"CLONING AND CHARACTERIZATION OF PROTEASE INHIBITOR GENE IN COTTON"

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DISSERTATION

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2009

DEDICATED TO
MY BELOVED PARENTS

CANDIDATE'S DECLARATION

I hereby declare that this dissertation or part thereof has not been previously submitted by me for the degree to any other University.

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CERTIFICATE-I

This is to certify that Mr. ASHISH KUMAR *has satisfactorily prosecuted his course and research for a period of not less than four semesters and that the dissertation* **"CLONING AND CHARACTERIZATION OF PROTEASE INHIBITOR GENE IN COTTON"** *submitted by him is the result of original research work and is of the award of the degree of MASTER OF SCIENCE (Agriculture) in the subject of AGRICULTURAL BIOTECHNOLOGY. I also certify that the dissertation or part thereof has not been previously submitted by him for a degree of any university.*

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CERTIFICATE - II

This is to certify that the dissertation entitled **"CLONING AND CHARACTERIZATION OF PROTEASE INHIBITOR GENE IN COTTON"** submitted by **Mr. ASHISH KUMAR** to the Marathwada Agricultural University, Parbhani in partial fulfillment of the requirement for the degree of ***MASTER OF SCIENCE (Agriculture)*** in the subject of **AGRICULTURAL BOTANY (Agril.Biotechnology)** has been approved by the student's advisory committee after oral examination in collaboration with the external examiner.

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ABBREVIATIONS

A	- Adenine
bp	- Base Pairs
C	- Cytosine
$CaCl_2$	- Calcium chloride
DNA	- Deoxyribose Nucleic Acid
dNTP	- Deoxyribose Nucleotide Triose Phosphate
EDTA	- Ethylene Diamine Tetra Acetic Acid
et al.	- et alia (and other)
G	- Guanine
hr	- Hour
IPTG	- Isopropyl-beta-thio galactopyranoside
kb	- Kilobase pair
LB	- Luria Bertani
$MgCl_2$	- Magnesium chloride
min	- Minutes
ml	- milliliter
mM	- Milli Molar
ng	- Nano gram
^{O}C	- Degree Celsius
PCR	- Polymerase Chain Reaction
pH	- log H ion concentration
PIs	- Protease Inhibitors
PVP	- Polyvinyl Pyrrolidon
RNAse	- Ribonuclease
rpm	- revolutions per minute
SDS	- Sodium deodecile sulphate
T	- Thymine
Taq	- *Thermus aqaticus* DNA polymerase
TBE	- Tris Borate EDTA
Tris	- Tri-(Hydroxy Methyl)- amino methane
Tris-Hcl	- Tris –Hydrochloride
U	- Uracile
U	- Units
UV	- Ultra Violet
X-gal	- 5-Bromo-4-chloro-Indoly-β-D-Galactoside
μg	- Microgram
μl	- Microliter
μM	- Micro Molar

INTRODUCTION

Chapter-I

INTRODUCTION

After food and shelter cloth is the third primary need for human beings. Because of providing this need to human beings cotton "***the white gold***" enjoys the predominant among all cash crops. In India, after agriculture textile industry is the second largest employment generative sector and cotton is the chief in providing raw material for this industry. So cotton is not only an agricultural crop it is also a socio-economic crops. Cotton plays a key role in the national economy in terms of both employment generation and foreign exchange earning. Cotton impacts the lives of an estimated 60 million people in India, including approx. 5.5 mill cotton producing farmers. By the way of export foreign exchange earning of cotton is about 30%(Rs. 7600 crore) of the total foreign earning of the country (Khadi *et al*. 2007).

In the world major cotton growing countries are China, India, USA, Pakistan, Uzbekistan and Brazil. India ranks first and second in area under cotton production respectively. India has largest cotton area in the world about 94.4lakh ha. accounting one fourth of the global cotton produce (16-18% of global cotton produce). India rank second in cotton production after China approx.5.35 MM tons lint verses China which has 7.91 MM tons and USA accounts cotton production of approx. 4.1-4.7MM tons in 2007.In India cotton productivity is 553 kg lint per ha. Now cotton production in India rose to 310 lakh bales in 2007. In India major states growing cotton are Maharashtra, Gujarat and AP. Maharashtra has larger area under cotton about 32-34 lakh ha

followed by Gujarat 17-19 lakh ha and AP 10-12 lakh ha with production of 45 lakh bales, in Gujarat followed by Maharashtra which is 25 lakh bales.

The cotton plant is deciduous, indeterminate perennial plant of family Malvaceae and genus *Gossypium* is native to sub-tropical climate. *Gossypium* consist of 45-50 species with 40-45 diploids (2n= 26) and five being allotetraploid (2n=52), designated 'A'5 through 'G' and 'K' on the basis of chromosomal pairing affinities. At tetraploid level, there are five species designated $(AD)_1$ through $(AD)_5$ for their genome constitutions. There are four cultivated species of *Gossypium* including two allotetraploid viz *G. hirsutum* and *G.barbadense* with genomic content of 2n=4x=52, called American cotton or new world cotton, and two diploid viz *G. herbaceum* and *G. arboreum* called old world or Asiatic cotton with genomic content of 2n=2x=26. *G. hirsutum* and *G.barbadense* have been domesticated independently through out the world. The most widely grown worldwide species is *G. hirsutum*, which is grown over 95% of the world cotton ha followed by *G.barbadense* upland cotton. *G. hirsutum* is native to Mexico and Pima. Egyptian, *G.barbadense* is native to South America. India is an exception to most countries with only 30% of its cotton production planted to *G. hirsutum*, 17% planted to *G. arboreum* 8% to *G. herbaceous* and remaining area under intraspecific and interspecific hybrids.

As cotton is commercially important crop, it is also affected by number of insect-pest and diseases, which not only cause large damage to cotton fiber quality but also to production as well. Major diseases affecting the cotton plant are seedling diseases; leaf spot and root rot etc.

Seedling diseases can be caused by several fungi commonly *Pythium and Rhizoctonia.* Leaf spot in cotton is caused by *Alternaria macrospora.* It is a major disease in all cotton growing areas. There are many insects, which damages to cotton by variety of ways by damaging leaf and more by damaging boll of cotton. The most important insect orders damaging the cotton are lepidoptera, coleopteran and dipteran. Bollworm is the most important pest of cotton. The world wide damage due to insect pest attack accounts for 15% despite of use of the use insecticides which represents over US $100 bill (Krattiger, 1997). By Gujar *et al.* (2000), the minimum loss were caused by sucking pest approx.4.6% where as bollworm cause loss about 51.6 %. Among these *H. armegera* causes US $ 290-350 mill worth of damage every year in India Globally 4.5% crop production lost due to lepidopteron insect (ISAAA, 2003). Average yield loss in cotton due to insect pest ranges from 50-60% (Dhaliwal *et al.*2004).

To overcome these problems farmer have only one choice to use chemical pesticides. The one important aspects of use of pesticides is non-specificity and broad range effect on insects. The cost of these pesticides is so high that majority of farmers cannot afford. The main drawback of these pesticides is the development of resistance in insects against these chemicals. Resistance strains can arise rapidly, killing susceptible populations and resistance populations being raised next year. Using these pesticides other biota's e.g birds, mammals also affected. The exclusive use of these pesticides not only results in rapid build up of resistance to such compounds but there nonselectivity affect the balance between natural predators and is generally in favor of pests (Metcalf, 1986).

The majority of proteinase inhibitors studied in the plant kingdom originate from three main families, namely *Leguminosae, Solanaceae,* and *Gramineae*. Plant proteinase inhibitors are well known to play a potent defensive role against predators and pathogens. Diverse endogenous functions for these proteins have already been proposed, ranging from regulators of endogenous proteinases to storage proteins, but evidence for many of these roles is partial or confined to isolated examples(Lawrence and Koundal, 2002; Birk, 2003). In addition, many plant protease inhibitors have been shown to act as defensive compounds against insects by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has accumulated consistently (Lawrence and Koundal, 2002). The role and mechanism of action for most of these inhibitors have been, or are being, studied in detail, and their respective genes have been isolated. These genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programs (Lawrence and Koundal, 2002). Given the number of pesticidal proteins involved in host plant defense, effective pest control by this strategy will presumably result from the co-expression of numerous determinants, each of which could be custom engineered by directed molecular evolution to maximize its effectiveness against specific pests. Protease inhibitor gene is classified into serine, cystein, aspartate and metalloprotease inhibitor depending upon type of protease they inhibit. Plant PIs are mostly abundant in storage organs like seeds tubers (Melville and Ryan, 1970) and endosperm (Svendsen *et al.* 1980,Richardson, 1991). These gene accumulate on attack by herbivorous and pathogens. These defensive genes are found on either

constitutive component in various plant tissue (Rhodes 1979, Mauricio *et al.*1997) or synthesized in response to attack by pest and pathogen (Fossom.1970, Green and Ryan, 1972 Carroll *et al.*1980). To date more than fourteen plant protease inhibitor genes have gene have been isolated and introduced in many crops.

The term protease refers to both endopeptidaes and exopeptidase. Exopeptidaes cleave the peptide bond proximal to amino or carboxyl termini of the substrate while endopeptidaes cleave peptide bond distance form termini. The plant protease inhibitors are endopeptidaes (Barrett, 1987 Ryan, 1990). The one property of plant defensive gene is their attack on different sites. The possible role of protease inhibitor gene in plant protection was first investigated in 1947 when Mickle and Standish observed that the larvae were unable to develop normally on soybean products.

Development of transgenic crop has come a long way from the first transgenic development (Hilder *et al.,* 1987). Many of these protease inhibitors are rich in cystein and lysine contributing to better and enhanced nutritive quality (Ryan, 1989). Protease inhibitor genes have practical advantage over genes encoding for complex pathways ie. they transfer single defensive gene from one plant species to another species and expressing them from own wound inducible or constitutive promoters thereby imparting resistance against insect pest (Boulter, 1993). This was first demonstrated by Hilder *et al.* in 1987 by transferring trypsin inhibitor gene from *Vigna unguiculata to tobacco* which conferred resistance to wide range of lepidopteron insect Protease inhibitor also exhibit a very broad spectrum of activity including suppression of pathogenic nematode (Williamson and Hussy, 1996)

inhibition of spore germination and mycelium growth of *Alterneria alternata* (Dunaevskii *et al.*1997).

These advances make protease inhibitor an ideal choice to be used in developing transgenic crop resistance to insect pest. Among different types of inhibitors, serine inhibitors PIs are exclusively studied because they are ubiquitous in plant and most lepidopteron pest like *H.armegera* and *S. exigna* depend upon serine proteases for food digestion. Many insects possess serine proteases to digest their food. Serine including trypsin chymotrypsin is the main intestinal digestive enzyme responsible for hydrolysis of food proteins. They hydrolyze peptide bond in which the carboxyl group is contributed by lysine and arginine residue (Brown *et al.* 1997). The most concerned plant protease inhibitor is serine type from potato, soybean and tomato. The potato and tomato protease inhibitor are most widely used and well characterized inhibitor and exhibit broad- spectrum activity Potato inhibitor-II family has been shown to inhibit serine proteases such as chymotrypsin, trypsin subtilisin, oryzin and elastase (Pearce *et al.*1991). So Protease inhibitors are highly specific and act as insect retardants. Protease inhibitor affect insect by reducing their capacity to assimilate plant protein and reduce insect feeding and lead them to starvation. So, the defensive capacities of PIs relay on inhibition of protease present in insect guts or secreted by microorganism, causing protease inhibitor using reduction in availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002).

Modern plant genetic engineering involves different technologies to transfer these genes to any crop. Plant biotechnology attempt to identify candidate gene for genetically modifying and enhancing traits in

all cultivated cotton plant. The present aim is to isolate and characterize traits, more specifically, genes responsible producing protease inhibitor in plants.

In present investigation the following objectives were set up to isolate, characterize and clone the protease inhibitor gene(s)-

1- Primer designing based on the gene sequence available in the public domain by using FastPCR.
2- Primer base amplification of Protease inhibitor gene from total genomic DNA of cotton (*G.hirsutum* L.)
3- Characterization and cloning of amplified fragment into appropriate vector.
4- Transformation of vector to host bacteria (DH5 alpha) and selection of transformants.

REVIEW OF LITERATURE

Chapter-II

REVIEW OF LITERATURE

'Protease' is an enzyme that catalyzes the hydrolysis of protein into its component amino acids and 'Inhibitor' that stops or slows down chemical reaction. So Protease inhibitor (PIs) is antimetabolic protein that interferes with digestion process of insects. It is one of the defense strategies existing in plant against predators. These defense chemicals in plant tissue are both developmentally regulated or induced in response to insect and pathogen attack. Proteins that form complexes with protease and inhibit their proteolytic activity, these inhibitors are widely distributed among different plant system. Read and Haas (1938) reported that Kunitz confirmed the presence of protease particularly in legume seeds and their biochemistry as protein molecules from soybean (Kunitz, 1945).

One of the recent developments in the field of plant genetic engineering is the manipulation of plant for disease and insect resistance. In an afford to develop insect resistance crop plant, the role of plant derived PIs was recognized early by first ever transgenic tobacco plant were reported by Hilder *et al.*(1987),using cowpea trypsin inhibitor cDNA clones. Richardson (1991) documented that PIs are the largest class of protein that have undergone extensive investigation and consequently their structural properties, function and metabolism. Jouanin *et al.* (1998) reported that occurrence classification

physiological significance effect of PIs on the digestive physiology of animals and role of the PIs as defensive proteins in plants against insect and microorganism.

Connors *et al.* (2002) studied and characterized PIs present in the leguminaceae, graminae and solanaceae.

2.1 Plant Protease Inhibitors

The possible role of protease inhibitors (PIs) in plant protection was investigated as early as 1947 when, Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products. Lipke *et al.* (1954) reported that the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum.* Hilder *et al.* (1987) first demonstrated by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including lepidopteron, such as Heliothis and Spodoptera, coleopterans such as Diabrotica, Anthonomnous and orthoptera such as Locusts. Ryan,(1989) reported many of protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality Further there is no evidence that it had toxic or deleterious effects on mammals.

Ryan (1990) gave the term protease includes both "endopeptidases" and "exopeptidases" whereas, the term "proteinase" is used to describe only "endopeptidases. Richardson (1991) predicted that majority of proteinase inhibitors studied in plant kingdom originates from three main families namely leguminosae, solanaceae and gramineae. These protease inhibitor genes have practical advantages over genes encoding for complex pathways. Boulter (1993) by

transferring single defensive gene from one plant species to another and expressing them from their own wound inducible or constitutive promoters thereby imparting resistance against insect pests. Williamson and Hussey (1996), showed protease inhibitors also exhibit a very broad spectrum of activity including suppression of pathogenic nematodes like *Globodera tabaccum, G. pallida,* and *Meloidogyne incognita* by CpTi. Dunaevskii *et al.* (1997) shows inhibition of spore germination and mycelium growth of *Alternaria alternata* by buckwheat trypsin/chymotrypsin. Joshi *et al.* 1998 shows cysteine PIs from pearl millet inhibit growth of many pathogenic fungi including *Trichoderma reesei.*

Koiwa *et al.* (1997) showed these inhibitor families that have been found are specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their "reaction center" are classified as serine, cysteine, aspartic and metallo-proteases. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems and the use of plants as factories for the production of heterologous proteins, Following these early studies, there have been many examples of protease inhibitors active against certain insect species, Pannetier *et al.* (1997) Koiwa *et al*, (1998) showed both in *in vitro* assays against insect gut proteases and in *in vivo* artificial diet bioassays (Urwin *et al.* 1997; Vain *et al.* 1998).). Several non-homologous families of proteinase

inhibitors are recognized among the animal, microorganisms and plant kingdom.

Joshi *et al.* (1998) reported cysteine PIs from pearl millet inhibits growth of many pathogenic fungi including *Trichoderma reesei*. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems and the use of plants as factories for the production of heterologous proteins Sardana *et al.* (1998).

2.2 General Properties of Plant PIs

Studies on the biosynthesis of several plant PIs demonstrated these PIs are synthesized as either prepro-proteins (Graham *et al.*, 1985a) or pre-proteins that are processed in vivo either during or after synthesis to produce the native PIs.

Richardson, (1991) reported plant PIs usually have a high content of cysteine residues that form disulfide bridges and confer resistance to heat, extremes in pH, and proteolysis (Richardson, 1991).Many PIs are produced in response to various stress conditions, e.g. pathogens, insects, wounding, and environmental stresses such as salt (Koiwa et al., 1997).

Hung *et al.,* (2003) reported plant PIs vary from 4 to 85 kDa, with the majority in the range of 8 to 20 kDa. For example, a trypsin inhibitor (BCTI) with molecular weight of 8 kDa was purified from seeds of *Brassica campestris*. The BCTI was found to be a thermostable Bowman-Birk type TI that inhibits trypsin at the molar ratio 1:1. The stability of BCTI is apparently related to the presence of the disulfide bridge (Hung *et al.,* 2003).

2.3 Families of inhibitors

Laskowski and Katol (1980) reported that PI are widely spread in the plant kingdom and their classification based on amino acid sequence homology, reactive site assignment and inhibitory mechanism Ryan *et al* (1981,1990) showed the role of PIs, seems to be control of endogenous protease during seed dormancy and protection against proteolytic enzyme present in the pest digestive system. These features have encouraged the development of pest resistance based on PIs expression in transgenic plant. There is no any other specific classification of PIs due to their great diversity in terms of source, structure, specificities and size. However Garcia *et al* (1987) grouped them in to four categories viz serine, cysteine metello and aspartate protease inhibitor. Among them potato inhibitor-I and II families, Brown-birk protease inhibitor family (BBI), and soybean trypsin inhibitor (Kunitz) family are very important due to their inhibitory potential on gut enzymes of major crops pests. Member of serine and cysteine protease inhibitor families have been more relevant to the area of plant defense.

2.4 Families of Plant Protease Inhibitor

2.4.1 Serine protease inhibitors

Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteinases is enormous. Serine proteinase inhibitors have been reported from a variety of plant sources and are the most-studied class of proteinase inhibitors. The role of serine PIs as defensive compounds against predators is particularly well established, since the major proteinases present in plants, used for processes such as protein mobilization in storage tissues, contain a cysteine residue as the catalytically active nucleophile in the enzyme active site.

Green and Ryan (1972) first reported that trypsin inhibitor (serine protease inhibitor) played a part in the defenses of plant against pest and pathogen. These authors demonstrated that leaves of potato and tomato accumulated proteinase inhibitor when mechanically wounded or chewed by insects. It is largest and the most widespread super family of PIs.

Laskowski and Kato (1980) reported that all serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism. Shulke and Murdock (1983) and Gatehouse and Boulter (1983) and observed that trypsin inhibitors at 10% of the diet were toxic to larvae of the *Manduca sexta* and *Callosobruchus maculatus* respectively. Serine proteases have been identified in extract from the digestive tract of insects of various orders.

Shulke and Murdock, (1983) Applebaum, (1985) showed that serine proteases inhibitors have anti-nutritional effects against several lepidopteron insects. Applebaum, (1985) reported that the order lepidoptera, which includes a number of crop pests, the pH optima of the guts are in the alkaline range of 9-11, where, serine proteinases and metallo-exopeptidases are most active.

Broadway and Duffey (1986a) compared the effects of purified SBTI and potato inhibitor II (an inhibitor of both trypsin and chymotrypsin) on the growth and digestive physiology of larvae of *Heliothis zea* and *Spodoptera exigua* and demonstrated that growth of larvae was inhibited at levels of 10% of the proteins in their diet. Garciaolmedo *et al.* (1987) reported that the serine classes of proteinases such as trypsin, chymotrypsin and elastase, which belong to a common protein super family, are responsible for the initial digestion of proteins in the gut of higher animals. *In vivo* they are used to cleave long, essentially intact polypeptide chains into short peptides, which are then acted upon by exopeptidases to generate amino acids, the end products of protein digestion.

Huber and Carrell, (1989) studies have provided a basic understanding of the mechanism of action that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well. Serine proteinases have been identified in extracts from the digestive tracts of insects from many families; particularly those of lepidoptera (Houseman *et al.* 1989) and many of these enzymes are inhibited by proteinase inhibitors. In contrast, a major role for serine PIs in animals is to block the activity of endogenous proteinases in tissues where this activity would be harmful,

as in case of pancreatic trypsin inhibitors found in mammals. In vivo they are used to cleave long, essentially intact polypeptide chains into short peptides, which are then acted upon by exopeptidases to generate amino acids, the end products of protein digestion.

Ryan (1990) reported that of digestive serine proteases are distinguished based on their specificity, trypsin specifically cleaving the C-terminal to residues carrying a basic side chain (Lys, Arg), chymotrypsin showing a preference for cleaving C-terminal to residues carrying a large hydrophobic side chain (Phe, Tyr, Leu), and elastase showing a preference for cleaving C-terminal to residues carrying a small neutral side chain (Ala, Gly). Inhibitors of these serine proteinases have been described in many plant species, and are universal throughout the plant kingdom, with trypsin inhibitors being the most common type. At least, part of this bias can be accounted for by the fact that (mammalian) trypsin is readily available and is the easiest of all the proteinases to assay using synthetic substrates, and hence is used in screening procedures. Because of these reasons the members of the serine class of proteinases have been the subjects of intense research than any other class of proteinase inhibitors.

Richardson (1991) reported that there are eight families of plant protease inhibitor viz, Bowmam-Birk, Kunitz, Potato I and II, cucurbit,cereal superfamily, ragi AI and Thaumatin-PR like families. Ravichandaran *et al.* (1999) and Mukhopadhyay (2000) showed recent X-ray crystallography structure of winged bean, Psophocarpus tetragonolobus Kunitz-type double headed alpha-chymotrypsin shows 12 anti-parallel beta strands joined in a form of beta trefoil with two reactive site regions (Asn 38-Leu 43 and Gln 63-Phe 68) at the external

loops. Structural analysis of the Indian finger millet (*Eleusine coracana*) bifunctional inhibitor of alpha-amylase/trypsin with 122 amino acids has shown five disulphide bridges and a trypsin-binding loop (Gourinath *et al.* 2000). These structural analyses would greatly help in "enzyme engineering" of the native PIs to a potent form, against the target pest species than the native PIs. Plant serpins have molecular mass of 39 - 43 kDa, with amino acid and nucleotide homology with other well-characterized serpins. Majority of serpins inhibit serine proteases.

Huntington *et al.* (2000) reported that serpins are irreversible 'suicide inhibitors'. The cleavage of an appropriate peptide bond in the active center loop of the inhibitor triggers a rapid conformational change so that catalysis does not proceed beyond the formation of an acyl-enzyme complex. Irving *et al.*(2002b,c) showed that two oat (*Avena sativa*) serpins have specificity for chymotrypsin and / or elastase, and another one has specificity for trypsin and chymotrypsin at overlapping loop sites.

Ligoxygakis *et al.*(2003), studied that squash serpin Cmps-1 also inhibits two overlapping sites. Plant serpins have been shown to inhibit model trypsinlike proteins Roberts *et al.*(2003), but there are no obvious targets for these inhibitors in plants, which may apparently be involved in inhibiting proteases of plant pathogens Hejgaard, (2005). Wieczorek and his coworkers have showed an inverse correlation between the up regulations of squash phloem serpin-1 (cmps) and aphid survival. Law *et al.* (2006), Vercammen *et al.* (2006) shows that the serpin 1 of Arabidopsis has been shown to act on metacaspase-like proteins *in vivo* and play a role in the plant immune response. It has been suggested that

rather than directly interacting with pathogens, plant serpins may have a role in the complex pathways involved in up-regulating the host immune response.

2.4.2 Cysteine protease inhibitors

Rele *et al.*(1980) reported cysteine protease inhibitor have been detected seeds of plant of all botanical family from gymnosperm to angiosperms. Cysteine proteases have been isolated from variety of sources namely plants, animals and microbes. The cysteine super family is composed of several families and includes proteins that are related in structure and function to an inhibitor of cysteine protease, the members of this family inhibit the activity of cysteine protease and are called cysteine PIs or cystatins. Abe and Arai (1985) reported rice cysteine protease inhibitors are the most studied of all cysteine protease inhibitors, which is proteineous in nature.

Barrett (1987) and Turk and Bode (1991) grouped these inhibitors into four families based on sequence relationships, molecular mass and disulfide-bond numbers and arrangements as cystatin, stafin kininogen and phytocystatins super families. Turk and Bode (1991) also reported that advances in enzymology has revealed the existence of a variety of cysteine protease resulting in their classification into several families namely Papain, clapin and asparagines. Cystanins have also been characterized from potato (Waldron *et al.* 1993), ragweed (Rogers *et al.* 1993), cowpea (Fernandes *et al.* 1993), papaya (Song *et al.* 1995) and avacado (Kimura *et al.* 1995).

2.4.2.1 Family-1 cystatins (stefin family)

Machleidt *et al.* (1983) and Stato *et al.* (1990) studied that the members of this group have a molecular mass of about 11 kDa. They are generally present in the cytosol and are devoid of any carbohydrate groups and disulfide bonds.

2.4.2.2 Family-2 cystatins (cystatin family)

These inhibitors consist of proteins having 120 – 126 amino acids and the molecular mass of 13.4 - 14.4 kDa. They also contain a signal sequence and are known to be secreted Abrahanson *et al.* (1987), Machleidt *et al.* (1983); Turk *et al.*(1997), reported these inhibitors contain two disulphide bonds but are devoid of any carbohydrate groups.

Grzonka *et al.* (2001) showed all the family-2 cystatin inhibitors contain a conserved tripeptide of sequence Phe-Ala-Val near the C-terminus and a conserved dipeptide, Phe-Tyr, near the N- terminus. These conserved sequences are important in binding to the target proteases.

2.4.2.3 Family-3 cystatins (kininogen family)

These inhibitors are glycoproteins and are of three different types. High Molecular Weight kininogens (HMW) with a molecular mass of 120 kDa and Low Molecular Weight kininogens (LMW) with molecular mass ranging between 60 and 80 kDa are known. A third type T kininogens with molecular mass of 68 kDa has also been reported. These proteins contain tandem domains that result from gene duplication of the family–2 cystatins.

2.4.2.4 Family–4 cystatins (phytocystatins)

Plant cystatins or phytocystatins are the second most-studied class of inhibitors and have been identified and characterized from several plants, viz., cowpea, potato cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, and Job's tears. Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants include those of sunflower, rice, wheat, maize, soybean, and sugarcane (Kuroda *et al.* 2001; Connors *et al.* 2002).

This family includes nearly all the cysteine PIs described in plants. They have been identified in rice Abe *et al.* (1987 a, b), soybean Hines *et al.* (1991) maize Abe *et al.* (1992), and several other monocotyledonous and dicotyledonous plants. Phytocystatins have sequence similarity to stefins and cystatins, but do not contain free cysteine residues reported by Fernandes *et al.* (1993). The unique feature of this superfamily, however, is a highly conserved region of the G58 residue, the glu-val-val-ala-gly(QVVAG) motif and a pro-trp (PW) motif. The studies on the papain inhibitory activity of oryzacystatin and its various truncated forms have identified the conserved QVVAG motif as a primary region of interaction between the inhibitor and its cognate enzyme.

Phytocystatins, on the basis of protein structure, can be divided into two groups. Walsh and Strictland (1993); and Bolter (1993) showed that one group contains multiple-domains and includes the cysteine PIs isolated from potato tubers and tomato leaves and another consists of single domain proteins and includes a majority of these inhibitors (Pernas *et al.*1998).

Of cysteine protease inhibitor from plant sources the rice cysteine proteinase inhibitors (Oryza cystatins) are the most studied of all the cysteine PIs, which is proteineous in nature. These are low molecular weight compound involved in biodefence of rice seeds.

Following is a partial chronological survey plant cystatins.

Rodis and Hoff (1984) showed that potato tuber contains papain inhibitors, which are present in the form of protein crystals with mass of 80kD, and inhibits also chymopapain and ficin. Abe *et al.*(1987) isolated a cDNA clone for oryzacystatin from a cDNA library of rice immature seeds. They obtain a full-length clone, which encode 102 amino acid residues. He also transcribed the gene for oryzacystatin into a single mRNA species of 700 nucleiotide. Abe *et al.*(1988) also expressed oryzacystatin cDNA and truncated fragments in *E.coli.*

Oliva *et al.*(1988) purified a cysteine inhibitor from *Enterolobium contortisiliquum* a beans. The inhibitor, with a single polypeptide chain with a molecular mass of about 60kD, inhibits papain and bromelain and is active in pH range 2-10 and at temp up to 60°C.

Zimacheva *et al.* (1988) reported a cysteine protease inhibitor of 6kD mass isolated form seeds of pumpkin. Kondo *et al.*(1989) reported the cloning and sequence analysis of genomic DNA fragment encoding oryzacystatin. Kondo *et al.*(1990) investigated two distinct cystatin species oryzacystatin-I and II in rice seed with different potencies and specifications of inhibition against cysteine inhibitor and in expression pattern of their mRNA in ripening stage of rice seeds. They isolated cDNA clones for oryzacystatin-II in rice seeds by screening with oryzacystatin-I cDNA probe. The newly isolated cDNA clone encodes

107 amino acid residues. Kondo *et al.*(1991) showed that he gene organization of Oryza cystatin-II is closely related to that of oryza cystatin-I, but different from those of animal origin.

Arai *et al.* (1991) showed that the Papain inhibitory activity of Oryza cystatin depends on the central Gln55-Val54-Ala56-Gln57 region conserved among cystatin super-family members. Abe *et al.*(1991) described two Oryza cystatins I and II that occur in mature seeds of rice. These inhibitors are highly homologous to each other and are significantly homologous to cystatin super family member of animal origin.

Abe *et al.* (1992) performed molecular cloning and expression studies of corn (maize) kernel cysteine protease inhibitor as a novel cystatin super-family member of plant origin. The protein named corn (maize) cystatin-I, is considered to be the member of the cystatin family since it contain commonly conserved Gln-Val-Val-Ala-Gly region that exist in most known cystatin. Abe *et al.*(1996) described the structural organization of the gene encoding maize cystatin.

Nagata *et al.* (2000), showed recent three dimensional structure analysis of Oryza cystatin OC-I by Tanokura's group using NMR has showed a well defined main body consisting of amino acids from Glu 13 - Asp 97 and an alpha helix with five stranded anti parallel beta-sheet, while the N terminus (Ser 2-Val 12) and C terminus (Ala 98-Ala 102) are less defined.

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2.5 Aspartic and metallo-protease inhibitors

Knowledge on the role of aspartic proteinases in insect digestion is limited than that of cysteine proteinases. Plants have also evolved at least two families of metallo-proteinase inhibitor.

Rancour and Ryan (1968) reported the metallo-carboxypeptidase inhibitor family in potato. Hass *et al.* (1975) reported that metallo-carboxypeptidase inhibitor families are small peptide inhibitor consisting molecular mass of about 4.2kD and 38-39 amino acid residues. Keilova and Tomasek, (1976) studied a Cathepsin D inhibitor family in potatoes.

Graham and Ryan (1981) reported that inhibitor also accumulates in potato leaf tissues along with inhibitor I and II proteins in response to wounding. Thus the inhibitors accumulated in the wounded leaf tissues of potato have the capacity to inhibit all the five major digestive enzymes i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects reported Hollander-Czytko *et al.* (1985).

Houseman and Downe (1983) reported that in species of six families of the order Hemiptera, aspartic proteases (cathepsin D-like proteinases) were found along with cysteine proteinases).

Havkioja and Neuvonen (1985) showed that the inhibitors of the metallo-carboxypeptidase from tissue of tomato and potato are polypeptides (4 kDa) that strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from yeast and plants. Potato tubers possess an aspartic proteinase inhibitor, cathepsin D (Mares *et al.* 1989)

that shares considerable amino acid sequence identity with the trypsin inhibitor SBTI from soybeans. The cathepsin D inhibitor (27 kDa) is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, rennin or cathepsin E. The inhibitor is found in tissues of potato tubers where it accumulates during tuber development along with potato inhibitor I and II families of serine proteinase inhibitor. The detailed structural analysis of prophytepsin, a zymogen of barley aspartic proteinase shows pepsin like bilobe and a plant specific domain. The N terminal has 13 amino acids necessary for inactivation of the mature phytepsin (Kervinen *et al.* 1999), and the aspartic PI cardosin A from cardoon shows regions of glycolylations at Asn-67 and Asn 257. The Arg-Gly-Asp sequences recogonizes the cardosin receptor, which is found in a loop between two-beta, strands on the molecular surface (Frazao *et al.* 1999). Aspartic PIs have been recently been isolated from barley (Kervinen *et al.* 1999) and cardoon (*Cyanara cardunculus*) flowers named as cardosin A (Frazao *et al.* 1999). The detailed structural analysis of prophytepsin, a zymogen of barley aspartic proteinase shows pepsin like bilobe and a plant specific domain.

Kervinen *et al.* (1999) reported that the N terminal has 13 amino acids necessary for inactivation of the mature phytepsin, and the aspartic PI cardosin A from cardoon shows regions of glycolylations at Asn-67 and Asn 257. The Arg-Gly-Asp sequences recogonizes the cardosin receptor, which is found in a loop between two-beta, strands on the molecular surface (Frazao *et al.* 1999). Park *et al.*, (2000); Lawrence and Koundal, (2002) described aspartyl PIs in sunflower, barley and cardoon (*Cynara cardunculus*) flowers and in potato tubers. The cathepsin D

inhibitor an aspartyl PI described in potato tubers shares considerable aminoacid sequence homology with soybean trypsin inhibitor. It is a 27 kDa protein and inhibits serine proteases, trypsin and chymotrypsin in addition to the aspartyl protease, cathepsin D, but does not inhibit pepsin, cathepsin E and rennin, which are all aspartyl proteases Lawrence and Koundal, (2002).

2.6 Other families of Plant Protease Inhibitor

2.6.1 Bowman Birk inhibitors (BBIs) family

On the basis of sequence homology, this forms another family of serine PIs. The family is named after D.E. Bowman and Y. Birk. Bowman (1946); Birk *et al.*(1963) who were first to identify and characterized a member of this family from soybean. The soybean inhibitor is now the most well studied member of this and is often referred as the classic BBI. BBIs have been classified on the basis of their structural feature and inhibitor characteristics. The inhibitor from dicotyledonous plant consists of a single polypeptide chain with molecular mass of 8kDa. These are double headed with two homologous domains each bearing a separate reactive site for cognate proteases. Odani and Ikenaka (1973a) reported that inhibitor consist of two tandem homology regions on the same polypeptide chain each with a reactive inhibitory site. Bode and Hubr (1992) reported that the BBI family of protease inhibitors contains a unique disulfide-linked nine-residue loop that adopts a characteristic canonical conformation. Chen *et al.* (1992); Lin *at al.* (1993) reported that the active site configuration in these inhibitors is stabilized by the presence of seven conserved disulfide

bonds. Lee and Lin (1995) observed that the loop is called protease-binding loop and binds the protease in a substrate-like manner.

BBIs from monocotyledons plant are of two types. One group consist a single polypeptide chain withy a molecular mass of 8kDa and have a single reactive site. Prakash *et al.*(1996) reported that other group has a molecular mass of 16kDa and consist two reactive site. Tanaka *et al.*(1997) Laing and McManus (2002) reported that the inhibitors have been found in legumes and cereals. Kennedy (1998) shows that soybean derived BBI with a well-characterized ability to inhibit trypsin and chymotrypsin is particularly effective in suppressing carcinogenesis in a variety of in vivo and in vitro systems. Qi *et al.* (2005) shows that the first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase.

Lin *et al.*(2006) reported that BBIs are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocot and dicot species they have been shown to act as anticarcinogenic compounds.

2.6.2 Potato inhibitor family

Large numbers of proteases have been isolated from potatoes and related plants. Potatoes inhibitor account for up to 25% of the soluble protein of the potato tuber. They exhibit a variety of inhibitor of serine endopeptidase, metallo carboxlypeptidase, papain, microbial protease and kallikreins with specificity of inhibition varying from 4 to 40kDa.The potato protease inhibitor that has been purified and characterized fall into three main categories viz potato inhibitor-I, II and carboxyl peptidase inhibitor.

2.6.3 Potato inhibitor-I

The inhibitor of this family is widespread in plant and have described min many species including potato tubers (Ryan and Balls, 1962), tomato fruit (Margossian *et al.*1988 and, Wingate *et al.*, 1989) and in tomato leave in response to wounding Lee *at al.*(1986). These inhibitors are nonnumeric with molecular weight of about 8 to 10kDa. This inhibitor family also referred to as chymotrypsin inhibitor-1 because its specificity is directed most strongly towards chymotrypsin although it inhibits subtilisin proteases as well as some other alkaline microbial proteases and it is also a weak inhibitor of trypsin. The inhibitor belonging to this family is noncovalent tetramer of four different subunits. Each of these protomers has a molecular wt of 10kDa and is comprised of a single chain with a small intrachain disulphide bridge. The amino acid sequence of four protomers display extensive homology with each other.

2.6.4 Potato inhibitor-II

Members of this group have been reported mainly from the members of Solanaceae family. Iwasaki *et al.*(1971); Pearce *et al.*(1993) reported that these inhibitor have been found in leaves,flower,fruit and phloem of other solanaceae family. The inhibitor –II has molecular weight of 20kD and is composed of dimer of four different promoters, which are not homologue to promoter that makes up to inhibitor-I. Each dimer inhibits two molecules of chymotrypsin, indicating that each of the subunit has a binding site for chymotrypsin. Antcheva *et al.*(1996) reported that Inhibitors in this family been known to inhibit

chymotrypsin, trypsin,elastase, oryzin, pronase E and subtilisin. Christeller and Liang,(2005) initially characterized it from potato tubers.

2.6.5 Carboxypeptidase Inhibitor

The first report on a polypeptide from potato a tuber that specifically inhibits the pancreatic metallo-carboxypeptidase came from C.A. Ryan laboratory in 1968, (Rancour and Ryan 1968) reported that the carboxypeptidase inhibitor has a molecular weight of 4.3kD. They showed that it inhibits potently and competitively mammalian pancreatic carboxypeptidase A and B from various animals and other metallo-carboxypeptidase from organs or fluids of animals and microorganism.

Hass *et al.*(1979) studied that CPI composed of three isoinhibitor, of which isoinhibitor CPI-II is the predominant species and CPI-I and CPI-III are minor isoinhibitor.The amino acid analysis of the isoinhibitor reveals that they contain neither met nor leu, and CPI-I lakes arg as well. CPI-I differs from CPI-II in amino acid sequence at two positions. CPI-III is identical to CPI-II except that it lakes the amino-terminal pyrollidone carboxylic acid and the adjacent glu residue.

Hass and Hermondson (1981) reported that six residue of half cysteine of CPI are found as three disulphide bonds (cys8-cys24, cys12-cys27and cys18-cys34). The three-dimentional structure of CPI-II, deduced from the X-ray diffraction analysis of the inhibitor-carboxypeptidase A complex, indicate that CPI-II has neither helical structure nor β-pleated sheets. The X-ray crystallography analysis also demonstrates that the inhibitor binds like an extended substrate and that the carboxyl terminal peptide val38-gly39 is cleaved in the enzyme inhibitor complex. The gly39 appears to be trapped in the binding

pocket of the enzyme, indicating that this residue is not required for enzyme inhibitor interaction (Hass and Hermondson 1981).

2.6.6 Squash inhibitor family

Squash-family inhibitors have been described only in plants and form yet another active against serine proteases. Hara *et al.*(1989) reported that these inhibitor have three disulphide bridges and fold in a novel knottin structure. Hojima *et al.*(1982); McWherter *et al.*(1989) reported that the small size of these inhibitors combined with potential activity against important biological molecules such as Hageman factor, human leukocyte elastase and cathepsinG, has made them particularly attractive for studying proteinase and inhibitor interactions.

Heitz *et al.* (2001); reported that the member of this family consist of a small single peptide containing between 28 and 30 amino acids with molecular mass of 3-3.5 kDa.

Lee and Lin (1995) and Felizmenio *et al.*(2001) reported that the member of this family have been described from many cucurbit families. Seven serine PIs belonging of this family have been isolated and characterized from seeds of wild cucumber (*Cyclanthera pedata*) (Kuroda *et al.* 2001).

Thaimattan *et al.* (2002) predicted the structure of squash inhibitors, and inhibitor and proteinase complexes have been determined by X-ray crystallography and NMR spectroscopy and showed that this inhibitor follows a standard mechanism of inhibition. Atiwetin *et al.* (2006) reported two different but inter-convertible (cis-trans isomer) inhibitors have been isolated and characterized from seeds of wax guard (*Benincasa hispida*).

Chiche *et al.* (2004) first introduced the squash inhibitor, a well-established family of highly potent canonical serine proteinase inhibitors isolated from Cucurbitaceae. The squash inhibitors were among the first discovered proteins with the typical knottin fold shared by numerous peptides extracted from plants, animals, and fungi.

2.6.7 Kunitz family

On the basis of sequence homologies Kunitz-type inhibitors form a separate family. The first plant protease inhibitor to be isolated and characterized was Kunitz soybean trypsin inhibitor (KTI). The purification, crystallization, kinetics of interaction and complex formation of KTI with trypsin (Kunitz 1947a, b) comprises a major landmark in the study of protein protease inhibitors. The numerous studies on KTI concerning specificity, stability, and physical kinetic and other properties have been complied and summarized by Birk (1976). KTI consist of two 181 amino acid residue and includes two disulphide bridges with one reactive site and molecular mass of 18-22 kDa.

Ishikawa *et al.*(1994) Laskowski and Kato,(1980) reported that the inhibitor in this family are widespread in plant and have been found describe in legumes, cereals and in solanaceous species.

Laing and McManus (2002) Park *et al.* (2005) studied that the member of this family are mostly active against serine proteases, but may also inhibit other proteases as trypsin chymotrypsin and subtilisin.

Park *et al.* (2005) Ledoigt *et al.*(2006) founded that kunitz type PIs are also produced under stress, and have been found in potato tubers. Wang and Ng (2006) reported a 20.5kDa kunitz-type trypsin inhibitor

with antifungal activity from the roots of punce ginseng (*Pseudostellaria heterophylla).*

2.7 Mechanism of toxicity

The mechanism of action of these proteinase inhibitors has been a subject of intense investigation. Knowledge on mechanisms of protease action and their regulation *in vitro, and in vivo*, in animals, plants, microorganisms and more recently in viruses have contributed to many practical applications for inhibitor proteins in medicine and agriculture.

Applebaum (1985) showed that the secretion of proteases has been attributed to two mechanisms, involving either a direct effect of food components (proteins) on the midgut epithelial cells, or a hormonal effect triggered by food consumption.

Brovosky (1986) Birk and Applebaum (1960), proposed models for the synthesis and release of proteolytic enzymes in the midguts of insects which reveal that ingested food proteins trigger the synthesis and release of enzymes from the posterior midgut epithelial cells. The enzymes are released from membrane-associated forms and sequestered in vesicles that are in turn associated with the cytoskeleton. Peptidases are secreted into the ectoperitrophic space between the epithelium, as a particulate complex from where the proteases move transversely into the lumen of the gut, where the food proteins are degraded. PIs inhibit the protease activity of these enzymes and reduce the quantity of proteins that can be digested, and also cause hyper-production of the digestive enzymes which enhances the loss of sulfur amino acids as a result of which, the insects become weak with stunted growth and ultimately die. The digestive proteolytic enzymes in the different orders of

commercially important insect pests belong to one of the major classes of proteinases predominantly.Coleopteran and hemipteran species tend to utilize cysteine proteinases (Murdock *et al.*1987) while lepidopteran, hymenopteran, orthopteran and dipteran species mainly use serine proteinases reported by Ryan, (1990); Wolfson and Murdock (1990).

2.8 Regulation of protease inhibitors

Plant protease inhibitor proteins that are known to accumulate in response to wounding have been well characterized. Earlier research on tomato inhibitors has shown that the protease inhibitor initiation factor (PIIF), triggered by wounding/injury switches on the cascade of events leading to the synthesis of these inhibitor proteins (Melville and Ryan, 1972; Bryant *et al.* 1976), and the newly synthesized PIs are primarily cytosolic (Hobday *et al.* 1973; Meige *et al.* 1976).

Delaney *et al.* (1994) reported that wound induction and pathogen defense pathways overlap considerably. Expression of wound and jasmonic acid inducible genes can be positively and negatively regulated by ethylene or salicylic acid (SA), both of which are components of the pathogen-induced signaling pathway Transgenic plants expressing prosystemin antisense cDNA exhibited a substantial reduction in systemic induction of PI synthesis, and reduced capacity to resist insect attack McGurl *et al.* (1994).

Malone and Alarcon, (1995) reported that there are four systemic signals responsible for the translocation of the wound response, which includes systemin, abscisic acid (ABA), hydraulic signals (variation potentials) and electrical signals.

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Hunt *et al.* (1996) reported that plants sometimes specifically forego one type of defense response for another. Salicylic acid (SA) and its methyl ester (Me-SA) are both defense compounds that potently induce systemic acquired resistance of plants against pathogenic microorganisms

Wasternack and Parthier (1997) reported application of jasmonate or its methyl ester, methyl jasmonate, strongly induces local and systemic expression of PI genes in many plant species, suggesting that jasmonate has an ubiquitous role in the wound response Analysis of a potato PI-IIK promoter has revealed a G-box sequence (CACGTGG) as jasmonate-responsive element (Koiwa *et al.* 1997).

Titarenko *et al.* (1997) developed the model for the wound-induced activation of the proteinase inhibitor II (Pin2) gene in potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) establishes the involvement of the plant hormones, abscisic acid and jasmonic acid (JA) as the key components of wound signal transduction pathway.

Levels of ABA have been shown to increase in response to wounding, electrical signal, heat treatment or systemin application in parallel with PI induction (Koiwa *et al.* 1997). Abscisic acid originally thought to be involved in the signaling pathway is now believed to weakly induce the mRNAs of wound response proteins and a concentration even as high as 100 mM induced only low levels of proteinase inhibitor as compared to systemin or jasmonic acid (Birkenmeiner and Ryan, 1998), suggesting the localized role of ABA.

Koiwa *et al.*(1997) showed that the current evidence suggests that the production of the inhibitors occurs via the octadecanoid (OD) pathway, which catalyzes the breakdown of linolenic acid and the

formation of jasmonic acid (JA) to induce protease inhibitor gene expression. These signal molecules are translocated from the wound site through the xylem or phloem as a consequence of hydraulic dispersal. The plant systemin an 18-mer peptide has been intensely studied from wounded tomato leaves which strongly induced expression of protease inhibitor (PI) genes. Systemin regulates the activation of over 20 defensive genes in tomato plants in response to herbivorous and pathogenic attacks.

Ryan (2000) reported that the polypeptide activates a lipid-based signal transduction pathway in which linolenic acid, is released from plant membranes and converted into an oxylipin signaling molecule, jasmonic acid. Ryan, (2000) reported wound-inducible systemin cell surface receptor with an molecular weight of 160,000 has also been identified and the receptor regulates an intracellular cascade including, depolarization of the plasma membrane and the opening of ion channels thereby increasing the intracellular Ca (2+), which activates a MAP kinase activity and a phospholipase A (2). These rapid changes, play a vital role leading to the intracellular release of linolenic acid from membranes and its subsequent conversion to JA, a potent activator of defense gene transcription

Moura and Ryan (2001) reported methyl ester positively affects plant defense through another defense mechanism involving tritrophic plant herbivore interaction. Different jasmonic acid-dependent and independent wound signal transduction pathways have been identified recently and partially characterized. Components of these signalling pathways are mostly similar to those implicated in other signalling cascades, which include reversible protein phosphorylation steps,

calcium/calmodulin-regulated events, and production of active oxygen species (León *et al.* 2001).

Rakwak *et al.*(2001) studies on the induction of PI proteins have indicated a *de novo* synthesis of proteins such as a Bowman Birk protease inhibitor (OsBBPI) from rice which was found to be rapidly induced in seedling leaf in response to cut, exogenous jasmonic acid (JA), and two potent protein phosphatase 2A (PP2A) inhibitors, in a light/dark, time and dose dependent manner but was completely inhibited by cycloheximide.

2.9 Structure of protease inhibitor genes

Odani *et al.* (1983) reported Bowman-Birk type double-headed protease inhibitors are assumed to have arisen by duplication of an ancestral single headed inhibitor gene and subsequently diverged into different classes i.e. trypsin/trypsin (T/T), trypsin/chymotrypsin (T/C) and trypsin/elastase (T/E) inhibitors. The structure of potato protease inhibitor-II gene was determined by sequencing a genomic fragment of about 2kbp containing entire RNA coding as well as about 900 nucleotides of the 5'-upstream and 250 nucleotides of 3'-downstream region. The transcription start site was determined by RNAase protection experiments. The composition of the genomic sequence with cDNA sequences reveals the presence of one intron with length of 117 nucleotides. The genomic clones contain an open reading frame of 462 nucleotide allowing for 154 amino acids.

The protease inhibitor-II gene displays typical feature of eukaryotic genes. The sequences TATAAA is found 26 nucleotides

upstream of the transcription start site and the sequence CAAAT at position-103.

Keil *et al.*(1986) reported that 3'region the sequences AATAA is found 33 nucleotides in front of the poly- A additional site. Potato protease inhibitor II is composed of two sequence repeats. It contains two reactive site domains.

Hilder *et al.* (1989) reported that the inhibitors are synthesized as precursors from which the leader sequence is cleaved and a long trait of leader-encoding sequence is present in soybean genomic clones. There is no significant homology in this region to other seed-expressed protein leader sequences, other than a high representation of hydrophobic residues. Multiple potential initiator codons are a common feature of legume seed protein genes exemplifying the high degree of evolutionary novelty, which appears to be tolerated within such seed specific secondary compound genes.

Ryan,(1990) reported many of these inhibitors are products of multigene families and the gene size and coding regions of the inhibitors are generally small with no introns (Boulter, 1993).Also they reported substitutions and deletion/additions appear to be very feasible in this region, provided that there is a cleavable serine or asparagine residue within 10-20 amino acids of the first cysteine. Kondo *et al.* (1991) reported second oryzacystatin, OC-II; present on chromosome 5 also has introns in the same positions thus suggesting deviation from the earlier PIs, which lacked introns. Kishimoto *et al.* (1994) reported analysis of oryzacystatin OC-I has revealed the presence of two introns; the first a 1.4 kbp region between Ala 38 and Asn 39 and a second region of 372 bp in the 3' non coding region.

The improvement of plant PIs by phage display is still an infant stage to be commercially important. Insect midgut contains an estimated 1020 different proteases (Bown *et al.* 1997), which are differentially regulated, and all cannot be inhibited by plant's PIs (Broadway, 1997). Therefore, to achieve an effective pest control strategy it is very important to achieve different inhibitors expression in a concerted manner.

MATERIALS AND METHODS

Chapter-III

MATERIAL AND METHOD

3.1 Plant Material

The plant materials used for the studies viz., *G. hirsutum* cultivars *viz*., Anjali (LRK – 516), LRA – 5166, were collected from Crop Improvement Division, CICR, Nagpur. Seeds were soaked in water for 24-36 hours and total genomic DNA was isolated from the seeds of these plants.

3.2 Chemicals

The tissue culture grade and analytical grade chemicals / enzymes from Himedia, Qualigens, Genetix, Qaigen and Sigma were used for media preparation and other molecular biology studies.

3.3 Glasswares

Rewarere (Shott), Borosil glassware, Himedia plastic wares were used for reagent preparation. The glassware cleaned with soap solution, treated with chromic acid and rinsed with distilled water. All the glassware was sterilized in an oven at 180° C for 3 hours.

3.4 Enzymes

Enzymes from Hi-media, Qaigen and Bangalore Genei are used for the gene amplification and elution.

3.5 Isolation of plant DNA

In the present investigation we followed the isolation method developed by Dellaporta *et al.* (1983).

Materials

1. Sample material

2. Extraction buffer

100mM Tris-HCl (pH7.8)	6.06g
10mM EDTA Na_2	9.3g
500mM NaCl	14.61g
Water	500ml

4. Suspension buffer

50mM Tris-HCl (pH 8.0)	0.61g
10mM EDTA Na_2	1.8g
Water	100ml

5. 7.5M Ammonium acetate

6. Phenol, chloroform, isoamyl alcohol mixture (25:24:1)

7. TE buffer

10mM Tris-HCl (pH 7.5)	0.12g
1Mm EDTA Na_2	0.37g
Water	100ml

Method

1. Seeds were soaked in water for 24 – 36 hours and seed coat were removed and 2-3 seeds were ground using a pestle – mortar.
2. Added 2 ml of extraction buffer in small aliquots and ground thoroughly.
3. 1 ml of suspension buffer was added to suspend the material.
4. Transferred the homogenate to a centrifuge tube and 80 μl of 20% SDS and 20% PVP were added and mixed the contents properly.
5. The tube was kept in water bath at 65°C for 30 min.
6. The tube was taken out and 3ml of 7.5 M Ammonium acetate was added and kept the tube in ice for 1 hour.
7. The content was centrifuged at 15000rpm, 4.0°C for 15 min.
8. To the aqueous layer, 6/10th volume of isopropyl alcohol was added and kept at -20°C overnight for complete precipitation of DNA.
9. Spinning at 15000rpm for 15 min pelleted the DNA.
10. The pellet was dissolved in 25 μl of TE buffer and 10 μg of RNase was added and incubated for 15 min at 37° C.
11. Spinning at 15000rpm for 15 min pelleted the DNA.
12. The DNA was dissolved with 100μl of TE buffer and added equal volume of phenol, chloroform and isoamyl alcohol mixture and mixed properly by inverting the tube 4 to 5 times.

13. Centrifuged it at 15000rpm for 15 min.

14. Carefully pulled out the aqueous layer in a fresh tube leaving the inter phase.

15. Added equal volume of ice-cold ethanol and pelleted the DNA by centrifugation.

16. Dissolved the pellet in suitable volume of TE buffer. Reprecipitated the DNA with ethanol, it is spinned and vacuum dried the pellet.

17. Dissolved the pellet in suitable volume of TE buffer.

18. Estimated the DNA content and checked the purity by UV spectrophotometer.

19. Checked the DNA by gel electrophoresis by resolving the sample on 0.8 or 1.0% agarose gel.

3.6 Quantification of DNA

The isolated DNA was quantified in UV-Spectrophotometer at 260 nm for DNA and 280 nm for protein. The extinction coefficient of DNA is 200 or DNA at a concentration of 50ug/ml shown as absorbance of 1.0 at 260 nm. The 260/280 ratios should be <0.50 for good quality DNA.

3.7 Agarose Gel Electrophoresis

The quality of the DNA was checked by agarose electrophoresis. Agarose gels separate and resolve DNA fragments from 0.1 Kb to 25 Kb.

3.7.1 Preparation of Agarose Gel

The percentage of the gel depends on the size of the DNA molecule to be separated. For checking the quality of DNA of the plasmid and cotton genomic DNA, 0.8% agarose gel was used, for electrophoresis of PCR amplified soybean genomic DNA products 1.5% agarose gel were used.

Materials

5 X TBE (Electrophoresis Buffer)	p^H 8.0
Tris HCL	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20.0ml
Distilled Water	1000ml.

The solution was prepared and autoclaved.

Method

1. The ends of the gel casting plate were sealed with cello-tape and kept on perfectly horizontal leveled platform.

2. Agar was melted in 1 x TBE buffer and allowed to cool to lukewarm temperature.

3. The agarose was poured into the gel casting plate and the comb was fixed on one end of the plate and the gel was allowed to solidify.

4. After solidification of the agarose, the comb was carefully removed from the gel plate without disturbing the well.

5. The casted gel was placed on the electrophoresis unit (Bangalore Genei Pvt. Ltd.) and submerged with 1 X TBE.

6. The DNA samples were mixed with the loading dye and carefully loaded into the wells.

7. The cathode and anode were connected to the power pack (Bangalore Genei) and the current was adjusted to 20 mA.

8. The negatively charged DNA molecules move towards the anode and they separate according to their molecular weight. The power was turned off when the tracking dye reached at about 2 cm. from the anode end.

3.7.2 Viewing Gel

1. The gel was then viewed on an UV transilluminator. Nucleic acids (DNA) on the gel appeared orange owing to the fluorescence of bound ethidium bromide. DNA was photographed the gel has been checked for the presence of bands. Used the photograph for further interpretation of band patterns.

3.8 DNA Amplification by PCR

Materials:

1. Sample DNA
2. 25m M DNTPs
3. MgCl2 (25mM)
4. 10 X PCR buffer
5. Primers
6. Sterile distilled water
7. Taq DNA polymerase (3U/μl)

The following of primers were designed to obtain the PI Genes.

Table1. Primer details

Oligo name	Sequence 5' to 3'	T_m°C (given)	T_m°C (calcu-lated)	Mer	%GC	MW (g/mol)	O.D.
TIPI 1F1	Forward Primer: TATGGCGAAAGCT-GAGGTTGTA	58.4 °C	64 °C	22	45	6855	3.5
TIPI 2R1	Reverse Primer: TCACTTGGTCTAAG-AAGCCATCCA	61.0 °C	70 °C	24	46	7337	4.4

PROCEDURE

1. The PCR tubes were labeled properly and arranged them open in a rack.
2. A master mix was prepared in one eppendorf as follows for a PCR reaction.

Table-2 PCR reaction mixture

Sr. No	Chemical/Enzyme	Quantity
1.	Sterile distilled water	9.5µl
2.	Taq buffer	2 µl
3.	25mM $MgCl_2$	3 µl
4.	10mM dNTPS	2 µl
5.	4µM Primes Forward Reverse	 1 µl 1 µl
6.	Taq polymerase (3U/µl)	0.5 µl
7.	DNA template	1 µl
8.	**Total**	**20.0µl**

3. Mixed the master mixture properly and distributed into 20µl to each tube containing DNA sample and mixed it well.
4. The PCR tubes were spanned and kept in the Thermal cycler.
5. PCR program was standardized at following programme.

Table-3 PCR Programme:

Steps	**Temperature (°C)**	**Time**
1.InitialDenaturation	94	5 min
2.Denaturation	94	45 sec
3.Annelaing	5	45sec
4.Initial Extension	72	1 min
33 cycles		
5.Extension	72	7 min
6.Holding	4	Variable

6. After completion of all the cycles the tubes were taken out and preserved at 4°C.
7. The amplified samples were resolved on agarose gel (1.5 %) and observe the banding pattern.
8. Documented the results by gel documentation system.

3.9 Checking of Amplified DNA by Gel Electrophoresis

Materials

1. Tris Borate EDTA (TBE) buffer (10X; pH 8.2)

0.9M Tris HCl	113.0 g
0.025 M EDTA Na_2	9.3 g
0.9 M Boric acid	55.0 g
Double distilled water	1000 ml

2. Agarose: 1.5% (w/v) in single strength TBE buffer. Boiled to dissolve the agarose; then maintained at 50°C in a flask until use.

3. Gel loading solution

Sucrose	30%
Bromo phenol blue (BPB)	0.25%
Xylene cyanole FF	0.25%

All w/v in single strength TBE buffer.

4. DNA preparations (plant DNA, plasmid DNA, amplified DNA etc.) Marker DNA for size determination (Lambda DNA EcoR I &Hind III digest).

5. Gel casting plate.

6. Gel tank.

7. Power pack.

8. UV transilluminator with photography system.

9. Safety glasses.

Procedure

1. Formed a wall around clean dry, gel casting glass plate (10X 60.6 cm) using zinc oxide tape. It has given a leak proof wall about 1 cm high all around the plate. Alternatively, the plate is placed in a suitable gel-casting tray purchased from a commercial supplier. Placed the set-up perfectly horizontal over a leveled plate.

2. Poured 30 ml of agarose solution maintained at 50°C onto the casting plate. Immediately placed a suitable well forming comb about 1 cm from one end of the plate. The teeth of the comb should not touch the wall of the glass plate. Allowed the gel to get set for 1 hr.

3. Removed the comb from the gel, carefully. Transferred the gel along with the gel plate to the electrophoresis tank in such way that the wells are near the cathode (positive electrode). Poured 1X TBE buffer into the tank until gel is submerged completely.

4. Connected the electrodes to the power pack properly.

5. Prepared DNA samples in the gel loading buffer (10μl DNA sample in 4μl gel loading buffer) and loaded approx. 5-20μl of sample using micropipettes.

6. Turned on the power supply and run at 50V. Monitored the progress of fast running dye(BPB) and tracking dye (Xylene

cyanol) during electrophoresis. Terminated the run when tracking dye is about to leave the gel.

7. Transferred the gel onto a thick plastic sheet, placed on an UV transilluminator and viewed the gel under ultra violet light (300nm). Nucleic acids (DNA) on the gel appeared orange owing to the fluorescence of bound ethidium bromide. Photographed the gel has been checked for the presence of bands.Used the photograph for further interpretation of band patterns.

9. Measured (from photograph) the distance moved by each band from the loading well.

3.10 Elution by MinElute Gel Extraction Kit

Electroelution is a process of eluting out DNA from the agarose gel. This protocol is designed to extract and purify DNA of 70bp to 4kb.

Materials

1 QG Buffer (pH<7.5)

2. Isopropanol.

3. PE Buffer.

4. EB Buffer (10mM Tris-Cl, pH-8).

5. 5M Sodium acetate.

Method

1. The PCR product was run on 1.5% agarose gel with ethidium bromide.

2. The separated bands were located by UV-light.

3. The required bands were located and cut out the slice of agarose containing the band using sharp razor blade.

4. Weighed 100 mg of the gel containing the amplified band.

5. Added 300 μl of Buffer QG in 100mg of agarose gel in a 1.5ml micro centrifuge tube.

6. The tube was incubated at 50ºC for 10 min till the gel was completely dissolved and vertex at every 2-3 min.

7. After the gel had dissolved completely the colour was checked of the mixture similar to QG buffer.

8. One gel volume of isopropanol to the sample was added and mixed by inverting tubes several times.

9. MinElute column was placed in a collection tube of 2ml vol in a 2ml collection tube.

10. Sample was added to the MinElute column and centrifused for 1 min.

11. Flow-through was discarded and MinElute column was placed back in same collection tube.

12. Added 500µl of Buffer QG to spin column and centrifuged for one min.

13. Flow-through was discarded and placed in the MinElute column in the same collection tube.

14. For washing, added 750µl of Buffer PE to the MinElute column and centrifuge for one min.

15. Flow-through was discarded and centrifuged the MinElute for one min at 10,000rpm.

16. MinElute column was placed into a clean 1.5ml microcentrifuge tube.

17. To elute the DNA, 10µl of Buffer EB to the membrane and column was allowed to stand for one min and then centrifuged for one min.

3.11 Cloning and Transformation

3.11.1 Cloning of amplified genes

Materials

1. QIAGEN PCR cloning kit.
2. Ligation master mix.
3. pDrive cloning vector.
4. Competent cells.
5. SOC medium.
6. Distilled water.

Method:

1. Thaw 2X ligation master mix, pDrive-cloning vector DNA, and distilled water (provided) placed on ice after thawing.
2. Ligation reaction mixture prepared according to the following scheme.

Table-4 Ligation reaction mixture

Components	Quantity
PDrive cloning vector	1µl
PCR product	2µl
Sterile water	2µl
Ligation master mix	5µl
Total	**10µl**

3. Briefly mixed the ligation reaction mixture and then incubated for 30 min at 4 to 16 ^{0}C in water bath, where the ligation takes place.

3.12 Transformation into *E.coli* cells

3.12.1 Preparation of competant cell ($CaCl_2$ method)

Materials:

1. Buffer and solution-

a) $CaCl_2.2H_2O$ (1M) Stock- during preparing competent cells, thawed 10ml aliquot of the stock solution and diluted it to 100 ml with distilled water.

b) Standard transfers buffer ($MgCl_2.CaCl_2$) solution.

2. Media-

   SOB media

   SOC media

   LB agar media.

3. Ligation master mix.

4. Centrifuge and Rotors, etc.

Method

1. The single bacterial colony (2-3mm) in diameter was picked from a plate that has been incubated for 16-20 hrs at 37°C. The colony was then transferred into 100 ml of LB broth.
2. The culture was incubated for 3 hrs with vigorous agitation, monitoring the growth of culture.
3. The bacterial cells were then transferred to a sterile, disposable ice-cold 50 ml polypropylene tube. The cultures were cooled to 0°C by storing the tube on ice for 10 min.
4. The tubes were centrifuged at 7300 rpm for 10 min at 4°C.
5. The medium was decanted from cell pellets. The tubes were allowed to stand in an inverted position on a pad of paper towel for 1 min to allow the last traces of media to drain away.
6. Each pellet was resuspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution.
7. The tubes were centrifuged at 7300 rpm for 10 min at 4°C.
8. The medium was decanted from cell pellets. The tubes were allowed to stand in an inverted position on a pad of paper towel for 1 min to allow the last traces of media to drain away.

9. The pellet was resuspended by swirling or gentle vortexing in 2 ml of ice cold 0.1M $CaCl_2$ for each 40 ml of original culture.

3.12.2 Transformation into *E. coli*

1. Taken 200µl of competent cells (DH5 α).

2. SOC medium was warmed to the room temperature.

3. By adding 5µl ligation reaction mixtures per tube of competent cells and mix gently and kept for incubation on ice for 30 min.

4. The tubes were heated at 42 ^{0}C in water bath for 90 second.

5. Rapidly incubated the tubes on ice for 2 min.

6. 800 µl of SOC medium was added in each tube.

7. The above culture was incubated for 45min in water bath at 37^{0}C.

8. Then 100µl of each transformation mixture was used for plating onto LB agar plates containing kanamycin (30 µg per ml) as selection marker and IPTG 50µM, and X-gal 80µg per ml for blue/white,screening of recombinant colonies.

9. The transformation mixture can be plated using sterile bent glass rod or a spreader.

10. Incubated the transformation mixture at room temperature. Then invert the plates and incubate it in an incubator at 37^{0}C over night (12-16hrs).

3.12.3 Selection of transformants

Materials-

1. Luria Bertani HiVeg Agar medium.
2. X-gal stock (40mg/ml).
3. IPTG stock (100mM).
4. Kanamycin stock (10mg/ml).

Method:

1. Dissolved 4 gm of LB agar in 100ml of water.
2. pH adjusted to 7.5.
3. Flask is autoclaved at 15 lb for 20 min.
4. After autoclaving media was allowed to cool at 55°C.
5. Added 300 μl of filter sterilized kanamycin stock solution.
6. For Blue /White colony screening added 200 μl of X-gal stock solution and 50 μl of IPTG stock solution.
7. Mixed and poured medium into agar plates, allowed to set, inverted and stored at 4 °C under sterile condition.
8. 100μl of each transformation mixture was used for plating on to LB agar plates.
9. Incubated at room temperature at 37°C, for 12-18 hrs and non-transformed / transformed (Blue /White) colonies were observed respectively.

3.13 Confirmation of gene inserted in pDrive

Procedure

1. Taken one toothpick of transformed bacterial colony in a 20μl of water.

2. Denatured these bacteria at 95°C for 5 min in thermal cycler.

3. Taken 1μl of these denatured bacteria as a template DNA.

4. PCR was carried out as follows:-

A. PCR reaction mixture

Sr. No	Chemical/Enzyme	Quantity
1.	Sterile distilled water	9.5μl
2.	Taq buffer	2 μl
3.	25mM $MgCl_2$	3 μl
4.	10mM dNTPS	2 μl
5.	4μM Primes Forward Reverse	 1 μl 1 μl
6.	Taq pol. (3U/μl)	0.5 μl
7.	DNA template	1 μl
8.	**Total**	**20.0μl**

B. PCR Programme

Steps	Temperature (°C)	Time
1.InitialDenaturation	94	5 min
2.Denaturation	94	45 sec
3.Annelaing	56	45sec
4.Initial Extension	72	1 min
33 cycles		
5.Extension	72	7 min
6.Holding	4°C	Variable

6. After completion of all the cycles the tubes were taken out and preserved at 4°C.

7. The amplified samples were resolved on agarose gel (1.5 %) and observe the banding pattern.

8. Documented the results by gel documentation system.

RESULTS

Chapter-IV

RESULTS

4.1 Plant materials

The seeds of *G. hirsutum* cultivars *viz*., Anjali (LRK - 516), LRA - 5166, were selected for genomic DNA isolation.

4.2 DNA Isolation

The total genomic DNA from seeds was isolated in all the selected cultivars. The DNA was purified and the intact DNA was subjected to quantitative and qualitative analysis.

4.3 Quantification of DNA

DNA, thus obtained was quantified with BIO-RAD Smart Spec. Plus UV-Spectrophotometer at 260 nm and results are presented in the Table 5. All the DNA samples invariably showed around 50 μg/ ml concentration of DNA. Pure and more quantity of DNA were isolated from seeds.

Table 5: Estimation of total genomic DNA content isolated from cotton species

S. No	DNA samples	O. D. at 260	Quantity (μg/ml)
1.	LRA-5166	0.009	466.19
2.	LRK-516 (Anjali)	0.007	363.53

4.4 Qualitative check

The quality of DNA was checked by 1% agarose gel electrophoresis as shown in the Plate1. In case of total genomic DNA,

the intact bands were observed. The result shows that the genomic DNA was isolated without contamination of proteins.

4.5 Primers

The primers were synthesized with the help of the primer designing software, the **FastPCR**. The reference sequence retrieved from the NCBI with the accession number EF643506 *Gossypium hirsutum* [gi: 153805693]

1. T1PI 2F1 Forward Primer TATGGCGAAAGCTGAGGTTGTA 22 mer

2.TIPI 2R1Reverse Primer TCACTTGGTCTAAGAAGCCATGCA 24 mer

The primers were synthesized (Qiagen) and used for amplification of the protease inhibitor genes in cotton.

4.6 Standardization of PCR program for amplification of PI genes

Using primers as mentioned above, the target gene of protease inhibitor was amplified in Whatman Biometra TProfesional thermocycler. The amplification program in the thermo cycler was standardized with different program, especially annealing temperature, which plays crucial role in the amplification with primers. The PCR program amplified the expected gene length is presented in the Table 6.

Then gradient PCR proved that the temperature for better amplification was from 55°C to 60°C. Hence several times PCR was done at different temperatures. It was found that the amplification was the maximum at the temperature of 56°C.

Table No 6: PCR Program standardized for annealing temperature

Steps	Temperature (°C)	Time
1. (Initial Denatuiration)	94	5 min
2. Denaturation.	94	45sec
3.Annealing	56	45sec
4.Extension	72	1 min
33-cycles		
5.Final Extension	72	7 min
6.Hold	15	Pause

4.7 Amplification results of PI gene

The template DNA of LRA-516 and LRK-5166 cultivars of *G. hirsutum* was amplified with the forward and the reverse primers and the product was resolved on 1.5% agarose gel which produced 600bp fragment along with the standard marker 100bp ladder (Quick Load Ladders of New England Biolabs). The results are shown in the plate (2).

4.8 Electroelution

The MiniElute Gel Extraction Kit was used to elute the amplified gene out from the agarose gel. DNA was suspended into TE buffer for further studies on DNA sequencing.

4.9 Cloning into pDrive vector (QIAGEN)

PCR amplicon of the target PI gene was eluted and fragments were subjected to cloning into QIAGEN cloning vector pDrive. The fragments approximately 600bp amplified with the primers used were successfully ligated in pDrive vector. The ligated product was confirmed

by gel electrophoresis and found that a single band of 4.45 kb was obtained on 1.5% agarose gel plate (3).

4.10 Transformation

The ligated pDrive vector was transformed in to competent cells (DH5 α). The transformed bacterial cells were selected on kanamycin medium (50 μg/ml). The transformed colonies showed white colour due to insertion inactivation of *lacZ* gene, whereas non-transformed bacterial colonies showed blue in colour by reacting with the substrate X-gal and IPTG. The results are presented in the plate (5). This result shows that successful cloning and transformation of PI gene in pDrive cloning vectors.

4.11 Confirmation of gene integration.

The inserted DNA in cloned pDrive vector was analysed by reamplification of PI gene using TIPI primers from transformed colonies. The size of amplified product was found to be 600bp confirming gene integration. The results are shown in plate 6.

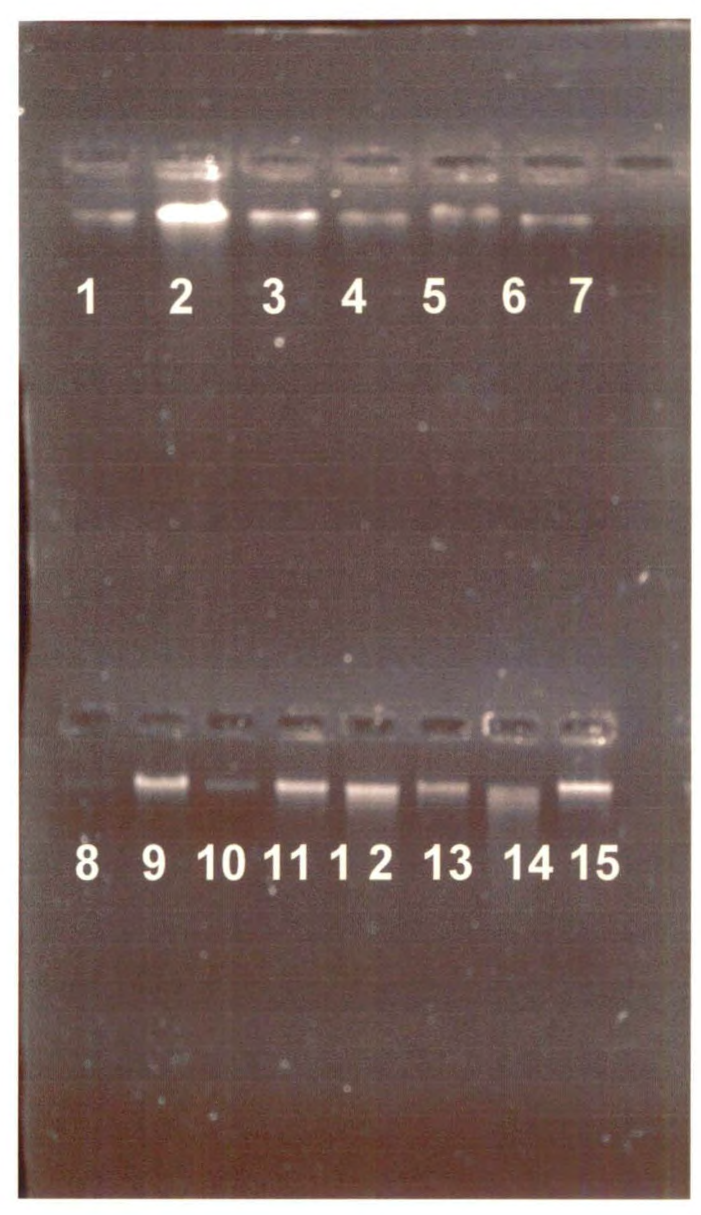


Plate-1 Total genomic DNA isolation from cotton varieties.

1-7 LRA 5166

8-15 LRK 516

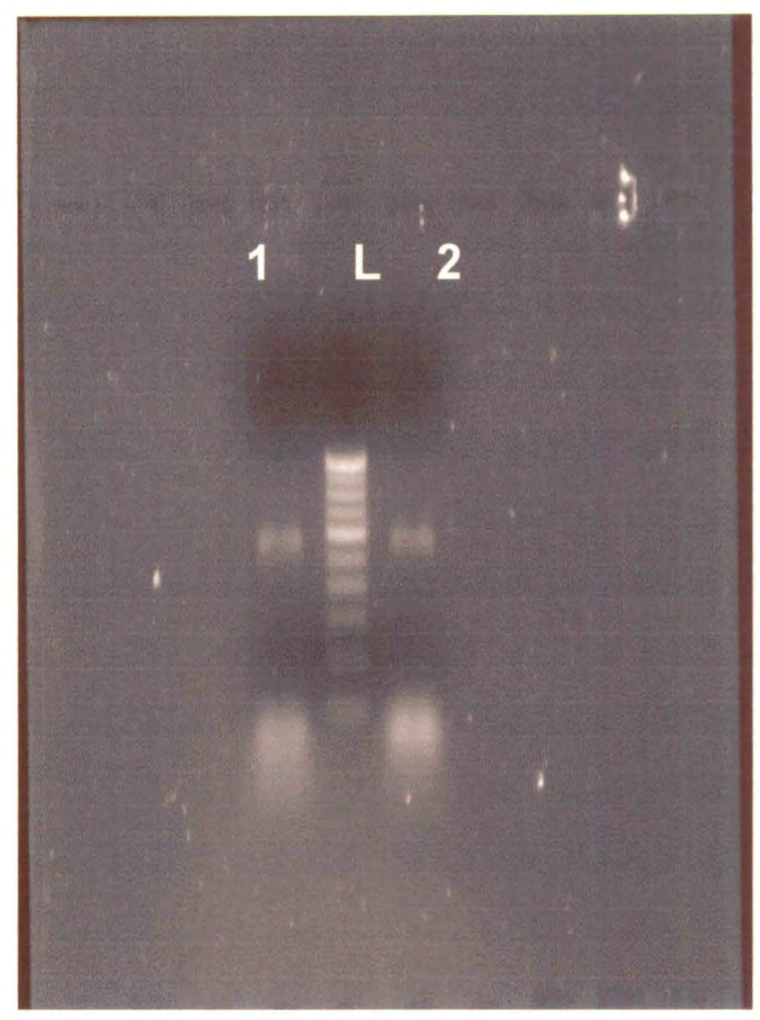


Plate-2 Amplification of PI gene from cotton variety using TIPI primer

1.LRA-5166
2.LRK-516
L-Ladder 100bp.

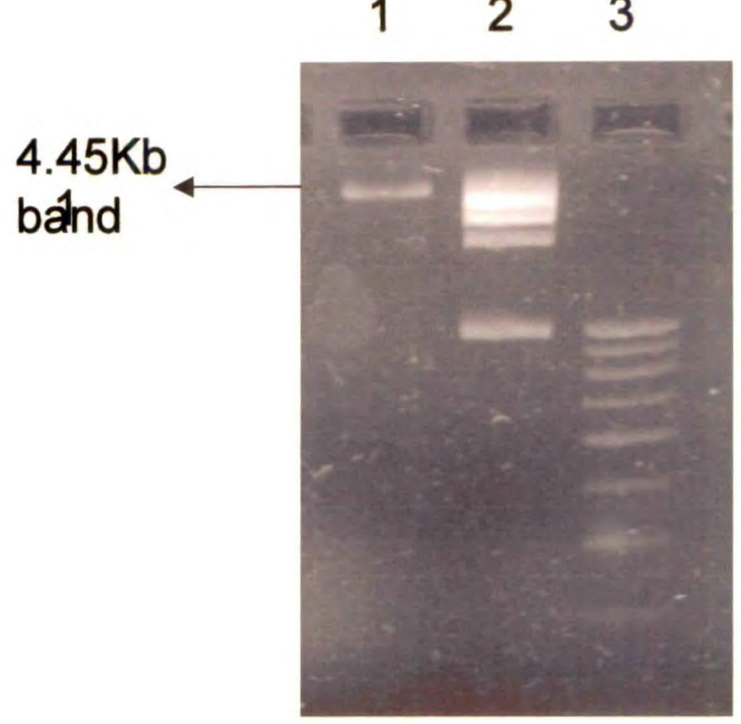


Plate 3 : The PCR Amplified product ligated to the pDrive Vector

Well No.1 = the ligated product
Well No.2 = 1Kb DNA Ladder
Well No.3 = 100bp λDNA Ladder
pDrive Vector = 3.85Kb
Amplified gene = 600 bp
Ligated Product = 4.45kb

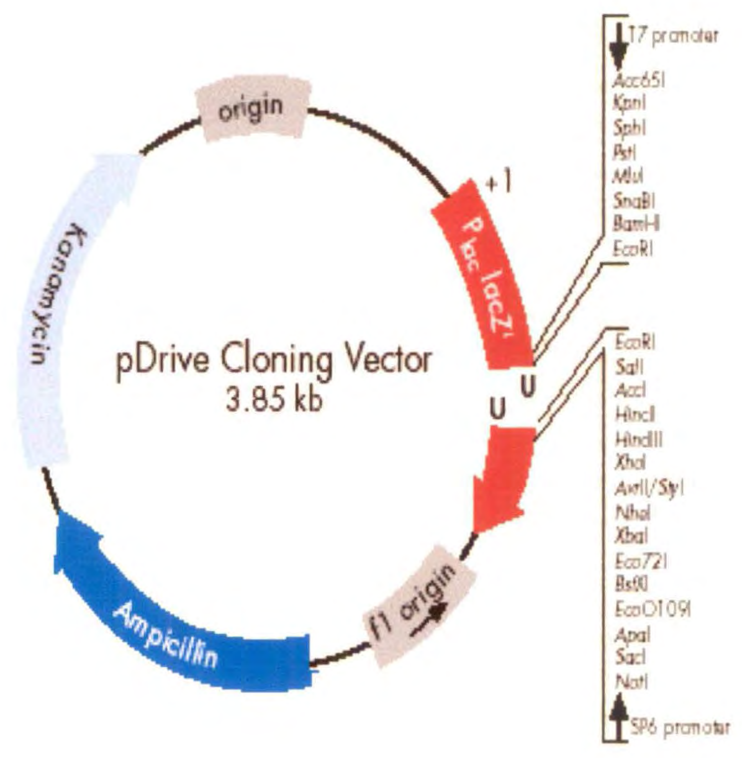
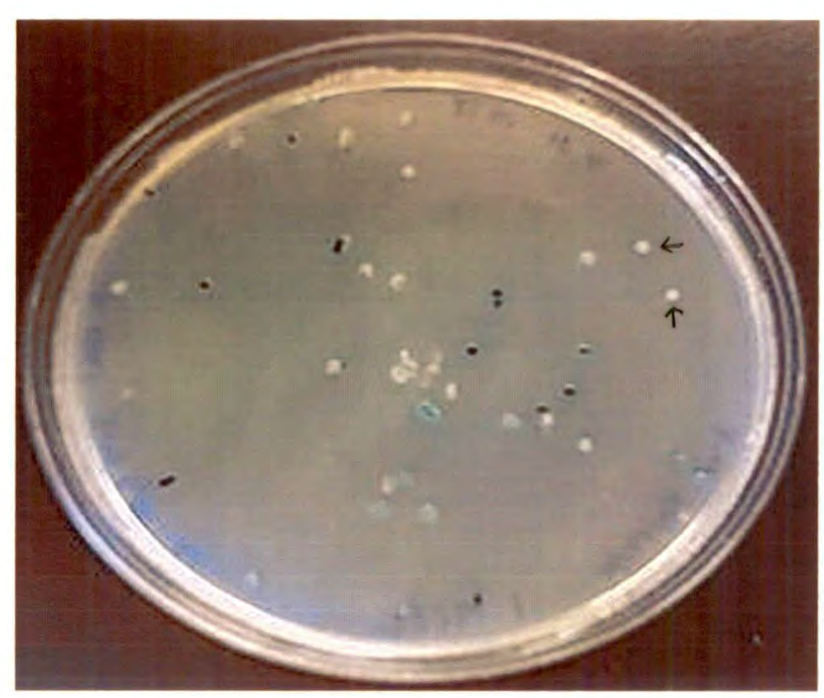


Plate-4 pDrive cloning vector



Arrow indicating transformed white colonies.

Plate no- 5 Transformed white-blue colonies.

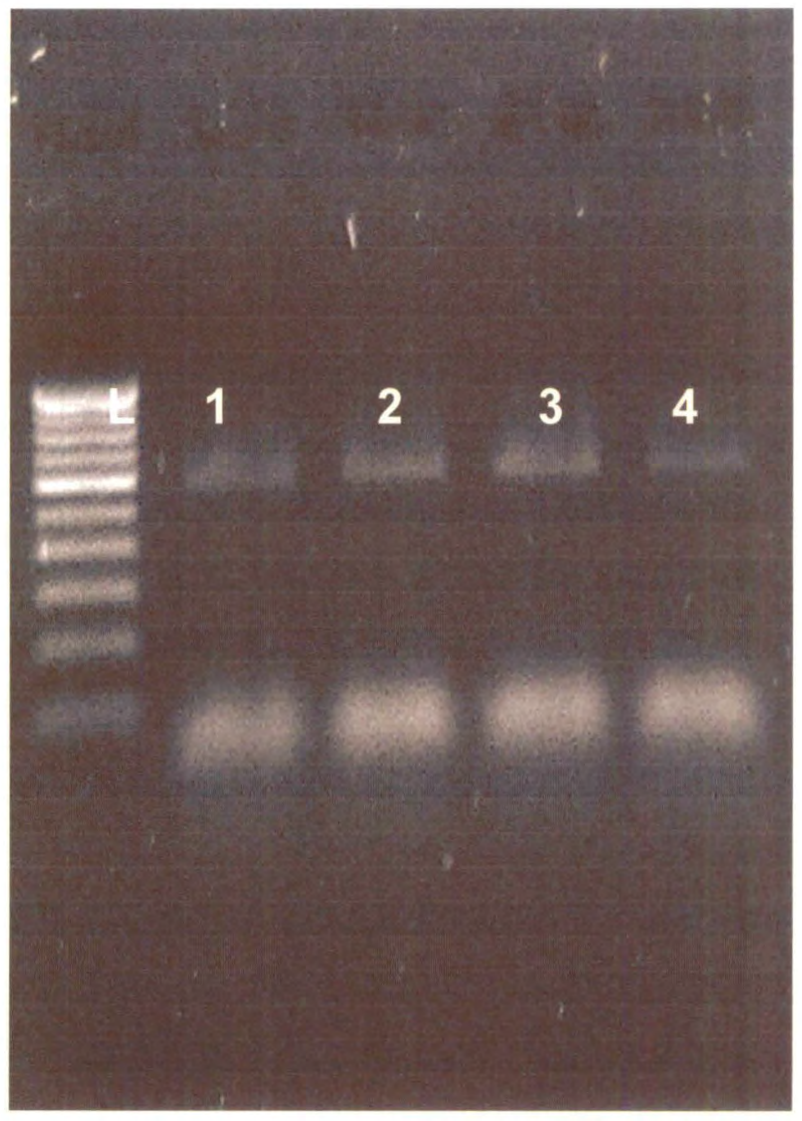


Plate-6 Reamplification of PI gene isolated from transformed (white colonies) *E.coli* cells.

L-Ladder 100bp.

DISCUSSION

Chapter-V

DISCUSSION

Cotton is very important commercial crop. Unfortunately it is susceptible for variety of insects and pests. So, the application of modern DNA technology is to develop the transgenic crops resistant to insects and pests. The production of transgenic crops has seen rapid advances during the last decade with the commercial introduction of *Bt* transgenics, but the major concern with these crops has been the development of resistance by pest and public acceptability. Hence, there has been a need to discover new effective plant genes, which would offer resistance/protection against these pests. **Plant protease inhibitors (PIs)** are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food.

5.1 Plant protease inhibitors (PIs),

A product of plant defensive gene (**PI gene**); have been well established to play a potent defensive role against predators and pathogens. Although diverse endogenous functions for these proteins has been proposed, ranging from regulators of endogenous proteases to act as storage proteins, evidence for many of these roles are partial, or confined to isolated examples. On the other hand, many PIs have been shown to act as defensive compounds against pests by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has been accumulated consistently. The role and mechanism of action for most of these inhibitors are being studied in detail and their respective genes isolated. These genes have been used for the construction of transgenic crop plants to be incorporated in

integrated pest management programmes. This article describes the classes of protease inhibitors, their regulation and genes used to construct transgenic plants against phytophagous insects.

The protease inhibitor genes have practical advantages over genes encoding for complex pathways *i.e.* by transferring single defensive gene from one plant species to another and expressing them from their own wound inducible or constitutive promoters thereby imparting resistance against insect pests (Boulter, 1993). It was first demonstrated by Hilder *et al.*(1987) by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of lepidopteran insect pests. Further, there is no evidence that it had toxic or deleterious effects on mammals. Many of these protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality (Ryan, 1989). Protease inhibitors also exhibit a very broad spectrum of activity including suppression of pathogenic nematodes (Williamson and Hussey, 1996), inhibition of spore germination and mycelium growth of *Alternaria alternata* (Dunaevskii *et al.* 1997). These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems and the use of plants as factories for the production of heterologous proteins (Sardana *et al.* 1998).

A large number of protease inhibitor genes with distinct modes of action have been isolated from a wide range of crop species. Development of

transgenic crops have come a long way from the first transgenic developed by Hilder *et al.* (1987). Further, the availability of diverse genes from different plant species makes it a possibility to use one or more genes in combination, whose products are targeted at different biochemical and physiological processes within the insect. These packages will not only contain protease inhibitor genes but also lectins, alpha-amylase inhibitors, or other plant genes encoding insecticidal proteins. This technology may not replace the use of chemical pesticides in near future but effectively complement it. However, in future non-scientific issues such as regulatory approval, propriety rights and public perception will be decisive in releasing crop plants produced by genetic engineering using recombinant DNA technology.

5.2 Isolation and Cloning of Protease Inhibitor gene

We isolated cotton protease inhibitor genes (cystein protease inhibitor) from of *G. hirsutum* cultivars like LRK - 516(Anjali) and LRA - 5166, using forward and reverse primers. We have amplified approximately a product of the size 600bp in each cultivars. The amplified gene was eluted efficiently, and utilized for further studies on DNA cloning and sequencing. Earlier studies show that same type of work has been also performed for other gene. Marchetti *et al.* (2000) isolated soybean Kunitz,(KTi_3),C-II and PI-IV inhibitor gene by PCR amplification, sequenced and cloned into *Agrobacterium tumefaciens* EHA-105. In our study we directly isolated Protease inhibitor (cystein type) of 600bp from cotton (*Gossypium hirsutum*) by designing specific primers (T1PI 2F1, 2R1, 22 and 24 mer respectively) and cloned into *E. coli* (DH5α), by using pDrive-cloning vector (QIAGEN). The pDrive cloning vector provides better ligation and allows easy analysis of

cloned PCR product. Earlier studies shows that same type of work has also performed by Shen Fafu and YU Shuxun (2004), by utilizing rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR), and a set of consensus oligonucleotide primers was designed to anneal the conserved sequences of plant cysteine protease genes (*Ghcysp*gene) of 1368bp from senescent leaves of *Gossypium hirsutum* and the full-length cDNA was gel-purified and ligated into the pGEM-T vector (Promega). Functional SBTI is coded by KTi3 gene in soybean (Jofuku *et al.* 1989). Same type of work also carried out by Nandi and Basu *et al.*.(1999) as they designed two primers from the available information on the nucleotide sequences of KTi3 gene and isolated SBTI gene of 650bp from soybean plant and cloned into a pUC18 vector and transformed into *E. coli*, DH5α. Colleen Murry and John T. Ccristeller reported PCR based amplification and characterization of a novel PI-II gene by designing a primer complementary to novel PI-II gene (Pin2-CM7) from potato genome.

5.3 Transformation

In the present study competent cells (DH5 α) were used as host for pDrive vector. The transformed colonies were observed as white and non-transformants were blue colonies. The transformed colonies with blue colour were found at low temperature incubation this results show that the enzyme activity on substrate might be temperature controlled.

Cysteine proteinases are important enzymes for digestion in many coleopteran pests, while vertebrates generally use proteinases from other classes for digestion (Reeck *et al.*, 1997) For this reason, the incorporation of genes encoding cysteine proteinase inhibitors into

transgenic grain has been proposed as a method to prevent seed damage from coleopteran pests while posing little or no side effects on vertebrates. Transgenic plants developed especially using proteinase inhibitors of insect digestive enzymes with the view to control crop pest or designed to retard their growth and development but not to kill the insects. These are the fundamental differences between proteinase inhibitor strategy with the chemical pest control or Bt-toxins, which aims to kill the insect's community. This will provide an efficient regulation mechanism of insect population at low profile instead of developing high resistant biotype of insects against the toxic proteins or chemicals.

Secondly, the results indicated that the proteinase inhibitor genes present in commercially cultivated cotton varieties of *G.hirsutum,* although the genes are present in the cotton plants but they are induced by insect attack or wounding. The main objective of the work is to isolate the proteinase inhibitor genes and attaching a constitutive promoter to the gene, which in turn produces throughout the plant parts during its life cycle, and control the bollworms at neonate larval stage.

This defensive protein present in plants systems helps us to understand the biophysical and biochemical rules that govern the activity of proteins and with advent of recombinant DNA technology would enable us to achieve efficient defensive mechanism in plant systems using proteinase inhibitors in biotechnology.

The further work on sequencing the amplified gene amplified in *G.hirsutum* would provide the complete knowledge of the presence of

various PI genes present in the cotton plants. The gene expression is found satisfactorily then the genes will be cloned for transformation into any crops.

Plant derived genes such as proteinase inhibitors will have advantage of efficient expression than bacterial genes especially Bt. The development of proteinase inhibitors genes construct would be relatively easy since modification of coding sequence is not necessary in this case, whereas if bacterial genes are used for the plant system, which needs modification of the coding sequence without modification of amino acid sequence of the protein.

Development of new types of proteinase inhibitors by protein engineering with altered affinity for different proteinase and incorporating multidomain proteinase inhibitors in a single gene are attractive strategies for developing insect-resistant transgenic plants in near future.

SUMMARY AND CONCLUSION

Chapter-VI

SUMMERY AND CONCLUSION

Biotechnology is a vast field of research to modify any organism like plants, animals and Microorganism. In past few years biotechnology has made remarkable progress especially in plant and health sciences. Agricultural biotechnology is one area involving application of biotechnology to improve economically important crops. The potential to improve the plants productivity and their proper use in agriculture relies largely on newly developed DNA technology. These techniques enable the selection of successful genotype, better isolation and cloning of favorable traits.

The plant protease inhibitor gene have practical advantages over genes encoding for complex pathways by transferring single defensive gene form one plant species to another and expressing them from their own wound inducible or constitutive promoter thereby imparting resistance against insects and pests. There are several types of protease inhibitor genes have been isolated from plants. In the present investigation, an attempt was made to isolate cysteine protease inhibitor gene from *G. hirsutum* cultivars using PIs primers. The expected fragment of corresponding genes was amplified, 600bp in all the cultivars. The isolated fragment was characterized by gel electrophoresis and the fragment was eluted and then purified and precipitated. The purified DNA was cloned into pDrive vector. The recombinant plasmid

was pushed into E. coli cells. The transformed bacterial colonies were screened in X-gal and IPTG plate. The transformed colonies were produced as white colonies whereas non-transformed colonies appeared as blue colonies.

After reamplification of isolated DNA from recombinant *E. coli* cells using same PI primers the expected fragments of 600bp were observed which confirm the successful cloning of inserted gene.

Conclusion

Our main aim was the isolation, cloning and confirmation of the protease inhibitor genes from cotton. We have shown a part of gene was isolated from cotton, which will gave way to move to target full-length DNA sequence and this gene would be designed with constitutive promoter and transfer into other crop plants. Although some data on the expression of this gene in plants show that it is not as effective as Bt genes, its utility as a supplementary gene to enhance the effect of other insecticidal genes cannot be overlooked. Such an approach is desirable to restrict developments of resistance by insect pests towards products of transgene.

At this point, we like to make a brief conclusion that the PI genes are present in cotton plants that need to be engineered for efficient expression to make viable transgenic cotton resistance against insect pest.

Although plant PIs have been isolated and characterized from a large number of sources, and that the natural inhibitors have been made

available by gene therapy and through transgenic plants over expressing specific inhibitors with therapeutic significance, the potential for the natural inhibitors in medicine and agriculture is enormous, awaiting full-scale exploration.

Finally we would like to conclude that the availability of diverse genes from different plant species makes it a possibility to use one or more genes in combination, whose products are targeted at different biochemical and physiological processes within the insect. These packages will not only contain protease inhibitor genes but also lectins, alpha-amylase inhibitors, or other plant genes encoding insecticidal proteins. This technology may not replace the use of chemical pesticides in near future but effectively complement it. The use of recombinant PIs may also be an attractive way to protect plants from fungal, bacterial and viral pathogens. Currently, two principal strategies are proposed to engineer effective pest control in plants: ectopic expression of pesticidal proteins, and induction of the plant natural defensive response. At present, screening gene pools without taxonomic constraint can help identify novel insecticidal determinants, but in future this approach will be augmented by directed in vitro molecular evolution. Given the number of pesticidal proteins that are involved in host plant defense, it is presumed that effective pest control by this strategy will result from the co-expression of numerous determinants, each of which could be custom engineered by directed molecular evolution to maximize its effectiveness against specific pests.

However, in future non-scientific issues such as regulatory approval, propriety rights and public perception will be decisive in

releasing crop plants produced by genetic engineering using recombinant DNA technology.

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APPENDIX

Cloning and characterization of protease inhibitor gene in cotton

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ABSTRACT

Biotechnology is a vast field of research to modify any organism like plants, animals and Microorganism. The plant protease inhibitor gene have practical advantages over genes encoding for complex pathways by transferring single defensive gene form one plant species to another and expressing them from their own wound inducible or constitutive promoter thereby imparting resistance against insects and pests. In present investigation we amplify cysteine protease inhibitor gene from cotton (*Gossypium hirsutum*), genome (variety viz LRK – 516 (Anjali) and LRA-5166) using forward and reverse primers. Total 600bps fragment was amplified and the amplified fragment was characterized by gel electrophoresis and amplified fragment was eluted and then purified and precipitated. The purified DNA was cloned into pDrive vector. The recombinant plasmid was pushed into *E. coli* cells (DH5α). The transformed bacterial colonies were screened in X-gal and IPTG plate. The transformed colonies were produced as white colonies whereas non-transformed colonies appeared as blue colonies. After reamplification of isolated DNA from recombinant *E. coli* cells using same PI primers the expected fragments of 600bp were observed which confirm the successful cloning of inserted gene.

Although plant PIs have been isolated and characterized from a large number of sources, and that the natural inhibitors have been made available by gene therapy and through transgenic plants over expressing specific inhibitors with therapeutic significance, the potential for the natural inhibitors in medicine and agriculture is enormous, awaiting full-scale exploration.

These protease inhibitors will not only contain protease inhibitor genes but also lectins, alpha-amylase inhibitors, or other plant genes encoding insecticidal proteins. This technology may not replace the use of chemical pesticides in near future but effectively complement it.

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