the plant's defense against fungi. In vitro studies demonstrated that WGA inhibits spore germination and hyphal growth of *Trichoderma viridae*. But subsequently it was demonstrated that the inhibition was due to contaminating chitinase in lectin preparation (Schlumbaum *et al.*, 1986). More definitive proof followed when it was demonstrated that chitinase-free lectin from stinging nettle (*Urtica dioica*) inhibited the growth of *Botrytis cineria*, *Trichoderma hamatum* and *Phycoyces blakesleeanus* (Broekaert *et al.*, 1989). It affects by disturbing chitin synthesis and/or deposition and thus the synthesis of cell wall is affected (Van Parijs *et al.*, 1992).

2.4 Mecahnism(s) of lectin toxicity

Despite attempts to demonstrate a correlation, the specificity of binding to carbohydrate residues for a given lectin (Table1) is not necessarily a good indicator of its potential insecticidal properties (Harper *et al.*, 1995) and thus it is still necessary to test each lectin against a target pest on a case by case basis. Since the mechanism(s) by which some lectins are toxic to higher animals are not fully elucidated, it is perhaps not surprising that mechanisms of lectin toxicity to insects are largely unraveled. Binding of lectin to cells lining the gut of insects has been demonstrated in a number of species, but it is also clear that binding is not in itself sufficient to produce toxicity (Gatehouse *et al.*, 1984, Harper *et al.*, 1995, Powell *et al.*, 1998). Most likely the toxicity of lectins based on a highly specific binding to receptor molecule, which are located somewhere in the gut of the insects. Unfortunately little is known with certainty about the nature of putative lectin receptor molecules in the insects or their exact location along the digestive tract. Theoretically several types of interactions are predicted: a) binding of lectins to the chitin in the peritrophic membrane (only for chitin binding lectins), b)binding of lectins to glycoconjugates exposed on the epithelial cells along the digestive tract, and, c)binding of lectins to glycosylated digestive enzymes.

First, the chitin in the peritrophic membrane, lining the mid gut of insects can be recognised and bound by Glc Nac or (Glc Nac)n binding lectins (Peumans and Van Damme 1995 a).This may cause physical blockage and as a result , the movement of

Table 1: Sugar specificity of few common lectins

Source of lectin	**Sugar specificity**
Arachis hypogea	Galactose
Bandeiraea simplicifolia	Galactose
Bauhinia purpurea alba	N-acetylgalactosamine
Dolichos biflorus	N-acetylgalactosamine
Glycine max	N-acetylgalactosamine
Lotus tetragonolobus	Fucose
Phaseolus lunatus	N-acetylgalactosamine
Phaseolus vulguris	Galactose
Ricinus communis	Galactose
Sophora japonica	N-acetylgalactosamine
Ulex europeus	Fucose
Vigna radiata	Galactose
Wisteria floribunda	N-acetylgalactosamine
Canavalia ensiformis	Glucose/mannose
Lens culinaris	Glucose/mannose
Pisum sativum	Glucose/mannose

either digestive enzymes or food components between the endo and exotrophic space may be altered (Eisemann *et al.*, 1994). Alternatively, the binding of the lectins can affect the structure of or the formation of the peritrophic membrane. Binding occurs to specific glycopolypeptides as shown by separation of brush border membrane by electrophoresis, followed by blotting techniques using labeled lectins (Harper *et al.*, 1995 and Powell *et al.*, 1998). At present the exact working mechanism of the chitin binding insecticidal lectins is not known, but there are indications that for instance wheat germ agglutinin has a deleterious effect on the peritrophic membrane (Murdock *et al.*, 1990).

A second type of interaction is the binding of lectins to glycoconjugates exposed on the surface of the epithelial cells along the digestive tract of insects. In principle, all lectins which posses a carbohydrate-binding site complementary to the sugar moieties of the glycoconjugates can bind to the epithelial surface in the prevailing conditions allow a specific lectin-sugar interaction. At present, experimental evidences for such a binding of lectin and the impact thereof on the insect gut or on the whole organism are very scarce.

Theoretically plant lectins can also interfere with the insect digestive system by a third type of interaction which consists of lectin binding to glycosylated digestive enzymes. Certain lectins may be stable enough to survive and bind carbohydartes under the conditions prevailing in the insect gut. In cases where they have the right specificity, these lectins can form right complexes with glycosylated digestive enzymes and inactivate them. At present there is no experimental evidence for a lectin-enzyme interaction, at least in insects (Peumans and Van Damme, 1995 b). Another important observation that lectins can be transported across the gut wall and can be detected in insect haemolymph (Fitches and Gatehouse, 1998) suggests that systemic effects may also be important in lectin toxicity.

In order to determine the mechanism of toxicity of *Phaseolus vulgyris* lectin (PHA) to the larvae of *Callosobruchus maculatus*, the possible binding of lectin to the luminal surface of larval gut was investigated (Gatehouse *et al.*, 1984). When larvae that had been fed on a 2% lectin diet for 48 h were examined, strong fluorescence was observed in the cell surface of the midgut epithelium, whereas in larvae fed on a control

diet, epithelial cells were devoid of any fluorescence. These results were analogous to those reported for rats (King *et al.*, 1980).

Eisemann *et al.*(1994) studied the effects of three different lectins—ConA, lentil lectin and wheat germ agglutininin the growth of *Lucilia cuprina* larvae. They proposed three mecahnisms of action: first, a reduced intake of diet, second, a partial blockage of the pores of peritrophic membrane and, third, direct binding of lectin to mid gut epithelium. The observation that ingestion of ConA , WGA and lentil lectin caused up to 80% to 90% weight loss in the larvae supported the first two mechanisms. The feeding deterrence caused by the ingested lectins, may be due to the binding of lectins to glycoproteins situated in dendrites of chemoreceptor neurons near the mouth parts of the larvae. A consequent disruption of the normal functioning of these neurons may give rise to abnormal sensory inputs to the central nervous system, resulting in a partial inhibition of feeding.

Like in insects, the epithelial cells along the digestive tract of higher animals are fully exposed to the content of the diet. Most of our present knowledge about the toxic properties of plant lectins towards animals and humans has been obtained from fedding experiments with PHA. Ingested PHA, which is highly resistant to gut proteases, binds to the brush border cells of the intestine, where it is rapidly endocytosed. When the lectin enters the cells it induces an enhanced metabolic activity that eventually leads to hyperplasia and hypertrophy of the small intestine (Pusztai *et al.*,1990). Moreover ingestion of PHA or raw beans causes acute nausea followed by vomiting and diarrhea. Although most of the research on toxic lectin has been done with PHA, there are plenty of evidences that other lectins provoke similar effects. It is important, however to realize that toxic lectins are much more wide spread among inedible plants. Extensive work by Pusztai's group has demonstrated that the lectins from *Glycine max, Canavalia ensiformis, Datura stromonium* and *Urtica dioica* bind to the small intestinal mucosa of rat (Pusztai *et al.*, 1990,1991,1992,1993). But the lectins from snowdrop (*Galanthus nivalis*) bulbs and pea (*Pisum sativum*) seeds were innocuous to mammals. The seed lectin from cowpea also has been reported to be harmless on oral uptake by rats (Pusztai

et al., 1990). To asses the health risk for humans and domestic animals from lectins present in seeds and vegetative tissues of plants, it is important to test each lectin on a case by case basis.

2.5 Lectin Genes

The structures of lectin genes have been determined in soybean (Vodkin *et al.*, 1983), French bean (Hoffman *et al.*, 1985), Pea (Kaminski, 1987), Canavalia (Yamauchi and Minamikawa, 1990). Lectins have probably evolved through gene duplication and the carbohydrate binding domains of lectins have been incorporated to give them a protective role (Chrispeels and Raikhel, 1991). Many abundant seed proteins are encoded by multigene families. Biochemical evidences indicate the presence of a number of lectin genes in the plant. (Hoffman *et al.*, 1982). Soyabean seed lectin is under the control of one structural loci, Le1. Le1 is devoid of introns and produces a 1.0 kb mRNA. It codes for a signal sequence of 32 amino acids and a mature protein of 253 amino acids. The presumed promoter for the lectin gene (TATAATA) resembles a eukaryotic promoter consensus sequence and is found 27 bases from the 5' end of the lectin message.

Although soybean seed lectin is produced by a single gene, another divergent gene, Le2, homologous to Le1 has been identified (Goldberg *et al.*, 1983). Con A, the gene coding for concanavalin-A also lacks intron as reported for other legume lectin genes. There are multiple transcription initiation sites with the majority located in nucleotides 62-64 upstream from the initiation codon. (Yamauchi and Minamikawa 1990). Molecular cloning of *Phaseolus vulgaris* lectin mRNA indicated that the 5' region of the lectin gene contains four AUG codons one of which may initiate synthesis of a 20 residue long signal sequence and a mature protein of 223 amino acids (Hoffman *et al.*, 1982). Cloning of the cDNA of arcelin revealed a nucleotide sequence that is 78% homologous to PHA. (Osborn *et al.*, 1988). A genomic library of *Phaseolus vulgaris* DNA was screened using a lectin cDNA clone that includes the entire coding region, to obtain a lambda clone containing the corresponding nuclear lectin gene. Comparison of the cDNA and genomic nucleotide sequence showed that the gene has no intervening

sequences (Hoffman, 1984). The 5' untranslated region was found to be only 10-16 base pairs long by S1 nuclease mapping. The lectin transcriptional unit is flanked by sequences which are exceptionally high (72-75%) in A + T content and its 5' flanking region contains potential transcriptional control elements

Gramineae lectins like WGA and barley lectins are synthesized as preproproteins; their signal peptides are removed cotranslationally. The mature proteins of all Gramineae lectins that have been studied consist of four homologus domains of 43 amino acids (Chrispeels and Raikhel, 1991), indicating that the genes arose through the duplication of a single domain. A characteristic 'chitin-binding domain' (Chapot *et al.*, 1986) is present in all the chitin binding lectins like hevein (Broekaert *et al.*, 1990), tomato lectin (Kilpatrick, 1980), thorn apple lectin (Broekaert *et al.*, 1987), and potato lectin (Allen *et al.*, 1978; Desai *et al.*, 1981). The presence of this common domain suggests that these proteins have evolved by gene fusion of the chitin-binding domain with unrelated domains.

2.6 Lectin cDNAs

Synthesis and cloning of cDNA has been one of the most challenging methods in molecular bology. Availability of complementary DNA (cDNA) copies of mRNA provides an extremely powerful tool for analyzing the structure, organizarion and expression of eukaryotic genes. Apart from the utility of cDNA in defining the initiation, coding and termination sequences of mRNAs, their use as hybridization probes makes it possible for, isolate, search and identify and characterize the corresponding genes from chromosomal DNA.

Since the first report on the molecular cloning of cDNAs (Rougeon and Mach, 1976; Efstratiadis *et al.*, 1976), this technology has undergone several modifications to become more efficient. A complex series of enzymatic reactions are involved in copying mRNA into double stranded cDNA and subsequently preparing the termini for vector ligation. To overcome the problem of low number and truncated cDNA clones, Okayama and Berg (1982) suggested the vector primed cDNA synthesis. Gubbler and Hoffman

(1983) suggested the vector primed first strand synthesis, followed by nicking of RNA molecules so as to generate RNA fragments, which could then serve as primers for second strand synthesis.

Depending on the expression pattern of the gene, the appropriate age and tissue is selected for isolating mRNA to construct cDNA library. In case of legumes, the seeds are abundant in lectins and these lectins are synthesized during seed development together with major storage proteins (Peumans and Van Damme, 1995 a). Hoffman *et al.* (1982) synthesized french bean lectin cDNA using size fractionated mRNA. A cDNA library was constructed in pBR322 vector at *Pst* I site. After screening with a pea lectin cDNA probe, 8 positive clones were obtained indicating a considerable sequence homology. The sequence of two overlapping cDNAs complementary to lectin mRNA were compared and found to have no stop codon between the coding sequences for the β and α subunits. The entire pea lectin sequence showed a high degree of homology with the purified amino acid sequence of lectins from lentil, broad bean and to a lesser extent with concanavalin A from Jackbean. A cDNA library was constructed from total poly (A) rich mRNA isolated from snowdrop ovaries. Recombinant lectin clones were screened by colony hybridization using 32P end labelled oligonucleotide probe derived from a particularly known amino acid sequence for the lectin (Van Damme *et al.*,1991). A comparison of the lectin amino acid sequence with the deduced amino acid sequence of a lectin cDNA clone revealed the lectin mRNA also encoded a 23 amino acid signal sequence and a C-terminal extension of 29 amino acids, besides the mature lectin polypeptide. In Canavalia gladiata, both, cDNA clone and genomic clones have been compared to show absence of intron in ConA gene. Lectin cDNA clones from Amaryllidiaceae and Alliaceae species were analyzed by Van Damme (1991) and subsequently these have been used for plant transformation (Gatehouse *et al.*, 1996). A cDNA clone encoding lectin was isolated by immunological screening of an expression library prepared from poly (A) RNA from the inner bark of *Robinia pseudoacacia.* The cDNA clone had a open reading frame of 858 bp that encoded a polypeptide with a predicted molecular weight of 31210 daltons (Yoshida *et al.,* 1994). It appeared that lectin was synthesized as a precursor that consisted of a putative signal peptide of 31

amino acids and a mature polypeptide of 255 amino acids. These workers (Yoshida *et al.*, 1994) observed the appearance and disappearance of the mRNA.

To construct plant transformation vector, Newell *et al.* (1995) have used a broad-host range plasmid pRK 290 (Ditta *et al.*, 1990) into which the T-DNA has been inserted at the *Eco*R I site to create plasmid pSLJ 1006. Two plasmids were used for transformation - pCTI 5 and pPCG 6. To construct plasmid pCTI 5, a 1.8 kb Hind III/partial *Eco*R I fragment carrying the CaMV 35 S promoter sequence, the cowpea trypsin inhibitor (CpTi) gene and the nopaline synthase (NOS) terminator was cloned into pBluescript SK. Plasmid pPCG 6 carries two insect resistance genes: the cowpea trypsin inbibitor gene (truncated at the 5' end) and the gene encoding snowdrop lectin (GNA). To create pPCG 6 a specially designed polylinker was used containing a 150 bp 'spacer' region and the two genes were oriented as a 'head to head' inverted repeat, limiting possibility of deletions. Both the expression cassettes, for CpTi and GNA were finally cloned into a single plasmid in such a way that GNA gene was proximal to the right border sequence. Hilder *et al.* (1995) developed transgenic tobacco plants expressing GNA, but they used a different binary vector pRoK 2 (Baulcombe *et al.* 1986), using the same promoter, CaMV 35-S. They were able to clone a near full-length cDNA clone of GNA. Gatehouse *et al.* (1996) also constructed two binary transformation vectors - pPWG 6 and pPBG 6. To construct pPWG 6, two different insecticidal genes - encoding wheat a-amylase inhibitor (WAI) and snowdrop lectin (GNA) were used. In case of pPBG 6 the genes were bean chitinase (BCH) and GNA. The coding region fragments of each gene were cloned individually between the cauliflower mosaic virus (CaMV) 35 S promoter sequence and the nopaline synthase (NOS) transcriptional terminator sequence to produce expression cassettes. Finally these pairs of expression cassettes were cloned into the T-DNA of binary vector pAPT 5 and transferred into *Agrobacterium tumefaciens*.

2.7 Genetic engineering of plants for insect resistance

Conventional plant breeding has been quite successful in providing insect resistant varieties of certain crops. But in most of the cases it has not been possible to introduce resistance by this means, due to the lack of variation in crossable material.

Genetic engineering of plants offer means by which characteristics can be freely transferred between species, providing that the genes that determine these characteristics can be isolated and thus allows transfer of insect resistance to crop plants. An advantage of using genetic engineering is that the gene pool that can be used is virtually limitless from which to select insect control genes and introduction into crop varieties (Gatehouse and Hilder, 1994). Genetic engineering allows production of crop varieties that are inherently resistant to or tolerant of insect pests and thus offers following comparative advantages e.g. season long and weather independent protection, action only against harmul insects and at their most sensitve stages, biodegradable and ecologically safe material.

Development in the field of plant tissue culture has enabled regeneration of insect resistant whole plants from the cells or tissues. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny. Alternatively once single transformed plant has been obtained, conventional plant breeding methods can be used to transfer resistance genes in agronomically superior varieties via crossing and backcrossing. Such intermediate methods will comprise the furthe steps of –

1.Sexualy crossing the insect resistant plant with plants from the insect susceptible taxon

2.Recovering reproductive material from the progeny of the cross

3. Growing insect resistance plants from the reproductive material

To substantially preserve the agronomic characteristics of the susceptible taxon repeated backcrossing with the insect resistant progeny must be desirable or necessary.

Two types of insect control agents have been developed and proven effective following introduction into plants. The main approach uses δ-endotxin coding sequences from the soil bacterium *Bacillus thuringiensis.* In another approach, genes of plant origin viz. protease inhibitors and lectins have been used to give protection to plants which lack these compounds or express in very low amount.

Bt toxin form an extensive range of preferred 'natural' insecticides. Spores of *Bt* contain a crystalline protoxin which on ingestion by insects is cleaved by digestive proteases in the insect gut to generate the active *Bt* toxin molecule (Choma *et al.*, 1990). The active toxin molecule on binding to specific glycoprotein receptors on insect gut cells form ion channels which destroy the difference in ion concentration across the membrane resulting in death and lysis of the cells lining the gut (Manthavan *et al.,* 1989).*Bt* toxin proteins are encoded by genes (cry) carried on a plasmid within the bacterium. Different strains of *Bt* contain plasmids encoding toxins with different sequences and different specificities of action against insects. Since the cloning and sequencing of the first insecticidal gene in 1987 (Vaeck, 1987), more than 100 crystal protein gene sequences have been published. Whereas the isolation of genes encoding *Bt* toxin was an easy task, subsequent of transgenic plants that express these toxins proved much less straightforward. In fact considerable modification to the *Bt* toxin gene has proved necessary in order to obtain adequate expression to confer insect resistance in transgenic plants. The necessary modification has fallen into two classes: alteration of the protein sequences of the *Bt* toxin and alteration to the gene sequence.

Expression levels of active toxin molecules were one to two order of magnitude higher when plants were transformed with truncated version of toxin gene that only encoded N-terminal region of the protein containing the active toxin gene. Genes encoding *Bt* toxins have been reconstructed by a combination of mutagenesis and oligonucleotide synthesis to produce synthetic genes which had codon usage typical for plant genes to enhance expression. Expression levels of *Bt* toxin from these synthetic genes was increased by nearly two orders of magnitude, measuring upto 0.3% of total protein (Perlak *et al.*, 1991). The development of *Bt* transgenic plants was among the first

Table 2 : Genetic Manipulation for Insect Resistance Using Plant Genes

Transgenic crop	Plant Gene Used	Target Insect
Tobacco	Cowpea trypsin inhibitor	*Manduca sexta*
	Tomato serine PI II	*Manduca sexta*
	Potato serine PI II	*Manduca sexta*
	Snowdrop lectin	*Myzus persicae*
Sweet potato	Cowpea trypsin inhibitor	*Lacanobia oleracea*
	Snowdrop lectin	*Myzus persicae*
Rice	Cowpea serine PI	*Sesamia inferens*
	Potato serine PI II	*Chilo suppresalis*
Pea	Bean α amylase inhibitor	*Callosobruchus maculatus*
	Wheat germ agglutinin	*Callosobruchus maculatus*

biotechnology products of commercial relevance. Three commercial transgenic crops have been introduced that contain *Bt* toxin encoding gene for insect control: cotton, maize and potato. The first reults concerning the transfer of *Bt* genes in tobacco and tomato were published in 1987 (Vaeck 1987, Fischoff 1987). Since then *Bt* genes have been transferred to a number of other crops such as rice, cotton, soyabean, potato etc. with lepidopterans as the main target pests.

While transgenic plants harboring *Bt* genes have been able to provide protection against several insect pests belonging to different orders, as more and more number of transgenics leave the controlled environment of laboratory and are put into field trial, there are widespread reports of resistance build up in insects. Higher plant genes encoding lectins and inhibitors of digestive enzymes in insects are viable alternative options for engineering insect resistance. The first gene of plant origin that was transferred to another plant species to result in enhanced insect resistance encoded a Bowman-Birk type serine protease inhibitor from cowpea (CpTi) (Hilder *et al.*, 1987). A simple construct was prepared in which a full length coding sequence derived from a cDNA clone was placed under the control of the constitutively expressed cauliflower mosaic virus (CaMV) 35 S promoter. Transgenic tobacco plants were produced by a standard *Agrobacterium tumefaciens* mediated transformation protocol using a binary vector system. Transformants were screened for CpTi expression, which showed that many of the resulting plants expressed CpTi at levels greater than 0.1% of the total soluble protein. This was clearly in contrast to the very low levels of expression observed for unmodified toxin genes of bacterial origin. Other serine protease inhibitor genes have also been tested as protective agents for crops. For example the tomato inhibitor II gene when expressed in tobacco was also shown to confer insect resistance (Johnson *et al.*, 1989). The wound inducible potato protease inhibitors (PI & PII) have been expressed in a range of crops where they have been shown to confer resistance (Mc Manus *et al.*, 1994, Duan *et al.*, 1996). The α amylase inhibitor gene of *Phaseolus vulguris* under seed specific promoter has been expressed in transgenic tobacco. Seeds from the transgenic plants expressed the α amylase inhibitor and contained inhibitory activity against mealworm, *Tenebrio molitor* (Altabella and Chrispeels, 1990).

2.8 Transgenic plants expressing insecticidal lectin genes

Despite its profound contribution towards crop improvement, conventional plant breeding suffers from many basic constraints in that only compatible cultivars can be crossed and also the time taken to establish a particular trait is very long. However, plant genetic engineering offers the advantage of speed, accuracy and variability. The technology allows insertion of defined genes into plant and also examines the effect of resulting change in phenotype. Recently several workers have reported the prospects of conferring insect resistance to plants using lectin genes (Boulter, 1993; Boulter, *et al.*, 1990 a; Kahl and Winter, 1995; Gatehouse *et al.*, 1996).

Lectins have been shown to confer insect resistance in transgenic plants. Among plant lectin genes most widely tested are - cowpea protease inhibitor (CPTI) gene; (Hilder *et al.*, 1987), Snowdrop lectin (GNA) gene (Sauvion *et al.* 1996); Phaseolus vulgaris lectin (PHA) (Gatehouse *et al.* 1991); Pea lectin (Edwards, 1988). In general, to confer resistance to an insect, to which the plant is susceptible, the selected lectin will not be native to the plant i.e. the lectin will come from a species other than the plant being transformed. However, in species, which produces toxic lectins but no tin suficient amount as to kill insects, it may be preferable to insert a gene of the native lectin under strong constitutive promoter to cause over production of the lectin, thus achieving insecticidal level. Alternatively, where a plant produces native lectin but the lectin is not produced or not distributed to tissues, which are normally infested by insects, a tissue specific promoter can be used to provide localized expression or overproduction of the lectin.

A gene encoding pea lectin (P-Lec) has been expressed at high levels in transgenic tobacco plants by *Agrobacterium tumefaciens* mediated transformation (Edwards, 1988). A simple construct was made where the complete lectin coding sequence was driven by constitutive CaMV 35 S promoter. Pea lectin expressing plants were then tested in bioassay for resistance to the budworm, *Heliothis virescens*. The results showed that in transgenic plants leaf damage as well as larval biomass was

reduced (Boulter *et al.* 1990 b). Transgenic tobacco plants containing both the cowpea trypsin inhibitor gene (CPTI) and the pea lectin gene were obtained by cross breeding plants derived from the two primary transformed lines. These plants expressing the two insecticidal genes were also screened for enhanced resistance to *H. virescens* and the insecticidal effects of these two genes were found to be additive, the leaf damage being the least on the double-expressing plants.

A gene encoding the snowdrop lectin (GNA) has also been engineered into transgenic plants; a cDNA clone described by Van Damme *et al.* (1991) was transferred to tobacco by making constructs similar to those described for P-Lec above. Constructs containing a complete coding sequence for the GNA gave rise to levels of GNA upto 1.5% of the total protein in leaf tissues of transgenic potato (Gatehouse *et al.* 1997). Hilder *et al.* (1992) demonstrated that lectins from snowdrop (GNA) is insecticidal to sap sucking insects e.g. rice brown plant hopper (*Nilaparvata lugens*) and rice green leaf hopper (*Nephotettix nigropictus*). In another experiment, expression of snowdrop lectin in transgenic tobacco plants were shown to result in added protection against peach potato aphid (*Myzus persicae*) (Hilder *et al.*, 1995). Gatehouse *et al.* (1996) developed transgenic potato plants with a different GNA construct and expression of GNA in transgenic potato plants showed deleterious effects on the development of M. persicae, resulting in significant reduction in population build-up. Reduction in fecundity of the cereal aphid *Sitobion avenae* has also been reported on transgenic wheat plants expressing GNA (Stoger *et al.*, 1999). European patent application publication number 351,924 describes a method of genetically modifying crops with foreign lectin genes. Plant cells were transformed with pRi T DNA for heterologous expression of lectin genes from *Phaseolus vulguris, Vicia faba* and *Lens culinaris*. Transformation of potato and tobacco was specifically described.

To establish a suitable transformation and regeneration system of transgenic plants, Newell *et al.* (1995) took sweet potato (*Ipomoea batatus*) as a model plant system. The transgenic sweet potato expressing both cowpea trypsin inhibitor and snowdrop lectin following transformation of storage root discs with *Agrobacterium tumefaciens*

have been regenerated successfully to whole plant. GNA expressing such transgenic potato plants have been shown to have enhanced resistance to the peach potato aphids.

Presently, the strategy employed by most of the workers is not only to use single genes, but also to use gene combinations whose products are targetted at different biochemical and physiological processes within the insect. These packages will not only contain lectin genes but also genes encoding other demonstrated insecticidal proteins such as enzyme inhibitors (Gatehouse *et al.*, 1992).

The use of this technology to control sap-sucking insects belonging to order Homoptera was not so far very successful. These insects with their piercing and sucking type mouthparts suck the sap from phloem tissue. Thus phloem specific expression was important rather than constitutive expression using CaMV 35 S promoter. For this purpose several alternative promoters have been tried of which the rice sucrose synthase 1 promoter appears very promising (Shi *et al.* 1994). The rice sucrose synthase gene RSs1 has been isolated, characterized and fully sequenced (Wang *et al.*, 1992). The RSs1 promoter, which include 1.9 kb of 5' flanking sequence, plus the transcription start site, first exon and first intron of the gene, has been tested in transgenic tobacco by means of a suitable reporter gene (gus) fusion (Shi *et al.*, 1994). A similar RSs1 promoter fusion to the GNA coding sequence was used to direct phloem specific expression of GNA. Transgenic tobacco plants expressing these constructs were shown to be expressing GNA in phloem cells by immunolocalization (Shi *et al.*, 1994). Transgenic rice containing a *RSs1-GNA* construct has been shown to accumulate GNA in vascular and epidermal tissues (Sudhakar *et al.*, 1998), and decreases survival of rice brown plant hoppers by upto 60% which are exposed to the plants (Rao *et al.*, 1998). Thus phloem specific promoters offer the advantage of confining expression of insecticidal gene to the site of insect attack only. Expression of GNA from maize ubiqutin promoter has been reported to cause feeding deterrence in rice brown plant hoppers (Powell *et al.*, 1995).

The levels of protection against pests observed in transgenic plants expressing foreign lectins are not high enough to provide complete resistance. However, the absence of genes with proven high insectcidal activity against homopteran pests may well mean that transgenic crops with partial resisatnce may still find significance in agriculture. This is especially true if the resistance produced by the lectin transgenes proves to be additive to the existing endogenous partial resistance genes, or other transgenes also conferring partial resistance.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

Seeds of cowpea (*Vigna unguiculata* L.) cv. V 130 were sterilized with 0.01% mercuric chloride and germinated on water soaked paper towels in dark. After one week, etiolated seedlings were harvested, frozen in liquid nitrogen and used for isolating genomic DNA. For isolating poly (A)+ RNA, immature, developing (15 DAF) cowpea seeds were used.

Brassica juncea cultivar RLM 198 maintained by selfing for 4-5 generations were used as plant material for transformation. Seeds were surface sterilized by washing with 0.1% mercuric chloride for 15 min and then thoroughly rinsed in sterile water. Seeds were germinated aseptically in culture tubes (approximately 3-4 seeds per tube) containing 15-20 ml modified MS medium (Murashige and Skoog, 1962) [½ MS, 1% sucrose, solidified with 0.8%(w/v) agar]. For first two days tubes were kept in dark and thereafter transferred to 14 hrs light /10hrs dark cycle and maintained at 26±2°C. Hypocotyls excised from 5 days old seedlings were used as explants. Hypocotyl segments (0.5-1.0cm) were cultured on 90mm petridishes containing MS medium supplemented with 1mg/l NAA and 1mg/l BAP. Medium was solidified with 0.8% agar (w/v) [Himedia, Mumbai]. Explants were pre-cultured for 2 days.

3.1.2 Chemicals

Most of the chemicals like - Tris-Cl; potassium acetate; EDTA; SDS; sodium chloride; PEG 8000; Agarose, IPTG were of molecular biology grade from Sigma Chemical Company; St. Louis MO, USA. Gelatin; Ampicillin, Kanamycin, Rifampicin, Ethidium Bromide, and Lysozyme were of regular grade and purchased from Sigma Chemical Company, USA. Yeast extract, NZ amine, Bacto agar and Bacto tryptone were from Difco Laboratories, Detroit, MI, USA. Luria broth and Luria Agar were obtained from Hi Media Laboratories, Mumbai. Chloroform Phenol, Isopropyl alcohols were procured from Qualigen, Mumbai and ethanol was from Bengal Chemicals, Kolkata.

3.1.3 Kits and Enzymes

Nick translation kit for radiolabelling the cDNA was obtained from Amersham International, UK. Wizard-minipreps plasmid DNA purification system and plasmid midi kit were procured from Promega corporation, Madison, USA. DNA sequencing kit was obtained from MBI Fermentas, Lithuania. All restriction enzymes were from Stratagene; Lajolla, USA. α-^{32}P dCTP was procured from M/s Jonaki Lab, BRIT, Hyderabad.

3.1.4 Bacterial strains

For phage lysis and library pouring, the *E. coli* strain XL1-Blue was used as host bacterial cell. The supercompetent *E. Coli* SOLR cells were used for transformation. Pea lectin cDNA clone pLG 4.10 containing 860 bp lectin insert in PUC 8 vector was obtained from Dr. R.R.D. Croy's Lab, University of Durham, UK.

Agrobacterium based binary vector pBinAR was modified to introduce the cowpea lectin cDNA fragment (Datta *et. al.,* 2000). Plasmid pSK4.0 was cut with *Bam* H1 and *Sal* 1 to take out the 776-bp long cDNA insert. The fragment was ligated to pBinAR restricted with same set of enzymes, to get pSKL. *Agrobacterium tumefaciens* starin GV2260 was transformed with recombinant vector pSKL, having cowpea lectin gene, by freeze thaw method (Holsters *et. al.,* 1978). The lectin gene was driven by CaMV 35S promoter and terminated by *nos*. The *npt*II gene present in the vector served as selection marker.

3.1.5 Other materials

Hybond N^+ nylon membranes and Hyperfilm X-ray sheets used for DNA blotting and plaque screening were purchased from Amersham International Inc., UK. Polaroid film type 667 (3000 ASA) were obtained from Polaroid Corporation, MA, USA. Developer and fixer solutions for developing X-ray films were purchased from Kodak Lab chemicals, USA. "Sequi-Gen" DNA sequencing apparatus was from BioRad Laboratories, USA. "LASERGENE" software for sequence analysis was obtained from DNASTAR*, USA. Siliconised plastic microfuge tubes, pipette tips and other plastic wares were obtained from Tarson Industries, Kolkata. The plasticwares and glasswares were siliconised and autoclaved at 15 lb/sq. inch pressure for 15 min.

3.2 Methods

3.2.1 Isolation and purification of total genomic DNA of cowpea

Genomic DNA from one week old etiolated seedlings of Cowpea cv V-130 was isolated following modified method of Dellaporta *et al.*(1983). Fifteen grams of seedlings were weighed and frozen quickly in liquid nitrogen and ground to a fine powder in a 3" mortar and pestle. The tissue was not allowed to thaw once frozen, until the buffer was added. The DNA extraction buffer (75 ml) was added and transferred to a 250 ml flask. SDS (20%) was added and mixed thoroughly by vigorously shaking for 20 minutes and the flask was incubated at 65°C for 10 min. 5 M potassium acetate (50 ml) was added to the flask, shaken vigorously and further incubated at 0°C for 20 min. Most of the proteins and polysaccharides were removed as a complex with the insoluble potassium dodecyl sulphate precipitate. The material was transferred to centrifuge tubes and spun at 25000 X g for 20 min (Sorvall SS 34 rotor). The supernatant was collected into a clean 30 ml tube through a mira cloth filter and 0.6 volume of isopropanol was added, mixed and kept at -20°C for 30 min. The DNA thus precipitated was pelleted at 20000 X g for 15 min, washed in 70% ethanol, dried and dissolved in 3.5 ml of 50 mM Tris : 10 mM EDTA (pH 8.0). The solution was transferred to an Eppendorf tube and spun for 10 min to remove any insoluble debris present. The supernatant was transferred to a new Eppendorf tube and DNA was reprecipitated with 2.5 volume of ethanol and 0.1 volume of 3M Sodium acetate. The tubes were then centrifuged for 1 min in a microfuge, the pellet was washed with 70% ethanol, dried and dissolved in 500 μl TE buffer.

The crude DNA was purified through cesium chloride gradient centrifugation. To 3 ml (1 mg) of DNA; 2.85 g of cesium chloride and 10 μl of (10 mg/ml stock) ethidium bromide was added and the volume was made upto 5ml with TE buffer. The solution was then transferred to quick-seal ultra-centrifuge tubes and centrifuged in Beckman VTi 65 rotor at 60000 rpm for 16 hrs at 20°C. The DNA band was collected using 18-gauge needle. Ethidium bromide was removed from the DNA by repeated extraction with n-butanol saturated with TE, dialyzed overnight against TE and precipitated using 0.1 vol of 3 M sodium acetate and 2 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in 100 μl of TE buffer (pH 8.0).

The yield of pure DNA was estimated by taking the absorbance at 260 nm, using Beckman DU-40 spectrophotometer. To calculate the yield of DNA, the relationship 1 A_{260} unit = 50 µg of double stranded DNA was used. A pure DNA sample has an A 260/A280 ratio ~ 1.8. Deviation from this ratio indicated the presence of protein, phenol or particulate contaminants, and therefore purified further.

3.2.2 Restriction endonuclease digestion of genomic DNA

Five microgram of purified genomic DNA was restricted with seven different restriction enzymes i.e. *Eco* RI, *Bam* HI, *Hind* III, *Xho* I, *Kpn* I, *Pst* I and *Xba* I (5 U/µl of DNA) at 37°C for 3½ hrs. Reactions were terminated by adding 2 µl of 0.5 M EDTA (pH 8.0).

3.2.3 Agarose gel electrophoresis of restricted genomic DNA

2 µl of Bromophenol blue-Xylene cyanol dye was added to each restricted sample and they were electrophorsed on 0.7% agarose gel at 30 V for 10 hrs using 1 x TAE buffer (Tris 2M; EDTA 0.05M; Glacial acetic acid 57.1 ml per 1000 ml of 50 x TAE) containing 0.05 µg/ml ethidium bromide at room temperature. The gel was photographed using Photodyne camera fitted with red orange filter (Kodak 23A wratten) 234 nm and polaroid type 667 (3000 ASA) film.

3.2.4 Southern Blotting: Transfer of DNA from agarose gel to nylon membrane

The agarose gel containing electrophoresed samples was blotted according to a modified protocol of Southern (1975). Prior to blotting, the gel was put in depurination solution (0.25 N HCl) for 15 min. This was then transferred to denaturing solution for 15 min. This step was repeated three times. The gel was then washed with sterile water and putting in neutralizing solution for 45 min did neutralization. Finally the gel was thoroughly washed 2-3 times with distilled water. All the above processes were carried out at room temperature. Denatured DNA was transferred from agarose gel to Hybond N^+ nylon membrane with 20 x SSC as transfer solution using capillary force. The membrane and 3 pieces of 3 mm Whatman filter paper were cut to the size of the gel. A wick was made using Whatman filter for transfer of solution through gel. A pile of filter papers was also cut. The gel was carefully placed on a raised platform, the DNA side facing up and then the membrane, presoaked with Milli Q water was put onto it, leaving

no air bubble trapped between gel and membrane. The Whatman paper and filter paper were put onto it. Whole thing was wrapped with cling film to prevent unwanted evaporation of transfer solution. A weight of ~ 500 gm was put on top of it and blotting was done for 16 hours.

After the transfer, the membrane was carefully removed and immersed in an excess of 0.4 N NaOH for 1 min, again neutralized using 0.2 M Tris-Cl, (pH 7.5), 2 x SSC for 15 min. It was then air dried, baked at 80°C for 2 hrs and stored at room temperature under vacuum in a dessicator for hybridization later.

3.2.5 Isolation and purification of pea lectin plasmid DNA

Chimeric plasmid, pLG 4.10 DNA was isolated using the protocol of Birnboim and Doly (1979) with slight modification. A single colony of the recombinant of *E. coli* was inoculated in 5 ml Luria broth (LB) containing ampicillin (100 μg/ml) and grown overnight at 37°C with shaking. 250 μl of this overnight grown culture was used to inoculate 250 ml LB containing ampicillin (100 μg/ml) in 1 litre flask and incubated overnight at 37°C with vigorous shaking. The cells were pelleted down at 5000 x g for 15 min at 4°C and the pellets were washed with 50 ml cold STE. The pelleted cells were again resuspended in 5 ml of lysozyme solution and lysis was allowed for 10 min at room temperature. Ten ml of 0.2 M NaOH and 1% SDS was added and kept in ice with occasional mixing for 15 min. Then 7.5 ml of chilled 3 M sodium acetate (pH 4.8) was added and mixed gently followed by incubation on ice for 30 min. The slimy precipitate was pelleted down by centrifugation at 22000 X g for 15 min. The supernatant was filtered through Mira cloth and DNA was precipitated with 0.6 volumes of isopropyl alcohol at room temperature for 1 hour. The precipitated DNA was collected by centrifugation at 12000 X g for 20 min at 4°C. The pellets were dried and dissolved in 3.5 ml of TE buffer. To this 4.5 g of CsCl was added, dissolved and loaded on to 5.0 ml quick seal tubes containing 100 μl of 10 mg/ml ethidium bromide. The tubes were centrifuged at 60000 rpm for 16 hours in a Beckman VTi 65 rotor using L8-80M Beckman ultra centrifuge. The DNA band was recovered from CsCl gradient, extracted with TE saturated butanol to remove ethidium bromide, dialysed overnight and ethanol precipitated. The precipitated DNA sample was pelleted and finally dissolved in 100 μl

TE buffer. DNA concentration was estimated by taking absorbance at 280 nm and found to be 0.9 µg/µl.

3.2.6 Recovery of pea lectin cDNA fragments

Restriction reaction was set up taking 5 µg DNA, 2 µl 10 x buffer, 20 units of *Eco* RI enzyme in a total volume of 20 µl. Restriction was carried out at 37°C for 3 hrs.

The restricted samples were loaded on 0.7% low melting agarose gel and electrophoresis was carried out at 4°C at 32 V for 3 hours. After staining with ethidium bromide when observed in UV, 2 bands were seen, the lower one representing the fragment band. This band was excised out by a sterile blade and transferred to Eppendorf tubes. The tubes were kept at 65°C for 5 min. The volume of the content of the tubes were made to 500 µl and again incubated at 65°C after adding 50 µl of 5 M NaCl. Equal volume of phenol, saturated with TE was added and vortexed briefly, the tubes were spun in a microfuge centrifuge for 5 min. The upper aqueous phase was carefully transferred to a new sterile Eppendorf tube and reextracted with phenol. The excess of phenol was removed by extracting it with two volumes of diethyl ether. The upper layer of ether was discarded and the tubes were kept at 65°C for another 5 min. with caps open to evaporate the diethyl ether; if any. The DNA fragment was precipitated with 2 volumes of chilled ethanol at -20 °C overnight. DNA was recovered by centrifugation at 12000 rpm for 20 min. at 4°C, washed with 70% ethanol, dried and dissolved in 10 µl of sterile water.

3.2.7 Preparation of probe: Radiolabelling DNA by nick translation

In vitro labelling of DNA was done based on the method of Sambrook *et al.*, (1989). The purified inserts isolated from cDNA clone, pLG 4.10 were radiolabelled using Amersham Nick Translation kit. Reaction mixture contained 0.5 µg DNA, 10 µl dNTP mixture (dATP, dGTP, dTTP), 5 µl Enzyme mixture (DNA polymerase 1 and DNase 1), 5 µl (50 µ Ci) of $\alpha^{32}P$ d CTP (specific activity 3000 µCi/ m mole) and sterile water to a total volume of 30 µl. This mixture was incubated at 15°C for 90 min after which the reaction was terminated by adding 2µl of 0.5 M EDTA. The labelled fragment was then purified from the unincorporated and free labelled nucleotides, passing it through a Sephadex G-50 column equilibrated with STE. Fractions of 500 µl each were

eluted in Eppendorf tubes and 1 µl of each fractions were spotted on a GF/C glass fibre paper, dried and placed in scintillation vials containing 5 ml of scintillation mixture. The incorporation of radioactivity in DNA was measured using LS 180 Beckman liquid scintillation counter. Fractions showing maximum incorporations were pooled and used for hybridization.

3.2.8 Southern hybridization of $\alpha^{32}p$ labelled probe to membrane bound restricted cowpea genomic DNA

(i) Hybridization

Southern hybridization was done according to the protocol of Kochert *et al.* (1989) with little modification. The baked blot was placed inside a hybridization bottle and 30 ml of prewarmed prehybridization buffer was added, so that each square cm. area of blot received ~ 100 µl of buffer. The caps were tightened and prehybridization was performed in a 65°C oven with shaking for 8 hrs. Excess of prehybridization solution was removed from the bottle, so that now it contains only 50 µl buffer / cm^2 of blot. Radiolabelled cDNA insert obtained from cDNA clone pLG 4.10 was denatured by placing in boiling water bath for 10 min and chilled on ice immediately. The probe was added in the prehybridization solution and caps were tightened properly. Hybridization was allowed to proceed for 24 hours at 65°C with shaking.

(ii) Washing of filters and autoradiography

After hybridization, the bottle was removed and hybridization solution was drained out carefully into a disposable bag. To remove free nucleotides and non-specifically bound probes, the filter was washed as follows

1st wash: 200 ml of 2 x SSC at 65°C for 30 min.

2nd wash: 200 ml of 1 x SSC + 0.1% SDS at 65°C for 30 min.

3rd wash: 200 ml of 0.5 x SSC + 0.1 SDS at 65°C for 15 min.

All washings were done with continuous shaking in a shaker. The washed filters were autoradiographed.

To locate ^{32}P labelled probe on membrane, autoradiography was done. The blot was placed on a support and covered with Saran wrap. One x-ray film was placed on the blot in the dark room. The cassette was closed tightly and wrapped in a black cloth to prevent light and exposed for 36 hours at - 70°C. The film was developed in Kodak developer at room temperature for 4 min, washed in water for 1 min and fixed in Kodak fixer for 3 min. It was then washed in running tap water for 20 min and air dried at room temperature.

3.2.9 Screening of cowpea cDNA library for the presence of lectin genes

The cowpea cDNA library constructed in our laboratory (Datta *et al.*, 2000) was used for screening in the present investigation. mRNA was isolated from immmature cotyledons and after double stranded cDNA synthesis,the library was constructed in λZAPII vector.

3.2.9.1 Plating and titer estimation of the library

The cDNA library was serially diluted to 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7} in SM buffer. The bacteria *E. coli* strain XL 1-Blue was used as host cell. A single colony of XL 1-Blue was inoculated in TB media supplemented with 0.2% maltose and 10 mM $MgSO_4$ and incubated overnight at 30°C with vigorous shaking. The cells were harvested by centrifugation at 100 x g for 10 min. The pellet was resuspended in 0.4 vol of 10 mM $MgSO_4$ so as to get an $A_{600} = 0.5$. 200 μl of this host cell suspension was mixed with 10 μl of serially diluted phage and incubated at 37°C for 15 min to facilitate adsorption. This infected culture was then mixed with 3 ml melted top agar, cooled to 45°C and plated onto 150 mm bacteriological plates having NZY medium. Plates were incubated at 37°C for 8 hrs until the plaques formed, of sufficient size, but well isolated.

The titer of the library was found to be ~ 2 x 10^8 pfu/ml.

3.2.9.2 Primary Screening

(i) Pouring plates and lifting the plaques

From 10^{-2} dilution of phage library, 20 µl of phage was mixed with 3 ml of top agar and poured on to NZY plates as described earlier. A total of 20 plates were poured for screening. Lifting of plaques onto nylon membrane and further processing were done as per Davis *et al.* (1986). After the plaques appeared, the plates were stored at 4°C for 2 hrs to avoid top agar from sticking to the membrane. Nylon membranes cut to the size of the plates were carefully placed on the plate and left undisturbed for 2 minutes. The membrane on the plate was punctured at 4 points with a sterile 18 G needle and were marked with a pencil so that these marks could be used later for superimposition and alignment. The nylon filters were peeled from the plates and put into denaturing solutions (1.5 M NaCl, 0.5 M NaOH) phage side up for 5 min. Meanwhile another filter was placed over the plate for 2 min. The filter was neutralized by dipping into 1.5 M NaCl + 0.5 M Tris-Cl (pH 8.0) for 3 min followed by rinsing in 2 x SSC. Similarly all other filters were processed. The filters were placed on a 3 mm. Whatman paper to remove excess moisture and then air dried. To fix DNA, filters were baked at 80°C for 2 hrs. These filters were used for screening the positive clones.

(ii) Preparation of cDNA probe for screening cDNA library

The plasmid clone pLG 4.10 containing pea lectin cDNA insert was isolated and purified. The cDNA inserts were purified from LMP agarose gel and radiolabelled as described earlierin section 3.2.7.

(iii) Prehybridization and hybridization

All the 20 filters were distributed to 4 hybridization bottles, each containing 5 filters, prehybridization solution at the rate of 25 ml per filter was added to the bottles and incubated at 65°C oven with shaking for 8 hours. Excess of prehybridization solution was removed so that the bottles contain 50 µl solution per sq. cm. of filter. The radiolabelled probe was denatured by boiling 10 min at 100°C water and immediately chilled on ice. The probe was carefully added to bottles, capped properly and kept for hybridization at 65°C for 24 hours.

(iv) Washing of filters and autoradiography

Filters were carefully removed from hybridization battles and hybridization solution discarded in a disposable bag. The filters were then washed thrice sequentially in 200 ml of washing solutions as described earlier in section 3.2.8.

Washed filters were exposed in different cassettes for 3 days at -70°C and autoradiogram was developed as explained earlier in section 3.2.5.4. Using a light box, the puncture marks on filters were aligned with the spots on autoradiogram. These were then superimposed to the master plates. From the marked areas, agar plugs containing 10-12 plaques were removed using a wide bore borosilicate pasteur pipette and put into an Eppendorf tube containing 1 ml of SM buffer with 50 μl of chloroform, vortexed and stored at 4°C for secondary screening.

3.2.9.3 Secondary Screening

For secondary screening, each of the selected clone was poured onto 90 mm plates containing NZY. After plaques of desirable size appeared, they were lifted on nylon membrane. Filters were processed by washing in 0.5 M NaOH, 0.5 M NaOH + 1.0 M Tris-Cl (pH 7.5).

After washing, the filters were dried and baked at 80°C under vacuum and used for prehybridization. The radiolabelled pea lectin cDNA probe was put into prehybridization solution after removing excess solution and hybridization allowed for 24 hours at 65°C. The hybridized filters were then washed and autoradiographed as described earlier.

3.2.9.4 Tertiary Screening

For further confirmation of the positive clones obtained after secondary screening, tertiary screening was done. 300 μl of overnight culture of *E. Coli* XL 1-Blue host cell was mixed with 3 ml top agar and poured onto NZY plates and incubated for 20 min. The plate was kept on a number grid and the positive clones obtained from secondary screening were spotted on the bacterial lawn. Plates were incubated at 37°C overnight. Next morning plaques were lifted on membrane, hybridized and autobiographed. The 8 positive clones identified were stored at 4°C in 1 ml SM buffer separately.

3.2.10 Isolation of Recombinant Phage DNA and Southern Hybridization

(i) Infection of λZAP and isolation of DNA

For isolating phage DNA from the positive clones, the protocol of Davis *et al.* (1986) was followed. 200 μl of overnight culture of XL 1- Blue cells grown in LB, supplemented with 10 mM $MgSO_4$ was mixed with 100 μl of phage suspension (10^7 pfu) and incubated at 37°C for 20 min for adsorption. The mixture was transferred to a 100 ml flask containing 10 ml LB and the flasks having different clones were shaken vigorously at 37°C with good aeration. After 4 hrs, the medium turned cloudy and gradually became clear in 5-6 hrs. Then 100 μl of chloroform was added and shaken for another 15 min at 37°C to lyse the intact bacterial cells if any. The bacterial debris was removed by spinning at 3000 x g for 10 min.

Supernatant was transferred in a new tube and 5 μl each of DNaseI and RNase were added (both stock 10 mg/ml), mixed well and incubated at 37°C for 30 min. 10 ml of phage precipitation solution (20% W/V PEG 8000; 2 M NaCl) was added and incubated overnight at 4°C. The precipitated phage particles were recovered by centrifuging at 10000 x g for 20 min of 4°C. The supernatant was removed and the tubes were kept in inverted position on a paper towel to allow the fluid to drain away. The phage pellet was suspended in 500 μl of SM buffer, transferred to 1.5 ml Eppendorf tube and equal volume of TE saturated phenol was added and spun for 5 min. The aqueous phase was collected in a new tube and extracted with phenol:chloroform (1:1) and with chloroform : isoamyl alcohol (24:1). After collecting the upper layer, 2 volume of ethanol was added and kept overnight at -20°C to precipitate DNA. Next day DNA was collected by centrifuging at 12000 x g for 20 min at 4°C, pellet dried and dissolved in 20 μl of TE. Concentration of DNA was estimated spectrophotometrically at 260 nm.

(ii) Agarose gel electrophoresis and Southern hybridization of phage DNA

0.7% agarose gel was prepared by boiling 350 mg of agarose in 50 ml 1 x TAE, cooled down to 50°C, 2.5 μl of ethidium bromide (10 mg/ml stock) added and poured in the gel casting tray. After solidification, the gel was transferred into a buffer tank and sufficient 1 x TAE was added as to submerge the gel. DNA samples were prepared by

taking 2 µl of DNA, 5 µl sterile water and 1 µl of 6 x loading dye and loaded into the wells. Electrophoresis was carried out at 32 v for 3 hrs at room temperature.

The gel was blotted onto Hybond N^+ membrane according to the method of Southern (1975). The membrane was baked at 80°C for 2 hrs to fix the DNA. The α-^{32}P dCTP labelled pea lectin cDNA was used as probe. The probe was denatured and added into prehybridization solution as described earlier. After the hybridization, the membranes were washed sequentially with 200 ml of following solutions: (i) 2x SSC/0.5% SDS, (ii) 1x SSC/0.1% SDS, and (iii) 0.1x SSC/0.1% SDS. All washings were done at 65°C in a rotary shaker.

3.2.11 In vivo excision of phagemid clones

For *in vivo* excision of pBluescript phagemids from positive clones obtained after final round of screening, Stratagene *in vivo* excision kit was used which makes use of super competent SOLR *E. coli* and Ex-assist helper phages. 200µl of XL1-Blue *E.coli* , 250µl of recombinant phage stock(10^5 phage particles) and 1µl of Ex assist helper phages (10^6 particles) were mixed and incubated for 15 min. at 37°C. To this infected mixture 5 ml of YT medium (Bactotryptone 0.8g + yeast extract 0.5 g + NaCl, 0.5 g + Bacto agar 0.6 g per 100 ml of medium) was added and incubated for 3 hrs at 37°C with shaking. Cells were spun at 1000 g for 15 min and the supernatant transferred to another tube, heated for 20 min at 70°C and again centrifuged at 4000 g for 15 min. 100 µl of this supernatant containing pBluescript phagemid as filamentous single stranded phage particles was mixed with 200 µl of freshly grown SOLR cells and incubated for 15 min at 37°C. From this mixture 10 µl and 50 µl were spreaded onto two different Luria broth agar plates (Tryptone 10.0 g + yeast extent 5.0 g + NaCl 5.0 g + Agar 15.0 g for 1000 ml) containing X-gal and IPTG. After blue-white selection of transformants, chimeric pBluescript DNA was isolated from white colonies.

3.2.12 Selection of recombinant plasmid DNA clones

(i) The transformed white colonies were streaked onto a fresh plate and incubated overnight at 37ºC. With a sterile toothpick the bacterial growth was scrapped and resuspended in 40 µl of 1 x STE solution. To this equal volume of TE saturated phenol was added, vortexed and spun for 30 sec. The supernatant was transferred to another Eppendorf tube and to this 2 µl of RNase A was added and waited for 2 min. From this, 20 µl alongwith dye was loaded on 0.8% agarose gel and electrophorsed. The colonies that gave plasmid preparation of higher molecular weight compared to the control pBluescript were selected for further analysis.

(ii) For large-scale preparation of plasmid DNA, from true transformants, single colonies were inoculated in 5 ml LB with ampicilin (50 µg/ml). Plasmid DNA was isolated as described in section 3.2.5.1.

3.2.13 Confirmation for the presence of inserts in the recombinant clones

The recombinant clones were confirmed for the presence of insert by restricting them simultaneously with *Eco* RI and *Xho* I. The restricted samples were run on 0.7% agarose gel, stained with ethidium bromide and photographed. The gel was blotted onto Hybond N^+ nylon membrane, hybridized with pea lectin cDNA probe and autoradiographed. The subclones that showed the presence of insert were stored carefully for further analysis and characterization.

3.2.14 Sequential deletion of lectin clones

Before nucleotide sequencing, the isolated cDNA clone pSK4.0 was sequentially deleted by Exonuclease III following the protocol of Henikoff (1984) using ExoIII Deletion Kit from MBI Fermentas. *Kpn*I and *Hin*dIII were used as site A and site B enzymes respectively. To take out the deleted inserts from transformants, combination of *Pst*I and *Bam*HI was used.

3.2.15 Sequencing of the lectin cDNA clone

Plasmid DNAs from deleted subclones of pSK4.0 were isolated according to the method of Stephen *et.al.,* (1990). Purified plasmid DNA (3-4μg) was alkali denatured (2M NaOH), neutralized with 3M sodium acetate (pH 4.8) and used for sequencing. DNA sequencing was done by the di-deoxy chain termination method of Sanger *et.al.,*(1977) using Reader™ DNA Sequencing kit (MBI Fermentas). DNA sequencing was done using both forward and reverse universal M13 primers. Reaction was terminated by adding 2μl of stop solution containing 0.5 M EDTA and the samples heat denatured at 90°C for 2 minutes. 2μl each of these samples were then loaded into lanes marked G, A, T, C in a denaturing polyacrylamide (8%) gel containing 8M urea. The gel was run at 2500V/50°C for 4 h in "Sequi-Gen" apparatus (Bio- Rad). After the completion of run, the gel was fixed in a fixer solution (15% methanol/10% acetic acid), dried for two hours at 80°C in a vacuum gel drier and autoradiographed. The nucleotide sequence was read from the autoradiograph and analysed using LASERGENE programe of DNASTAR*. Homology search was done in BLASTN search package using Netscape Communicator from the Internet.

3.2.16 Transformation and regeneration of Brassica juncea

i) *Agrobacterium* mediated transformation

Agrobacterium tumefaciens strain GV2260 carrying the plasmid pSKLwas grown for 2 days at 28°C in 5ml liquid YEM medium (0.2g/l $MgSO_4$, 7H2O, 0.5g/l KH_2PO_4, 0.1g/l NaCl, 1g/l yeast extract, 10g/l mannitol, 15g/l agar) containing 50mg/l rifampicin and 50μg/l kanamycin. Bacteria were pelleted and suspended into liquid MS media. The density of the bacteria was adjusted to A_{600}=0.6. Pre-cultured explants were inoculated with *Agrobacterium* for 10 min, blotted dry and co-cultured at 25°C in petriplates containing modified MS media (MS, 2% sucrose, 3.5mg/l $AgNO_3$, 250mg/l Cefotaxime). After 3 Days of co-cultivation, explants were transferred to shoot regeneration medium (MS, 2% sucrose, 1mg/l BAP, 0.1mg/l 2,4-D, 3.5mg/l $AgNO_3$, 250mg/l Cefotaxime).

ii) Selection of putative transformants and their regeneration

Hypocotyl segments cocultivated with transformed *Agrobacterium* GV2260 as previously described were transferred to selection media containing 250mg/l cefotaxime and 20mg/l kanamycin. Green plantlets that differentiated on selection media were transferred to shoot regeneration media. For optimizing shoot regeneration, medium was supplemented with 3.5mg/l $AgNO_3$. Well shooted plants were separated from rest of the calli and transferred to rooting media (MS, 2% sucrose, 1mg/l IBA). Properly rooted plants after removing all traces of agar were kept for hardening.

3.2.17 Molecular analysis of transformants

i) Isolation of Genomic DNA and Southern Hybridization

Genomic DNA was isolated from leaves of five putative transformed plants and an un-transformed plant (as control) following the method of Dellaporta *et al* (1983). DNAs were purified by RNase treatment and phenol:chloroform (1:1) extraction and dissolved in 100μl of TE buffer (10mM Tris,1mM EDTA). For Southern hybridisation (Southern 1973), DNA was restricted with *Eco* R1 for 6hrs at 37°C, run on 0.8% agarose gel and blotted onto Hybond N^+ membrane (Amersham) using 20X SSC (0.3M Na-citrate, 3M NaCl) as transfer solution. The cowpea lectin cDNA insert was recovered by restricting pSK4.0 with *Eco* R1 and *Xho* 1, electrophoresing at 0.7% low melting agarose gel and eluting from gel by melting. The cDNA fragment was radiolabelled with α-^{32}P dCTP using Amersham Nick Translation Kit following the manufacturer's protocol and Southern hybridization was done according to the protocol of Kochert *et. al.,* (1989) as described earlier.

ii) Isolation of RNA and Northern Hybridization

Total RNA was isolated from leaves of plants, which showed presence of transgene in Southern hybridization. RNA was isolated following the protocol of Chomczymski and Sacchi (1987). Extracted RNA from each sample was run on 1.2% formaldehyde agarose gel, and blotted onto Hybond N^+ membrane (Amersham) according to the protocol of Davis (1986). Pre-hybridisation was performed at 42°C in the presence of 50% formamide. Cowpea lectin cDNA labelled with α-^{32}P dCTP was used as probe. After hybridization, membranes were washed sequentially at 42°C by 2X SSC, 1X SSC/0.5% SDS and 0.5X SSC/0.1% SDS and autoradiographed as described earlier.

4. RESULTS AND DISCUSSION

4.1 Isolation and restriction of genomic DNA of cowpea

To check the homology of pea lectin gene with the other legume lectin genes, the total genomic DNA from one-week-old seedlings of cowpea was isolated as described in "Materials and Methods". Seedlings were grown in etiolated condition so as to avoid any chloroplast DNA contamination. The isolated DNA was purified in CsCl gradient and A_{260}/A_{280} ratio of 2.0 was obtained giving purified preparation of DNA. Approximately 5 μg of this purified DNA was used for complete digestion with 20 units each of different restriction enzymes. Restricted samples along with unrestricted DNA were separated on 0.7% agarose gel (Plate 1a). The DNA fragments were blotted onto Hybond N^+ nylon membrane and DNA was fixed on the membrane by baking and used for hybridization.

4.2 Isolation of pea lectin Plasmid DNA and recovery of cDNA insert

The plasmid DNA from the *E. coli* strain DH5α that contained pea lectin cDNA insert (pLG 4.10) was isolated as described in "Materials and Methods". The clone contains an 860-bp pea lectin cDNA insert at *Eco* R1 site of PUC 8 vector (Fig 1). The plasmid DNA isolation method yielded ~ 4 μg of DNA per 10 ml overnight culture. This purified plasmid DNA was restricted with *Eco*RI to excise cDNA insert. The uncut plasmid along with restricted samples were fractionated on a 0.7% low melting agarose gel along with the λ DNA markers. The isolated DNA had absolutely no contamination of RNA and bacterial genomic DNA. Plate 2 shows a complete restriction, components resolving into an upper vector band and a lower insert band. When compared with λ marker, the fragment was found to be ~ 850 bp which closely resemble the actual size of 860 bp (Gatehouse *et al.* 1987). The gel piece containing this insert band was cut and fragment was eluted from low melting agarose as described in 'Materials and Methods'. From 15 μg of chimeric plasmid DNA restricted, 2 μg of fragment was recovered and used for preparing probe.

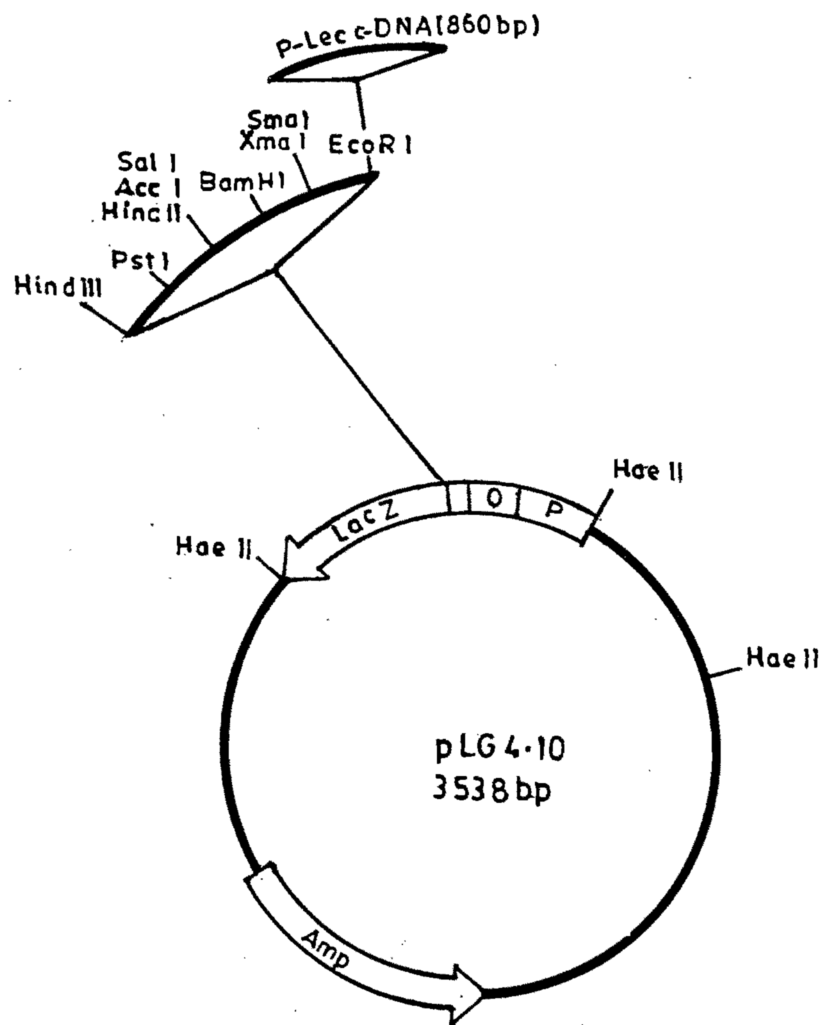


Figure 1 **Map of pea lectin clone pLG4.10.**

PLATE 1

a) Agarose gel electrophoresis of genomic DNA of cowpea digested with different restriction enzymes.
The digested samples were run in 0.7% agarose gel and stained with ethidium bromide as described in 'Materials and Methods'.

Lanes 1&6- Unrestricted cowpea genomic DNA
2- Genomic DNA digested with *Eco* R1
3- Genomic DNA digested with *Bam* H1
4- Genomic DNA digested with *Hind* III
5- Genomic DNA digested with *Xho*I
7- Genomic DNA digested with *Kpn* I
8- Genomic DNA digested with *Pst* I
9- Genomic DNA digested with *Xba* I

b) Southern hybridization of restricted genomic DNA of cowpea with pea lectin cDNA probe.

Lanes 1&6- Unrestricted cowpea genomic DNA
2- Genomic DNA digested with *Eco* R1
3- Genomic DNA digested with *Bam* H1
4- Genomic DNA digested with *Hind* III
5- Genomic DNA digested with *Xho*I
7- Genomic DNA digested with *Kpn* I
8- Genomic DNA digested with *Pst* I
9- Genomic DNA digested with *Xba* I

PLATE 2

Agarose gel electrophoresis of restricted pLG 4.10

Restriction was carried out at 37°C for 3 hours. The restricted samples were loaded on 0.7% low melting agarose gel and electrophoresis was carried out at 4°C at 32 V for 3 hours.

M- Marker, λ DNA digested with *Eco*RI + *Hind* III

Lane 1- Purified undigested pLG 4.10 cDNA clone

Lanes 2,3,5,6- pLG4.10 cDNA clone digested with *Eco*RI

Lane 4- Purified cDNA insert from pLG4.10 after restriction and elution from gel

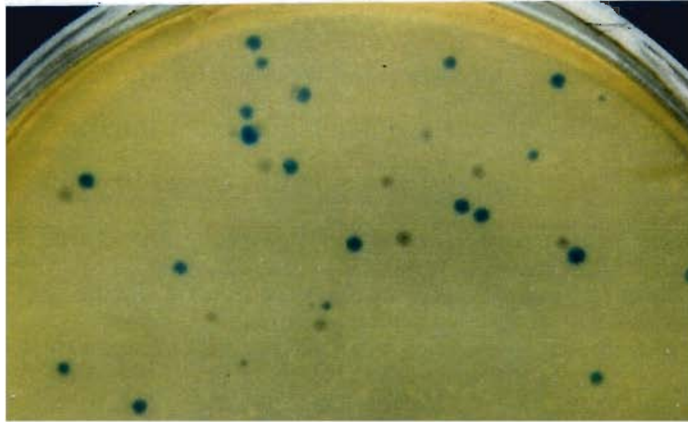
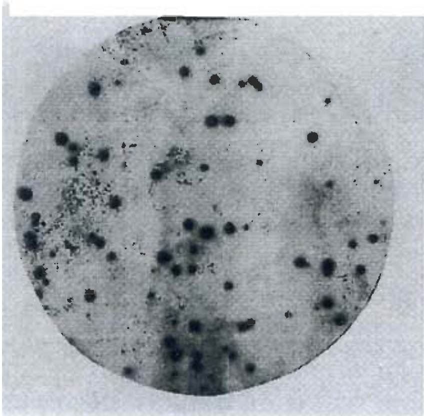
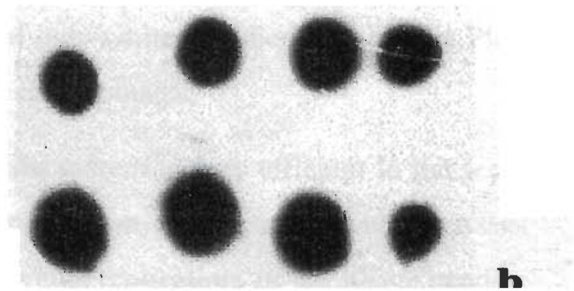


PLATE 3





a **b**

PLATE 4

4.3 Southern hybridization of pea lectin cDNA to genomic DNA of cowpea.

The southern blot prepared with the restricted genomic DNA of cowpea was hybridized with pea lectin cDNA probe. Though the probe DNA was heterologous it showed intense hybridization sugesting strong homology between pea and cowpea lectin genes. The stringency used (0.5 x SSC at 65°C for 30 min) was high enough to remove the nonhomologous DNA sequences from the membrane. As can be seen in plate 1b variation in hybridization indicates presence of sites for different restriction enzymes in the coding sequence of this gene. After establishing this homology between pea lectin and cowpea lectin gene, the pea lectin cDNA probe was further used for screening the cDNA library of cowpea for the presence of lectin clones.

4.4 Screening of cowpea cDNA library

(i) Primary Screening

For primary screening, 10^{-2} dilution of this library was used and approximately $2x10^6$ phages were plated on twenty 150 mm plates with varying efficiency. It was found that as many as 30000 plaques per plate can be screened without no apparent loss of signals on autoradiogram. This plaque density was found to give best signal. Plaques were lifted from all of these plates on Hybond N^+ nylon membrane and filters were always prepared in duplicate, which matched in their signal pattern. After hybridization and autoradiography, the autoradiogram was studied and the black spots were identified as positive signals. Initially a high number of clones were giving positive signal, which could be due to non-specific hybridization to 'spurious' clones. Altogether 50 plaques from 20 plates, corresponding to positive signals were picked up. Autoradiogram of one of the filters has been shown in Plate 4a. To separate out the real positive clones further from the mixture of primary clones, secondary screening was done.

(ii) Secondary screening

As can be seen in the plate, all the primary clones did not give equally intense signal. From the 50 such clones, 20 were selected showing most intense signals. These 20 plaques were individually poured onto 90mm plates after estimating their titer, so as to get well separated plaques. Out of 20, twelve showed strong positive signals when

probed with pea lectin cDNA. Four representative positive plaques from each of the 12 plates were picked up for a final round of tertiary screening.

(iii) Tertiary screening

Finally for further confirmation, the secondary screened clones were spotted on a bacterial lawn and the plaques thus formed were lifted, hybridized and autoradiographed as described in 'Materials and Methods'. This was done in duplicate. The autoradiogram showed 8 clones hybridized very strongly with pea lectin cDNA (Plate 4b). These positive clones were designated CL 1 to CL 8.

The results thus obtained clearly indicate that there is considerable degree of homology in lectin gene between different legumes. Earlier other workers have also reported homology between various legume lectin genes (Foriers *et al.,* 1979, Gatehouse *et al.,* 1987, Vodkin *et al.,* 1983 and Yamauchi, *et al.,* 1988).

4.5 Isolation of recombinant λZAP clones and hybridization

DNA was isolated from eight positive clones (CL 1 to CL 8). Initially there were difficulties in getting a good DNA preparation. Therefore, preliminary experiments were done to standardize proper bacterial cells: phage ratio for achieving good lysis. A ratio of 100:1 was found to be the most suitable one. Thereafter every time lysis was done using this ratio. The method yielded a pure preparation of DNA with absolutely no RNA and bacterial chromosomal DNA contamination when electrophorsed in 0.7% agarose gel. (Plate 5a). When the gel was blotted onto Hybond N^+ nylon membrane and hybridized with pLec cDNA probe, the clones hybridized very strongly. The autoradiogram (Plate5b) shows hybridization of all the clones with the probe. To distinguish whether this was due to nonspecific hybridization with the vector DNA, clones were excised *in vivo* as described below and when restricted samples were hybridized only the inserts gave signal with the probe.

PLATE 3

Blue –white screening of cowpea cDNA library for recombinants.
The library was poured on NZY plates containing X-gal and IPTG.

White plaques are recombinants.

PLATE 4

Autoradiogram showing screening of cDNA library of cowpea.

Library was poured on NZY plates, plaques lifted onto nylon membrane and hybridized with pea lectin cDNA probe.

a) Primary screening

b) Tertiary screening

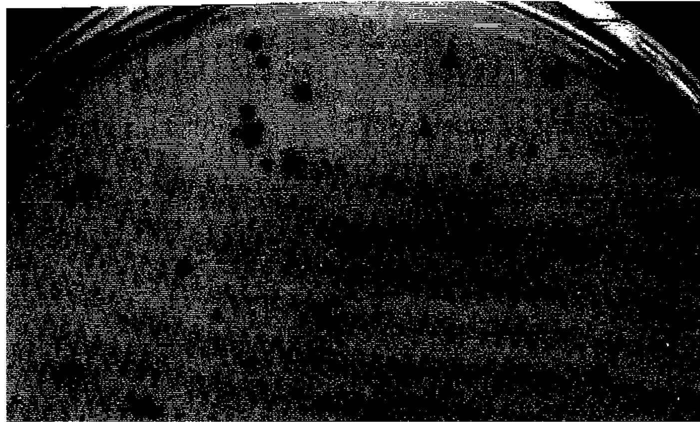
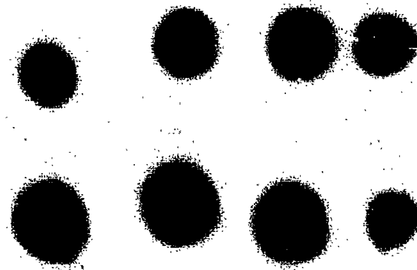


PLATE 3



a b

PLATE 4

4.6 *In vivo* excision and selection of transformants

The λZAP II vector system provides an easy subcloning of inserts into plasmid vector. *In vivo* excision of pBluescript phagemid allows characterization of insert into a plasmid system. The polylinker of pBluescript phagemid has 21 unique cloning sites flanked by T_3 and T_7 promoters and 6 different primer sites for DNA sequencing. Fig 2 summarizes the excision process. The plasmid has fl bacteriophage "origin of replication". When superinfected with fl derived 'helper phage', inside *E. coli,* the 'helper proteins' recognize the initiator DNA that is within the lambda vector. These proteins then nick one of the two strands and new single strand of DNA synthesis begins and continues through the cloned insert until a termination signal positioned at 3' is encountered. The single stranded DNA is circularized by another fl protein (gene II product). This conversion is the 'subcloning' step, since all sequences associated with normal lambda vector are positioned outside of the initiator and termination signals and are not contained within the circularized DNA. Using *E. coli* strain XL 1-Blue host cells and 'Ex Assist' helper phage, the pBluescripts were excised *in vivo* from all of the eight positive clones as described in 'Materials and Methods'. To plate the excised phagemids, phage supernatant was mixed with supercompetent *E. coli* strain SOLR, plated on X-gal-IPTG plates. Insertion of a cDNA clone into the polylinker region within the *lac Z* gene destroys the functioning of the *lac* Z gene resulting in white colonies. Colonies with functional *lac Z* gene remain blue and presence of only white colonies as shown in Plate 6 indicates *in vivo* excised clones came only from recombinants.

The 'Ex Assist' helper phage - SOLR strain system is very efficient in the sense that the problem associated with helper phage coinfection is eliminated. The Ex Assist helper phage contains an amber mutation that prevents replication, of the phage genome in non-suppressing *E. coli* strain SOLR. This allows only the excised phagemids to replicate in the host, removing the possibility of coinfection from the Ex Assist helper phage. Therefore, white colonies were further used for DNA isolation.

PLATE 5

a) Agarose gel electrophoresis of DNA purified from λZAP II clones.

Phage DNA from the positive clones was isolated and run on 0.7% agarose gel.

M - Marker, λ DNA digested with *Eco*RI + *Hin*dIII

Lane1 - λ ZAP DNA purified from CL 1

Lane2 - λ ZAP DNA purified from CL 2

Lane3 - λ ZAP DNA purified from CL 3

Lane4 - λ ZAP DNA purified from CL 4

Lane5 - λ ZAP DNA purified from CL 5

Lane6 - λ ZAP DNA purified from CL 6

Lane7 - λ ZAP DNA purified from CL 7

Lane8 - λ ZAP DNA purified from CL 8

b) Southern hybridization of restricted DNA from positive lambda clones

The gel was blotted onto Hybond N^+ membrane and hybridized with pea lectin cDNA probe as described in 'Materials and Methods'.

PLATE 6

Blue white screening of *in vivo* excised clones.

After transformation, cells were plated on Luria broth agar plates containing X-gal and IPTG.

White colonies are transformants of recombinant plasmids.

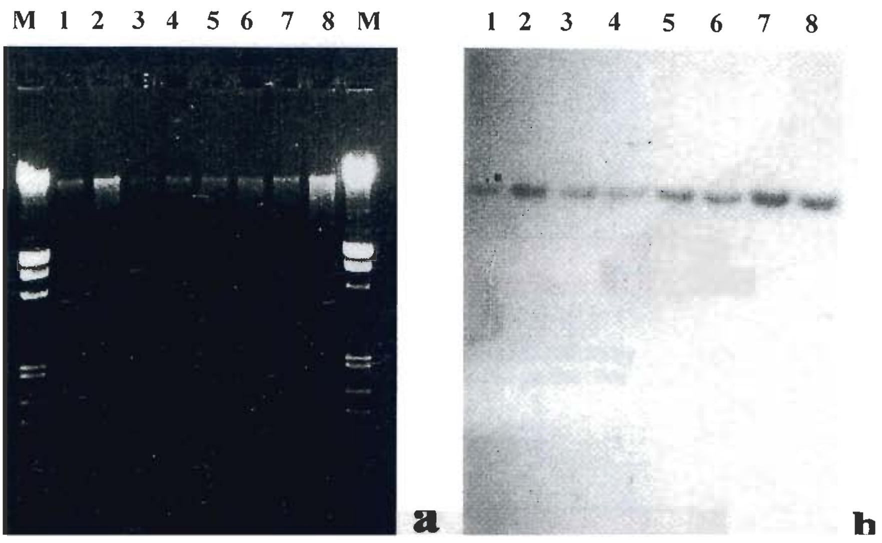


PLATE 5

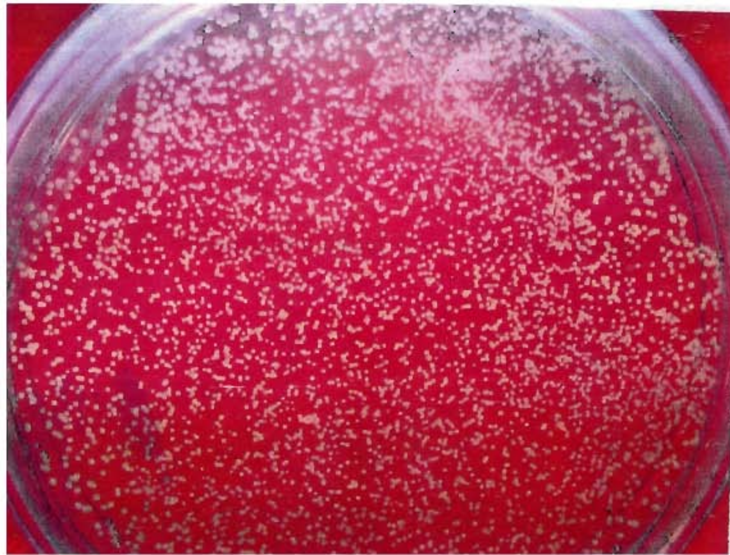


PLATE 6

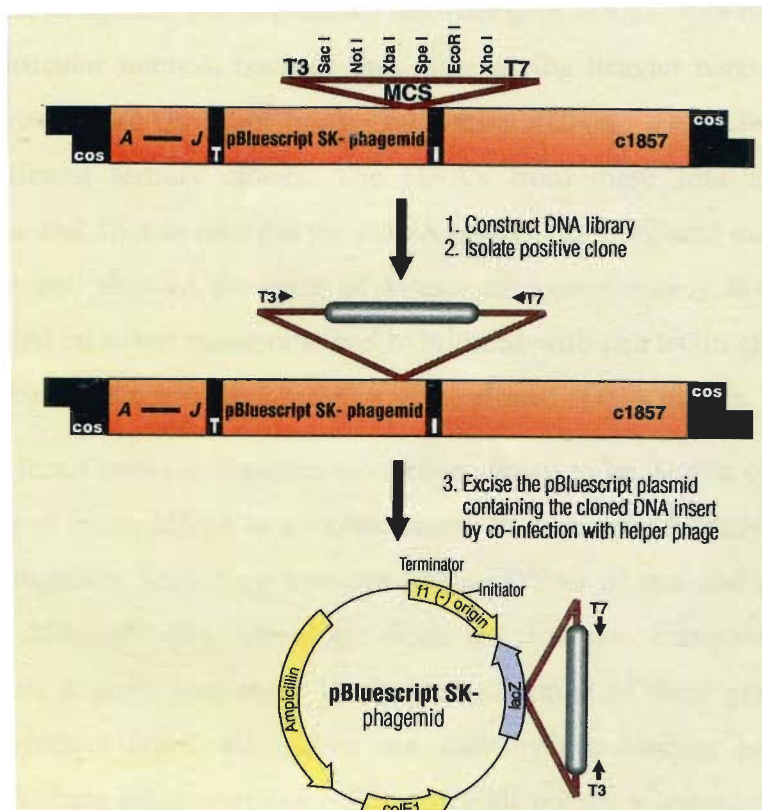


Figure 2 Summary of *in vivo* excision strategy.

4.7 Restriction of cowpea lectin plasmid DNA and confirmation of the cDNA inserts

The plasmid DNA was isolated from white colonies as described in 'Materials and Methods' and run in agarose gel to examine the quality of DNA. Out of these, four colonies were of particular interest, because they were giving heavier bands indicating presence of larger inserts, which might be the full-length cDNA. These four colonies were from four different tertiary clones. The DNA's from these four clones were restricted with *Eco*R1 and *Xho*I to take out the cDNA inserts.The restricted samples when separated in agarose gel, showed presence of inserts of approximately 800 bp (Plate 7a).The gel was blotted on nylon membrane and hybridized with pea lectin cDNA probe. Plate 7b shows the distinct hybridization patterns of the cloned cDNA inserts.

The seed lectin genes in legumes have been shown to be devoid of any intron and thus comparison of lectin cDNA is a viable means to search for homology in these genes. In this investigation, homology between lectin cDNAs of pea and cowpea has been established. Although this homology does not indicate complete sequence similarity, nevertheless it gives idea about the conserved nature of these genes through evolution. At the product level, all lectins are carbohydrate-binding proteins, the specificity for carbohydrate being variable. Thus they all posses a considerable no. of conserved residue which ultimately gives homology at gene level.

When the restricted genomic DNA of cowpea was blotted and hybridized to pea lectin cDNA probe, the autoradiogram showed variability, but not much variation of bands were found. In case of at least four restriction enzymes, the main band corresponded to the total genomic DNA. This might be due to the absence of sites for these enzymes in the coding region of the gene. During screening, initially a high number of clones were giving signal which was rather unusual, but through secondary and tertiary screening the number of positive clones came down to eight only. After *in vivo* excision when plasmid DNA was isolated, initially there was problem in getting a good DNA preparation. After several trials, the ratio of phage stock: helper phage: bacterial cells was standardized and thereafter pure DNA was obtained. The transformants when plated on LB-Amp agar plates, a large number of white colonies were obtained, from which one

PLATE 7

The recombinant clones were confirmed for the presence of insert by restricting them simultaneously with *Eco* RI and *Xho* I. The restricted samples were run on 0.7% agarose gel and hybridized with pea lectin cDNA probe.

a) Agarose gel electrophoresis of cowpea lectin cDNA clones digested with *Eco*RI & *Xho*I.

Lanes : **1** (pSK2), **2** (pSK4), **3** (pSK7), **4** (pSK8).

b) Southern hybridization of cowpea lectin cDNA clones of Fig.7a with pea lectin probe.

PLATE 8

ExoIII- SI deletion of pSK4.0.

The isolated cDNA clone pSK4.0 was restricted with *Kpn*I and *Hin*dIII, and sequentially deleted by Exonuclease III for different times. After religation, inserts were taken out by restricting with *Pst*I and *Bam*HI.

Lanes : M : Marker, λ DNA digested with *Eco*RI + *Hin*dIII
1-6 : Deletion for different time intervals.
1 (0 sec.), **2** (30 sec.), **3** (60 sec.), **4** (90 sec.), **5** (120 sec.), **6** (150 sec.).

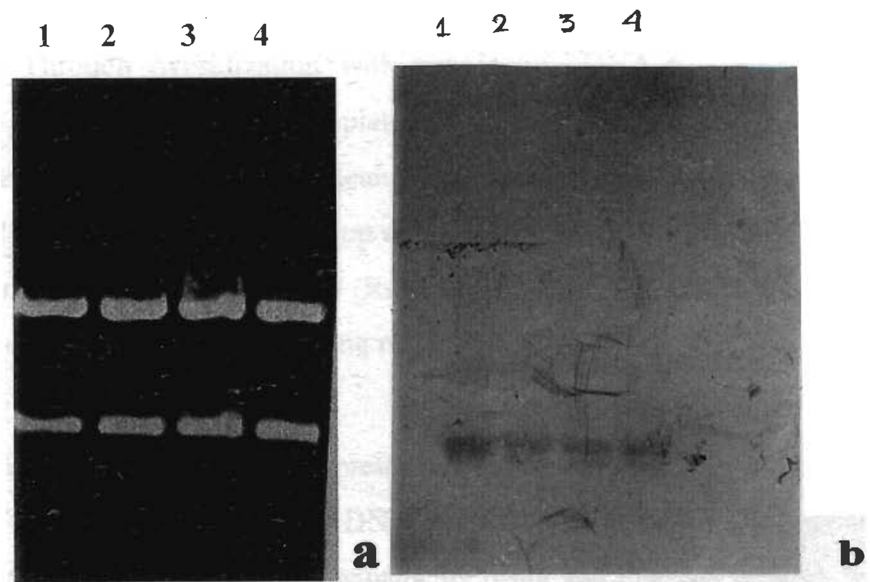


PLATE 7

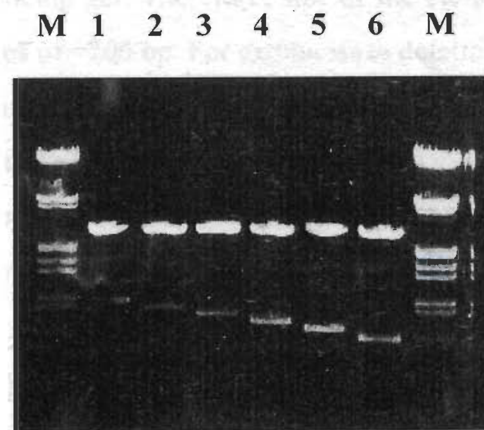


PLATE 8

from each plate was selected and DNA isolated and checked. Four of these clones appeared very promising when on restriction they showed presence of insert with an intense band in gel. Through hybridization with pea lectin cDNA this was further confirmed. The sizes of the clones are in complete agreement with other legume lectin cDNAs like pea (Gatehouse *et al.,* 1987), soyabean (Vodkin *et al.,* 1983) and french bean (Hoffman *et al.,* 1992). Cowpea lectin is made up of a mature protein of 225 amino acids with an approximate molecular weight of 25kD (Roberson *et al.,* 1983) and thus a cDNA of ~ 800 bp might be containing the whole coding region of cowpea lectin.

4.8 Sequencing of the lectin gene

All the four *in-vivo* excised clones showed presence of an insert of ~ 0.8Kb, therefore, one of he cDNA clone (pSK4.0) was selected for DNA sequencing. The quality, integrity and amount of DNA are critical parameters for any sequencing result and integrity of DNA was checked in agarose gel and was found to consist mostly of supercoiled DNA. This DNA was used for sequencing. The size of the insert was well above 500 bp, which is the usual limit that can be read on a sequencing gel. Therefore, size of the clones were reduced by ExoIII deletion to overlapping clones of ~200 bp. For exonuclease deletion it is absolutely necessary that the plasmid preparation has more than 80% supercoiled DNA, otherwise ExoIII digests double stranded DNA creating random nicks (Barnes *et.al.,*1983). ExoIII was preferred for deletion because it has a uniform rate of deletion compared to Bal31 or DNaseI (Putney *et.al.,*1981, Weiss 1976). When DNA from deleted clones digested with *Pst*I and *Bam*HI were run on 0.7% agarose gel showed uniform deletion of ~0.2 kb with the vector band appearing on the top (Plate8).

The deleted subclones were directly used for nucleotide sequencing. The sequences were aligned using "Megalign" program and the sequence obtained was found to consist of 776 nucleotides (Fig.3). Furtrher sequence analysis was done using "LASERGENE" program from DNASTAR* in a personal computer. When analyzed for the presence of open reading frames, the longest open reading frame was found to be between 1-675 nucleotides in the first reading frame (Fig.4). On translation this gives putative protein product having 224 amino acids (Fig.3) and the molecular weight was found to be 24.895 kD which fairly correlates with other legume lectins (Young and Oomen 1992). The total number of bases translated in the ORF is 675 with A+T (57.93%) and C+G (42.07%),

```
  1  ATG AAC TTC ATG TGC TGC ATA TTC TCG AAT GGT GGA TCG TGT CTA TCG
  1  Met Asn Phe Met Cys Cys Ile Phe Ser Asn Gly Gly Ser Cys Leu Ser

 49  ATT CCT ATC CGT CTC AGG AAC AAC ATC CTT CAA TCA GTC AAC GAC GGT
 17  Ile Pro Ile Arg Leu Arg Asn Asn Ile Leu Gln Ser Val Asn Asp Gly

 97  GAG TAT AAC TAC TTT CTT ACT CTT CTT CAA GTG AAT TCG GAT AGG TCA
 33  Glu Tyr Asn Tyr Phe Leu Thr Leu Leu Gln Val Asn Ser Asp Arg Ser

145  AAA CTA ACA GTT ACA CAT TAC TAC GAG CAA AAA ATG CTA TCA CAA ACT
 49  Lys Leu Thr Val Thr His Tyr Tyr Glu Gln Lys Met Leu Ser Gln Thr

193  AGC CTC TTC ACC ATT CTG CAA CTT CCA GGT CAT ACA CTT AAG CGA TTA
 65  Ser Leu Phe Thr Ile Leu Gln Leu Pro Gly His Thr Leu Lys Arg Leu

241  GTA ACT GTT CAA GGG TTT CCA AAA TGT CTA TCT CTT TTG CTG GGA CCC
 81  Val Thr Val Gln Gly Phe Pro Lys Cys Leu Ser Leu Leu Leu Gly Pro

289  CAT GCA GCT TTA TCA AAA GGG CAA AAC TCC AAA GCA ACA TTT TGG GTT
 97  His Ala Ala Leu Ser Lys Gly Gln Asn Ser Lys Ala Thr Phe Trp Val

337  TTT TTA TCA TAC TCT GGG CTT TTG AAA ACT CCG AGT ATC GCC CCC GGC
113  Phe Leu Ser Tyr Ser Gly Leu Leu Lys Thr Pro Ser Ile Ala Pro Gly

385  CCT CTC CCC TTT AGT TTC AAG GGG TCC GAT GAA AAA GGA AAA CCG TGG
129  Pro Leu Pro Phe Ser Phe Lys Gly Ser Asp Glu Lys Gly Lys Pro Trp

433  GCA ACC TTG GAA CTT TTT GGG CAA TTT TTG AAA AAA GTG GGG GAA GAA
145  Ala Thr Leu Glu Leu Phe Gly Gln Phe Leu Lys Lys Val Gly Glu Glu

481  AAA AAA TTA GAA ACA TTC CCC TTT TTC TCT ATC GGA GAT ATC GAT AGG
161  Lys Lys Leu Glu Thr Phe Pro Phe Phe Ser Ile Gly Asp Ile Asp Arg

529  TTA GCG ATG ATC GAC TTA TTA GAA CTT GAA GTT CAT TCC TTG GTC AGT
177  Leu Ala Met Ile Asp Leu Leu Glu Leu Glu Val His Ser Leu Val Ser

577  GTC AGC TTC TCA TTT GTG GTA CAG CCA TCT CCT TGG AAG CTT TAC GTA
193  Val Ser Phe Ser Phe Val Val Gln Pro Ser Pro Trp Lys Leu Tyr Val

625  TTC GTG GGC GGG GCT GAT CTT GGA GTT CAT GAA GCT AGT GGT TTC AGT
209  Phe Val Gly Gly Ala Asp Leu Gly Val His Glu Ala Ser Gly Phe Ser

673  TGA GTT CTC CTT GTT GTA ATC GTT TGT TGT TAA GTG AAT CTA TAG TAA
225  ***

721  CAT CGC AAT TAT CCT GAT CAT ATC GGA TTG AAC ACT ACC CAT ATT TGG

769  TTA TAA TT
```

Figure 3 Complete cDNA sequence and putative amino acid product of pSK4.0.

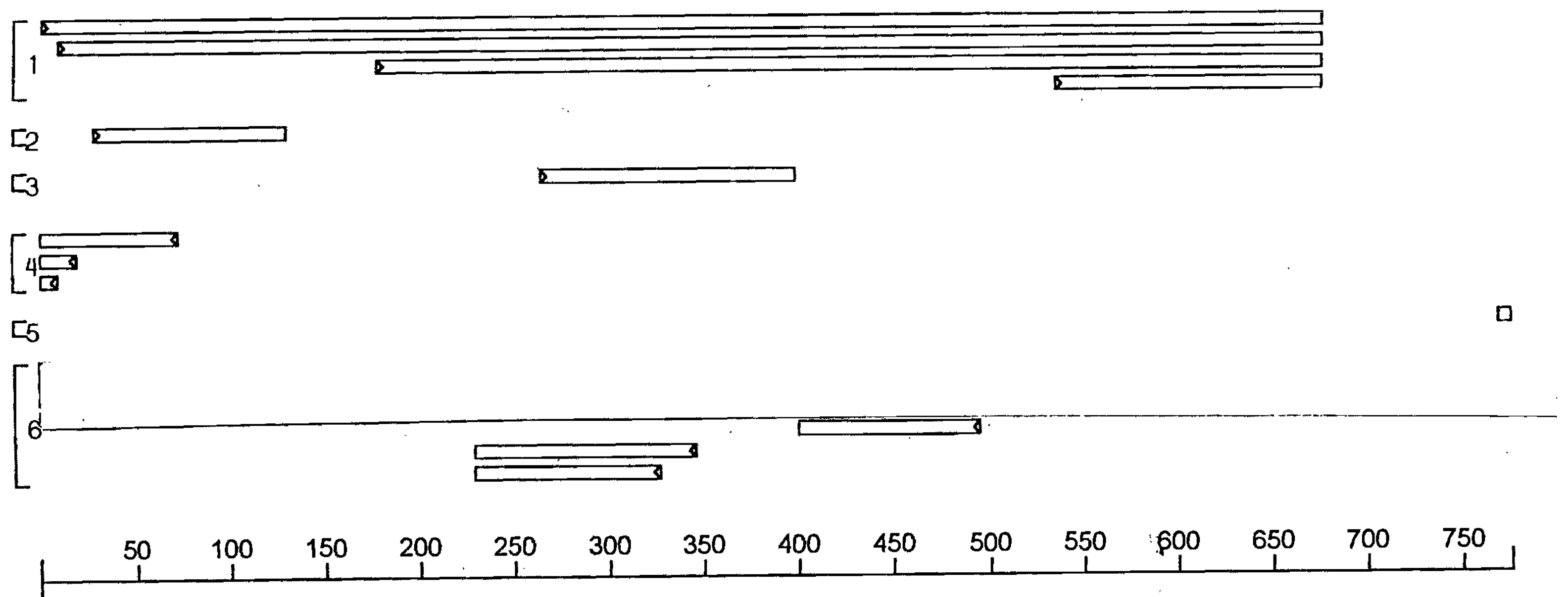


Figure 4 **Map of open reading frames (ORFs) of pSK4.0.**

which is in conformity with other legume lectin genes (Sharon and Lis,1990, Arango *et al.,*1992, Sharma and Surolia,1997). The nucleotide sequence is indexed in the EMBL Nucleotide Sequence Database under the accession number AJ277588.

The sequenced fragment shared considerable homology ranging from 83% to 92% with other legume lectin genes. *Medicago truncatula lec2* gene showed 86% homology. Though the sequence showed even 100% homology with *Medicago sativa lec2* gene, but this was with a relatively short stretch of sequence. This could be a consensus sequence in the sugar binding region. This also shared a homlogy of 83% with *Vicia cracca* lectin gene and 85% homology with the *Trifolium pratense* lectin gene. However maximum homology was found to be with *ps-lec* gene from *Pisum sativum* (Fig.5).

4.9 Transformation and regeneration of *Brassica juncea*

(i) *Plant materials and expalnts*

An efficient tissue culture system for high frequency plant regeneration from cultured tissue is a prerequisite for the success of plant transformation mediated by *Agrobacterium tumefaciens*. Although *Brassica* species in general are highly amenable to *Agrobacterium* mediated transformation and transformed *Brassica juncea* plants have been reported by several authors (Barfield and Pua 1991, Mathews *et al.,* 1990, Pental *et al.*, 1993), the transformation and regeneration frequency varies widely among cultivars. In *Brassica juncea* variety RLM 198, regeneration of complete plants with marker genes have been reported (Pental *et al.*, 1993). In the present investigation we report recovery of transgenic plants transformed with an insecticidal lectin gene isolated from cowpea.

Surafce sterilization of seeds with 0.1% mercuric chloride for 15 min followed by thorough washing in water was sufficient to eliminate any seed borne infection. Germination media did not have any plant hormones, and sucrose served as the carbon source. The cut in the hypocotyl explants made them highly susceptible to *Agrobacterium*. Introduction of NAA and BAP in the preculture media helped in better infection by *Agrobacterium*.

Hypocotyl explants of *Brassica juncea* are reported to be amenable to regeneration (Kirti and Chopra, 1989) and 0.5-1.0cm segments from top ¾ portion

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```

Figure 5 **Analysis of sequence homology of cowpea lectin cDNA with pea lectin gene.**

resulted in high callus induction and regeneration. Induction of BAP in the preculture medium was found to promote *Agrobacterium* infection.

(ii) *Agrobacterium mediated transformation*

The type of *Agrobacterium* strain used has been shown to affect the genetic transformation of several plant species (Moolney *et al.*, 1989, Zhan *et al.*, 1988). Previously Mathews *et al.* (1985) obtained transgenic shoots regenerated spontaneously from tumors of *Brassica juncea* transformed with wild type *Agrobacterium tumefaciens*, but induction of root formation was not successful. Similar results have also been reported in *Brassica napus* (Fry *et al.*, 1987) and *Brassica oleracea* (Srivastava *et al.*, 1988). Since the wildtype T-DNA caries the oncogenes carrying the phytohormone biosynthetic enzymes (Deblaere *et al.*, 1985), it probably causes an imbalance of phytohormones. This hormonal imbalance might be responsible for preventing transgenic shoots from root induction. In this study, *Agrobacterium* strain GV2260 carrying disarmed Ti vector was used which has earlier been successfully used in many transformation experiments (Lawrence and Koundal, 2000, Rathore and Chand, 1997). From the results it is evident that the *Agrobacterium* strain GV2260 is able to successfully transform *Brassica juncea* with good regeneration frequency.

Cultivar RLM198 has earlier been reported amenable to *Agrobacterium* mediated transformation (Pental *et al.*, 1993). Explants cocultivated with *Agrobacterium* strain GV 2260 carrying binary plasmid pSKL (Fig.6 and Plate 9) were put on modified MS media containing 250mg/l cefotaxime to kill the bacteria. All subsequent subculturing steps were also done in presence of cefotaxime to get rid of *Agrobacteria*.

(iii) *Selection of putative transformants and their regeneration*

After one week, explants had just starred callusing (Plate 10a) and were transferred to media having 20mg/l kanamycin along with 1mg/l NAA, 1mg/l BAP, 3.5mg/l $AgNO_3$ and 250mg/l cefotaxime. The calli resuming within one week were kanamycin resistant. These calli retained their green color even up to four weeks in this media (Plate 10b). The calli that were either completely white or white and purple were discarded. The green calli were transferred to shoot regeneration media. As reported by

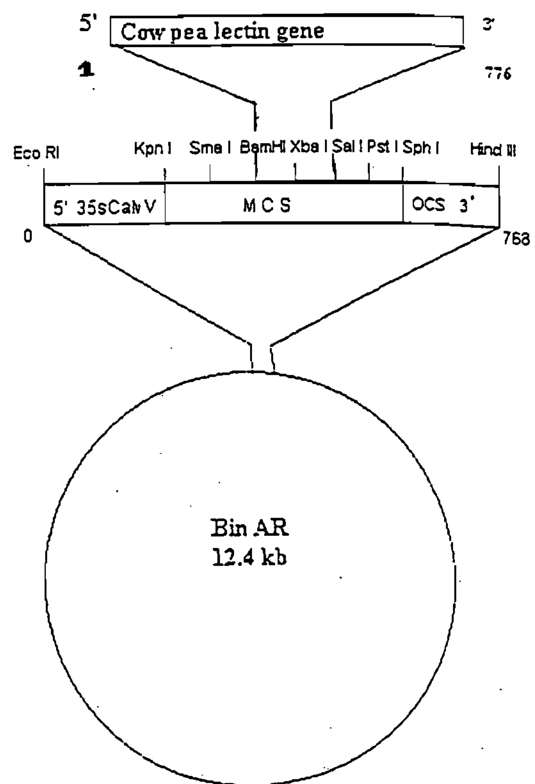


Figure 6 **Cowpea lectin cDNA cloned in pBinAR binary vector.**

PLATE 9

Agarose gel electrophoresis of restricted DNA from recombinant binary vector pSKL having cowpea lectin cDNA insert.

***Lanes* : M** - Marker, λ DNA digested with *Eco*RI + *Hin*dIII
1 - Unrestricted pSKL
2 - pSKL restricted with B*am* HI + *Sal* I
3 - cDNA insert eluted from restricted samples

PLATE 10

a) Precultured hypocotyl explants used for cocultivation.

b) Selection of green calli on MS + Kan selection medium.

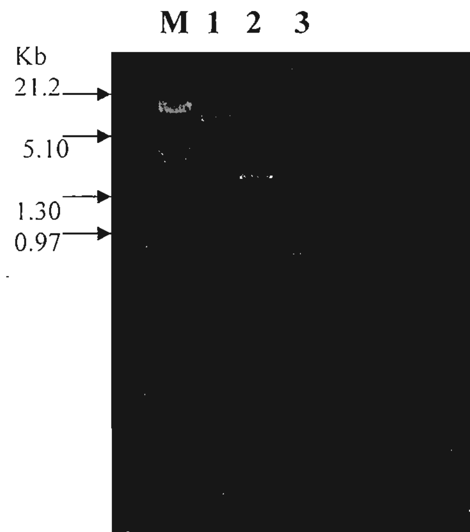
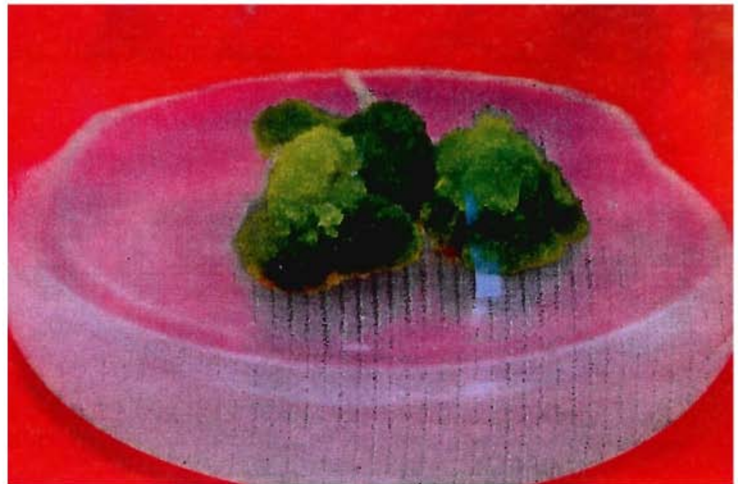


PLATE 9



a **b**

PLATE 10

other workers, introduction of $AgNO_3$ in media gave high frequency of shoot regeneration. Emerging shoots were visually screened and shoots that were either completely green or green with white patches were cut longitudinally and the green segments were subcultured (Plate 11a). Fourteen shooted calli obtained were put onto media with 2,4-D (0.1mg/l). After 3-4 weeks, shoots of 3-4 cm length were obtained (Plate 11b). The shoots were maintained in the media and after another 4-5 weeks leaves started emerging (Fig.11c). Well shooted plants with green leaves were rooted on medium with 2mg/l IBA and without kanamycin. Out of the 14 green shoots, only 5 of them developed root (Plate 11d).

Table 3 shows the frequency of callusing and shoot regeneration. Out of the 780 explants cultured, about 183 developed into green calli and callus induction ranged from 11% to 37% in different batches with a mean of 23.4%. Upon transfer to shoot regeneration media, out of the 183 green calli, only 14 regenerated well-developed shoots with an average regeneration frequency of 7.6%. The well shooted plants when transferred to root induction medium, only five plants developed roots with a rooting frequency of 35.7%.

Extensive studies carried out on the kanamycin tolerance of different explants of *Brassica juncea* have clearly demonstrated that 20mg/l kanamycin is sufficient to suppress shoot regeneration from untransformed explants (Mathews, 1988). Accordingly screening of transformants were done at 20mg/l kanamycin for co-cultured hypocotyl explants. Cefotaxime was added to the media to get rid of any *Agrobacterium* infection. The use of silver nitrate has been shown to be essential for high frequency shoot regeneartion from hypocotyl explants of *Brassica juncea* (Barfield and Pua, 1991). Silver nitrate is an inhibitor of ethylene, which is involved in the regulation of *de novo* shoot organogenesis (Chi *et al.*, 1991). In addition, the presence of silver nitrate greatly enhanced shoot regeneration from seedling explants of several recalcitrant genotypes of *Brassica juncea* (Chi *et al.*, 1991), although the mechanism of ethylene regulation in *in vitro* shoot regenerattion is not clear.

PLATE 11

a) Initiation of shoot bud emergence from transformed calli.

b) Shoot emergence from transformed plants.

c) Green leaves emerging from well shooted plants.

d) Emergence of roots from *in vitro* cultured plants.

a

b

c

d

PLATE 11

Table 3 : Frequency of transformation and regeneration from hypocotyl explants of *Brassica juncea*.

Sl.No	No of explants taken for co-cultivation	No of green calli on MS+Kan medium	Percentage of callus induction	No. of calli with green shoots	Percentage of shoot induction	No. of rooted transgenic plants	Percentage of root induction
1.	95	35	37.2	2	5.7	0	0
2.	107	30	28.4	3	10	1	33.3
3.	87	28	33.0	0	0	0	0
4.	96	23	24.1	1	4.3	0	0
5.	118	25	20.9	4	16	2	50
6.	93	13	13.6	2	15.3	1	50
7.	89	18	20.0	1	5.5	1	100
8.	95	11	11.0	1	9.0	0	0
Total	780	183	23.4%	14	7.6%	5	35.7%

4.10 Molecular analysis of transformants

(i) *Southern hybridization of transformed plants*

Southern blot analysis (Plate 12) shows that all *Brassica juncea* tarnsformants carry the transgene as evident from the presence of hybridization bands. Because the banding patterns of all the transgenic plants are different, it may be inferred that they have resulted from independent insertions. Only in two cases band sizes matched: a 21 kb band was present both in lane 1 and 5, and also presence of a 18kb band in both lane 4 and 5. The present study also shows integration of multiple copies of transgene into *Brassica juncea* genome. Evidence showed that up to 50 copies of T-DNA could be integrated into plant genome (Hebertle-Bors *et al.*, 1986). The presence of multiple copies of transgenes into plant genome is believed to be due to the replication and repair of the transgene during integration, or ligation of the replicated transgene prior to integration (Zambryski *et al.*, 1983). Our results indicate that multiple gene copies may have integrated at different loci in the genome. In *Lycopersicon esculentum* (Mc Cormick *et al.*, 1986) and *Arabidopsis thaliana* (Schmidt and Willmitzer, 1988), transgenes have been shown to be integrated at different chromosomal location.

(ii) *Northern analysis of transformed plants*

The RNA blot results (Plate 13) using the same probe revealed that the transgene expressed at least in three transformed plants. All the three transformed plants showed a signal at about 700 nt which fairly correlates with the mRNA size of cowpea lectin (Roberson and Strength 1983). Transgenic plants have been used as tools for studying regulation and organization of plant genes (Topfer *et al.*, 1987, Schell, 1987). However plants transformed with the same gene construct have been reported to vary considerably in gene expression pattern within and between plant species (Jain *et al.*, 1988). The present study also shows variation among individual transgenic plants of *Brassica juncea* in expression of cowpea lectin gene. The size of the transcript in all the three transformed plants was about 700 nt. The results clearly indicate that coding regions of legume lectins are fairly small and devoid of any introns. According to Vancanneyt *et al.* (1990), the quantitative control of foreign gene expression is regulated by its chromosomal location

PLATE 12

a) Agarose gel electrophoresis of restricted genomic DNA of *Brassica.*

Lanes M: Marker, λ DNA digested with *Eco*R1 and *Hind*III

C : Control, Genomic DNA from untransformed plants restricted with *Eco*R1

1-5: Genomic DNA from transformed plants restricted with *Eco*R1

b) Southern hybridization of genomic DNA of *Brassica* with cowpea lectin. probe

Lanes M : Marker, λ DNA

C : Control, (untransformed plant)

1-5: transformed plants

PLATE 13

a) Formaldehyde agarose gel electrophoresis of total RNA isolated from *in-vitro* cultured plants.

Lanes 1-5 : total RNA from transformed plants.

b) Northern Hybridization of total RNA from transformed plants with cowpea lectin cDNA probe.

Lanes 1-5 : total RNA from transformed plants.

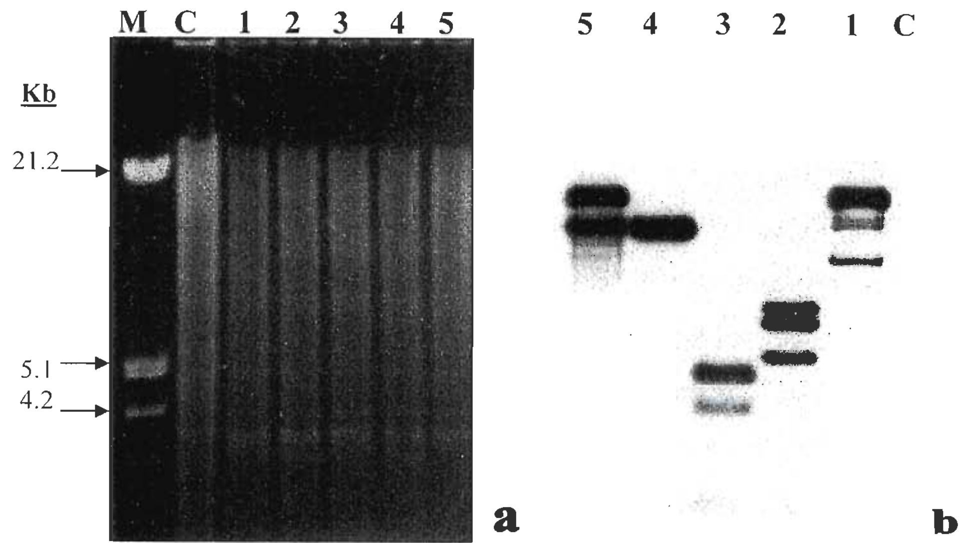


PLATE 12

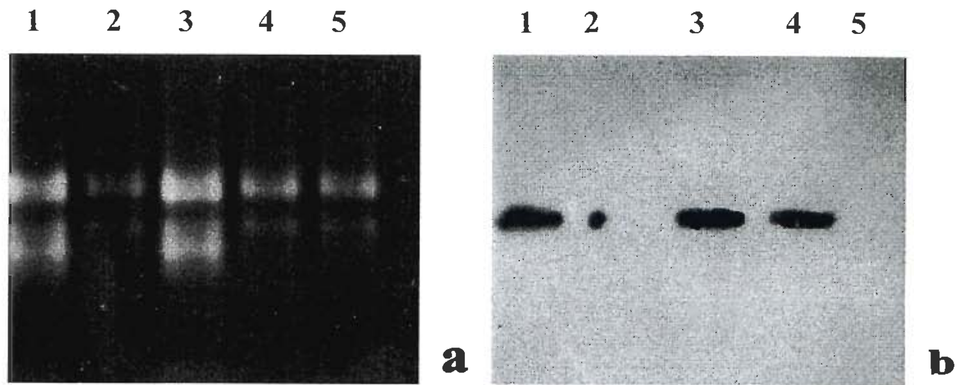


PLATE 13

and the surrounding DNA sequences. The transgene copy number, methylation pattern and the efficacy of the trans-acting factors in a heterologous system are also determinant of transgene expression. The variations in the present case may be due to one or combination of these factors, but exact cause of variation is not clear.

As the full sequence of the cowpea lectin gene is now known, this gene can now be used to construct several expression cassettes for plant transformation. This gene in combination with other insecticidal genes can be introduced into crops to produce stable homozygous lines with resistance to a wide range of insects (Chrispeels and Raikhel, 1991). Being of plant origin, lectin genes have high degree of compatability with the metabolic system of transgenic host plants and are expected to give sustained protection against sap sucking insects.The present study clearly demonstrates the efficient transfer of foreign genes into *Brassica juncea* using *Agrobacterium tumefaciens* mediated transformation. The present process of transformation is simple and rapid. Selection of appropriate explant and choice of proper *Agrobacterium tumefaciens* strain with suitable media and hormone combinations are the major determinants for the success of any transformation and regeneration system.The transfer of insecticidal lectin gene into *Brassica juncea* has great potential to reduce loss due to aphids. By introducing other insect resistance and agronomically important genes into major crops, the agricultural production and economic benefit for farmers can be increased.

5. SUMMARY AND CONCLUSIONS

The present study was undertaken to isolate and characterize the cDNA clones encoding lectins from cDNA library of cowpea with the aim to transfer them into *Brassica juncea* for conferring resistance against sap sucking insects like aphids. The cowpea genomic DNA was isolated, Southern blotted and hybridized with the heterologous pea lectin cDNA probe. Using the same probe, primary, secondary and tertiary screening of cowpea cDNA library were carried out to identify and ascertain the positive clones. Employing *in vivo* excision strategy eight clones were further subcloned in pBluescript and used to transform *E. coli* SOLR cells. The transformants were confirmed for the presence of lectin cDNA insert by rectriction and Southern hybridization. The positive clones, which hybridized strongly to pea lectin cDNA probe, were further analysed. Nucleotide sequencing was done by Sanger's di-deoxy chain termination method and sequence was analyzed using LASERGENE software in a personal computer. The lectin gene was cloned into pBinAR binary vector. The recombinant vector was transferred to *Brassica juncea* through *Agrobacterium mediated* transformation. In the present investigation, we report recovery of transgenic plants transformed with insecticidal lectin gene isolated from cDNA library of cowpea.

The main conclusions of the present study are summarized below:

1. Southern blot hybridization of cowpea genomic DNA with pea lectin cDNA probe indicated presence of lectin gene in cowpea genome. Hence, this heterologous probe was used to screen cowpea cDNA library for lectin clones.

2. Eight positive clones were obtained after primary secondary and tertiary screening of $\sim 2.0 \times 10^6$ plaques from the cowpea cDNA library using pea lectin cDNA probe.

3. From these recombinant clones phage DNA was isolated and when checked in agarose gel DNA was found to be intact and pure.

4. The eight positive clones were subcloned into pBluescript and recombinants selected through blue-white screening. White colonies were the true transformants.

5. The plasmid DNA was isolated from the transformants (white colonies) and run on the 0.7% agarose gel to test the quality of DNA.

6. Four of clones showed the presence of heavier inserts when their plasmid DNA was isolated and restricted with *Eco*R1 and *Xho*1 and analysed on 0.7% agarose gel.

7. Cowpea lectin cDNA fragments of all the four clones strongly hybridized with the pea lectin cDNA probe.

8. The clone pSK4.0 was completely sequenced by Sanger's di-deoxy method and the coding sequence comprises 675 nucleotides.

9. The cowpea lectin protein is made up of 224 amino acids with a molecular weight of 24895 D.

10. The lectin gene from cowpea shares considearble homology ranging from 83% to 92% with other legume lectin genes. Maximum homology was found to be with the *ps-lec* gene from *Pisum sativum*. The fragment also possessed a short stretch of conserved sequence in the sugar-binding region.

11. A recombinant binary vector pSKL based on pBinAR was constructed having cowpea lectin cDNA fragment.

12. The recombinant vector was introduced into hypocotyl explants of *Brassica juncea* cultivar RLM 198 through *A. tumefaciens* mediated genetic transformation and the *A. tumefaciens* strain GV 2260 gave high frequency of transformation.

13. To suppress shoot growth from non-transformed plants, 20mg/l kanamycin was sufficient. Inclusion of silver nitrate in the media promoted shoot regeneration and medium containing 2mg/l IBA gave high frequency of root induction in transformed plants.

14. The Southern hybridization results of the putative transformants showed that the transformation resulted in multiple copy and multi-location integration of lectin transgene into *Brassica juncea* genome.
15. The northern analysis showed that the regenerated transgenic plants varied among them in expression of lectin gene.

The present study clearly demonstrates that it is possible to isolate genes of eukaryotic organisms by screening the complementary DNA libraries with suitable radioactive probes (homologus or heterologus). The comparative study of cDNAs with corresponding genomic DNAs will be helpful to understand the structural organization and functional properties of plant lectin genes. Detailed characterization with complete DNA sequencing has established the homology of lectin genes among legumes. It is now established beyond doubt that legume lectins are encoded by relatively smaller genes with no intervening sequences. These informations are essential before attempting genetic manipulation of these genes for generating insect resistant transgenic plants.

The studies with genetic transformation showed the efficient transfer of foreign genes into *Brassica juncea* using *Agrobacterium tumefaciens* mediated transformation. The present process of transformation is simple and rapid. Selection of appropriate explant and choice of proper *Agrobacterium tumefaciens* strain with suitable media and hormone combinations are the major determinants for the success of any transformation and regeneration system. The transfer of insecticidal lectin gene into *Brassica juncea* has great potential to reduce loss due to aphids. This gene in combination with other insect resistance and agronomically important genes can be introduced into crops to produce stable homozygous lines, which are expected to provide sustained economic benefit.

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APPENDIX

List of the Buffers and Solutions

Denaturing solution

NaOH	0.4 N
NaCl	0.6 M

Depurination solution

0.25 N	HCl

DNA extraction Buffer

Tris-Cl	100 mM
EDTA	50 mM
NaCl	500 mM
DTT	150 μg/ml

GTE Solution

Glucose	50 mM
Tris-Cl (pH 8.0)	25 mM
EDTA	10 mM
Lysozyme	2 mg/ml

Neutralization solution

NaCl	1.5 M
Tris-Cl (pH 7.5)	0.5 M

NZY medium/litre

NaCl	5 g
$MgSO_4.7H_2O$	2 g
Yeast extract	5 g
Casein hydrolysate	10 g
Agar	15 g
pH	7.5

Prehybridization buffer

SSC	5X
Denhardt's solution	2.5X
Sodium phosphate buffer (pH 7.0)	0.05 M
Salmon sperm DNA	100 μg/ml

Scintillation mixture/litre

PPO	4 g
POPOP	100 mg
Toluene	1 litre

SM buffer

NaCl	100 mM
$MgSO_4$	10 mM
Tris-Cl (pH 7.4)	20 mM
Gelatin	0.1%

20 X SSC/ litre

NaCl	175.3 g
Sodium citrate	88.2 g
pH	7.0

STE buffer

NaCl	100 mM
Tris-Cl (pH 8.0)	10 mM
EDTA	1 mM

TAE buffer 50 X (per litre)

Tris-Base	242 g

Glacial Acetic acid	57.1 ml
0.5 M EDTA (pH-8.0)	100 ml

TB/10 ml

Bactotryptone	1.0 g
NaCl	0.05 g
pH	7.4

TBE buffer

Tris-Borate 0.5 X	45 mM
EDTA (pH 8.0)	1 mM

TE buffer

Tris-Cl (pH 8.0)	10 mM
EDTA	1 mM

Terrific Broth/Litre

Bactotryptone	12 g
Bacto yeast extract	24 g
Glycerol	4 ml

10X ExoIII Buffer

Tris-Cl	0.66mM
$MgCl_2$	6.60mM

10X Ligase Buffer

Tris-Cl (pH 7.6)	0.5mM
$MgCl_2$	100mM
DTT	100mM
BSA	500μg/ml

S1 Nuclease Mix

Deionised water	172μl
7.4X S1 buffer	27μl
S1 Nuclease	60 U

YEM agar (1 litre)

$MgSO_4$, $7H_2O$	0.2g
KH_2PO_4	0.5g
NaCl	0.1g
Yeast extract	1.0g
Mannitol	10g
Agar	15g

MS media (1 litre)

I. Macro elements	
NH_4N0_3	1650mg
KNO_3	1900mg
$MgSO_4$, $7H_2O$	370mg
KH_2PO_4	170mg
II. $CaCl_2$,$2H_2O$	440mg
III. Micro elements	
KI	0.83mg
H_3BO_3	6.3mg
$MnSO_4$, $4H_2O$	22.3mg
$ZnSO_4$, $7H_2O$	8.6mg
Na_2MoO_4, $2H_2O$	0.025mg
IV. Copper cobalt	
$CuSO_4$, $5H_2O$	0.025mg
$CoCl_2$, $6H_2O$	0.025mg
V. Iron-EDTA	
$FeSO_4$, $7H_2O$	27.8mg
Na_2EDTA, $2H_2O$	37.3mg

VI. Organic			**Antibiotic stocks**
Myoinositol	10.0mg	Ampicilin	100µg/µl in sterile water
Nicotinic acid	0.5mg	Cefotaxime	400µg/µl in sterile water
Pyridoxin-HCL	0.5mg	Kanamycin	100µg/µl in sterile water
Thymine-HCL	0.1mg	Rifampicin	50µg/µl in methanol
Glycine	2.0mg	Tetracyclin	25µg/µl in 70% ethanol

Plant Growth Regulators

IAA/ BAP	2mg/ml in dilute NaOH

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Advisory Committee Members	:	Dr. Srinivasan Dr. S.K.Mishra Dr. B.D.Kaushik
Title of the Thesis	:	**Isolation, characterization and transformation studies with lectin genes from cowpea (*Vigna unguiculata* L.Walp.)**

ABSTRACT

The cDNA library of cowpea constructed in λ ZAP II vector was screened for the presence of lectin genes using pea lectin cDNA probe. Initially, to demonstrate the presence of lectin genes in the cowpea genome, cowpea total genomic DNA was isolated, Southern blotted and hybridized with the heterologous pea lectin cDNA probe. After primary, secondary and tertiary screening, eight positive clones were obtained. When subcloned into pBluescript, out of these eight clones, only four showed presence of heavier of cDNA inserts. In order to confirm the homology, when the DNA of these positive clones was Southern blotted and hybridized, they showed strong hybridization with the probe DNA. One of the clones was sequenced completely and the nucleotide sequence showed strong homology with other legume lectin genes. The cowpea lectin cDNA was cloned into pBinAR binary vector and this was introduced into *Brassica juncea* cultivar RLM 198 through *Agrobacterium* mediated genetic transformation. From hypocotyl explants, five transgenic plants were regenerated. Calli resistant to 20mg/l kanamycin were regenerated and shooting frequencies ranged from 4% to 16%. MS media supplemented with 1mg/l BAP and 0.1mg/l 2,4-D gave high frequency of shoot regeneration. Inclusion of silver nitrate in the media promoted the regeneration frequency. For rooting of regenerated shoots, MS media with 2mg/l IBA was used. Genomic DNAs from well-rooted plants were isolated and on Southern hybridization with cowpea lectin gene, showed presence of transgenes into *B. juncea*. Northern blot results showed variable expression of the transgenes.

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