1. INTRODUCTION

The losses due to pests and disease have been estimated as 40% the total agriculture production. Pre-harvest losses due to insect pest are about 15% of total production (Krattiger, 1997). The insect pests are an important constraint for legumes. The agriculture is posed with a great challenge of meeting the ever-increasing food demand. Crop improvement and management have been proved handy earlier to keep pace with the demand. Also, many of the crop varieties developed in the past under modern high intensity agriculture included high yielders, nutritionally rich, adapted to environmental conditions and with low mammalian toxicity resulting in tremendous increase in food production for feeding the ever-growing world population. Farmers rely on the use of pesticides for crop protection against insect pests. Considerable amount of pesticides are used to managing insect pest. The indiscriminate application of pesticides has in some cases resulted in secondary pests becoming primary pests with even more devastating effects. So the control of insect pests has become an integral part of the development of agricultural practices, as crop damage caused by insects is a major economic factor in agriculture in tropic and temperate regions of the world.

Therefore, it is necessary to develop more efficient and environment friendly agriculture, which will have decreased inputs in energy and chemicals and will not generate harmful output such as pesticide residues (Jouanin et al., 1998). The molecular biology and genetic engineering approaches allow harnessing and development of insecticidal molecules in crop plant in a safe and sustainable way. Most of these have potent effects on insect pest, low mammalian toxicity, lack of neuro-toxic activity, low persistence in the environment and biodegradability (Jacobson, 1989). This approach will keep away from the tones of chemical pesticides used globally in agriculture.

The production of transgenic crops has emerged with the introduction of Bacillus thuringiensis (Bt) gene (Kumar et al., 1996). The scientists have used (Bt) gene for producing insect resistant transgenic plants in several crops some are commercialized as Bt cotton, maize and soybean. Since Bt toxin is of non-plant origin and also the codon bias is noticed when a prokaryotic gene is expressed in a eukaryotic system. So, there is a need to discover new effective plant genes, which would offer resistance or provide protection against these pests. In a co-evolving system of plant-insect interactions, plants synthesize a variety of toxic proteinaceous and non-proteinaceous molecules for their protection against insects. They have evolved various complex chemical weapons of defense which include antibiotics, alkaloids, terpenes as well as proteins such as enzymes, enzyme inhibitors and lectins (Rhodes, 1979; Baldwin and Semultz, 1983). These inhibitors also have the properties to
prevent uncontrolled proteolysis within the cells, organelles or fluid (Ryan, 1980; Ryan 1990). A 'copy nature' strategy for insect pest control has been presented aiming to be relatively sustainable with the idea that the above mentioned compounds contribute to the resistance against insects, pests and pathogens (Boulter, 1993; Bate and Rothstein, 1998). Proteases and \( \alpha \)-inhibitors serve as one of the defense mechanisms in plants against invading pests. Protease inhibitors are generally low molecular weight proteins that make complexes with proteases and reduce their proteolytic activities. Evidences indicate that the presence of protease inhibitor proteins in plant leaves can reduce predation by insects.

Since protease inhibitor (PI) gene(s) are of plant origin, these have practical advantages that they are eukaryotic in nature and are non-injurious to pollinators, predators, economic insects, natural enemies of the pests. By transferring single defensive gene from one plant species to another with higher expression using their own wound inducible or constitutive promoters would impart resistance against insect pests (Boulter, 1993; Gatehouse et al, 1997) and can be used as biocontrol option in an IPM strategy. The insect resistant transgenic crops not only eliminate the use of chemical pesticides but also provide a season long protection to crop against insect pests. In view of this, it is worthwhile to identify and isolate the inhibitor proteins encoding genes from Indigenous legumes, so that these genes will be free from IPR issues and transgenic crops can be developed and commercialized (Koundal et al., 2003).

Therefore, the present research proposal has been envisaged with the following objectives-

- Isolation and characterization of protease inhibitor proteins from mature and developing seeds of pigeonpea
- Bioassay of the purified protease inhibitor protein on the *Helicoverpa armigera*
- Construction of cDNA library of pigeonpea and isolation of protease inhibitor genes from cDNA library
- Characterization of protease inhibitor genes by nucleotide sequencing and sequence analysis through using bioinformatics tool
2. REVIEW OF LITERATURE

Insect pests are important constraints for crops production. These constraints include flowers and pod feeding Lepidoptera such as Helicoverpa armigera, Maruca vitrata, Etiella zinckenella, Lampides spp. and pod-sucking Hemiptera that cause 30-70% loss in yield (Minja, 2002). The losses of crop production without the use of pesticides and other non-chemical control strategies have recently been estimated to be almost 70% of the total production. The pre-harvest losses due to insect pests are about 15% of total production (Krattiger, 1997). The exclusive use of chemical pesticides not only results in rapid build-up of resistance to such compounds, but their non-selectivity also affects the balance between pests and natural predators, and that is generally in favor of pests. Though exogenous natural pesticidal agents such as Bt toxin are also effective in deterring plant predators, they create environmental safety and consumer health debates in future in addition to a multitude of ethical concerns (Gatehouse et al., 1998). New options to control pest losses must be on priority. A novel approach is to use plant defense proteins that are more specific against insect pests.

Plants synthesize a wide range of complex chemical molecules to defend themselves against insect attack (Bate and Rothstein, 1998). These weapons of defense include antibiotics, alkaloids, terpenes as well as proteins such as enzymes, enzyme inhibitors and lectins. Proteinaceous proteinase inhibitors (PIs) active against insect proteolytic enzymes are key players among these molecules (Ryan, 1990; Koiwa et al., 1997). The presence of inhibitors of digestive proteases in plants particularly in legumes has been known since 1938 (Read and Hass, 1938). PIs in large amounts have been detected in legume seeds and other storage organs of plants. These could therefore be excellent alternatives to the toxins from Bacillus thuringiensis. The toxins of plant origin conferring resistance when expressed in transgenic plants include tobacco, rice, cotton, strawberry and pea (Ussuf et al., 2001).

2.1 General properties of protease inhibitors

Proteolytic enzymes, also called “proteases” that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins and are widely distributed in nearly all plants, animals and microorganism (Haq et al., 2003; Lawrence and Koundal, 2002; Ryan, 1990). The “protease” includes both “endopeptidases” and “exopeptidases” whereas the term “proteinase” is used to describe only “endopeptidases” (Ryan, 1990). Being essentially indispensable to the maintenance and survival of their host organisms, proteases play key roles in many biological processes (Habeeb and Fazili, 2007). One important mechanism involves interaction of active enzymes with proteins that inhibit their activities. These inhibitors form less active or fully inactive complexes with their cognate enzymes and are
called protease inhibitors (Pis). Pis are of very common occurrence; the majority of Pis studied in plant kingdom are from three main families namely, *Leguminosae*, *Solanaceae* and *Gramineae* (Richardson, 1991). There have also been many examples of Pis active against certain insect species, both in *in-vitro* assays against insect gut protease (Pannetier *et al.*, 1997; Koiwa *et al.*, 1998) and in *in-vivo* artificial diet bioassays (Vain *et al.*, 1998).

Protease inhibitors are small protein generally present in high concentration in storage tissues, contributing up to 10% of the total protein content (Habeeb and Fazili, 2007). They vary in size from Mw 4kD to 80kD, but are usually in the range of 8kD to 20kD. This is particularly true for the legumes serine Pis. Many of these inhibitors, especially those that have a molecular weight of around 10kD are capable of inhibiting simultaneously two molecules of enzymes per inhibitor molecule. These may be present as dimers or tetramers. The specificity of inhibitors is due to the active sites for the inhibition of proteolytic enzymes. The Trypsin specific inhibitors always have either a lys-X or arg-X sequence at the binding site whereas Chymotrypsin specific inhibitors usually have a leu-X at their active centers.

Pis are able to express at levels from 0.5 to 1.0% of total soluble proteins that confers protection to the plants (Gatehouse and Gatehouse, 1998). These inhibitors are found constitutively in various parts of plant or may be induced in response to herbivore attack. Leaves normally contain very low PI levels, but Green and Ryan, (1972) found that Pis are actively induced to high levels in leaves when they attacked by insect or mechanically damaged. Signals from the wound site were transported through the phloem and stimulated the synthesis of Pis throughout the plant. At the same time they become more in concentration at the site of injury.

### 2.2 Classification of protease inhibitors

Pis are distributed throughout the plant kingdom. These are anti-metabolic proteins that form complexes with proteinase and inhibit their proteolytic activity. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as Pis, which inhibit different types of enzymes. Their occurrence in the aerial part of plants, as a consequence of several stimuli has also been widely documented. Koiwa *et al.*, (1997) described that in plants more than ten protease inhibitor families have been recognized which fall in the four mechanistic classes of proteolytic enzymes based on the active amino acid in their reaction center (Table-I). There are several non-homologous families of protease inhibitors recognized among animals, microorganisms and plant kingdoms (Laskowski and Kato, 1980). These inhibitor families that have been found are specific for each of the four mechanistic classes of proteolytic enzymes. These are classified as serine, cysteine, aspartic and metallo proteases.
Table 1. Families of plant protease inhibitors with their sources

<table>
<thead>
<tr>
<th>CLASS OF ENZYME</th>
<th>FAMILY</th>
<th>CROPS REPORTED</th>
</tr>
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<tbody>
<tr>
<td>Serine Protease</td>
<td>Soybean Trypsin Inhibitor (Kunitz) (SBTI-K)</td>
<td>Pea, Soybean, Cowpea, Castor-Bean, Pigeonpea, Spinach, Tobacco</td>
</tr>
<tr>
<td></td>
<td>Bowman-Birk Inhibitor (BBPI)</td>
<td>Vigna mungo, Soybean</td>
</tr>
<tr>
<td></td>
<td>Barley Trypsin Inhibitor</td>
<td>Barley</td>
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<tr>
<td></td>
<td>Potato Inhibitor I</td>
<td>Tomato, Potato</td>
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<tr>
<td></td>
<td>Potato Inhibitor II</td>
<td>Tomato, Potato</td>
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<td></td>
<td>Squash Inhibitor</td>
<td>Pumpkin</td>
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<tr>
<td></td>
<td>Ragi 1-2/Maize Bifunctional Inhibitor</td>
<td>Maize, Ragi, Wheat</td>
</tr>
<tr>
<td>Cysteine Protease</td>
<td>Cysteine Protease Inhibitor (Cystatins)</td>
<td>Pineapple, Potato, Corn, Rice, Cowpea, Mungbean, Rye, Millet, Wheat, barley</td>
</tr>
<tr>
<td>Aspartic Protease</td>
<td>Aspartyl Protease Inhibitor</td>
<td>Potato</td>
</tr>
<tr>
<td>Metallo Protease</td>
<td>Carboxypeptidase A, B Inhibitor</td>
<td>Potato, Tomato</td>
</tr>
<tr>
<td></td>
<td>Cathepsin D</td>
<td>Potato</td>
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</table>

2.2.1 Serine protease inhibitors

This is the largest and most widely spread super family of the PIs. Serine PIs are irreversible suicidal inhibitors. They play the role of defensive compounds mainly against predators. The major proteases present in plants are used for processes such as protein mobilization. They contain a cysteine residue as the catalytic active nucleophile within the enzyme active site. The major role of serine PIs in animals is to block the activity of endogenous proteases in tissues where this activity would be harmful. All serine proteases inhibitor given in Table-1 are competitive inhibitors. The serine and metallo proteases are most active at pH optima of 9-11, which is found in the guts of many Lepidoptera insects (Ryan, 1990). Serine proteinase inhibitors found in plants are typically polypeptides composed entirely of L-amino acids linked through peptide bonds. These inhibitors generally contain high percentage of half-cysteine residues, present as disulfide cross-links. They lack or have very low amounts of methionine, histidine and tryptophan but are rich in aspartic acid, serine and lysine residues. Serine proteinase inhibitors vary in size from 4 to 60 kDa. They are generally stable molecules and are often resistant to heat, extremes of pH and
proteolysis by proteases. This stability is partly due to the high degree of cross-linking through disulfide bridges and significantly contributed by non-covalent interactions.

(i) Kunitz inhibitors

This was the first plant inhibitor, which was characterized as soybean trypsin inhibitor. Kunitz in 1947 isolated, crystallized and studied the inhibitor chemistry. Kunitz soybean inhibitor had 181 residues and only two disulfide bridges and single headed active site (Koide et al., 1973). Kunitz inhibitor is resistant to denaturing agents such as urea and guanidinium chloride (Blow et al., 1974). An acid stable and heat labile protease inhibitor found in leaves of spinach was isolated by sequential chromatography and preparative isoelectric focusing by Satoh et al., (1985). The isolated inhibitor was competitive in nature and specific to serine protease. It had a single reaction site and strongly inhibited trypsin. The pure trypsin inhibitor isolated form cowpea was reported to be an anti-nutrient agent against wide range of insects including larvae of Heliolthis, Spodoptera, Diabrotica and Tribolium all agronomically important insect pests (Gatehouse and Boulter, 1983). Kunitz-type inhibitors are made up of one polypeptide chain or two disulphide linked chains containing an arginine residue in their single reaction site. In spite of differences in primary structure and topology, the reaction centre structure and mechanism of action are well preserved among serine protease inhibitors.

(ii) Bowman-Birk inhibitor

The family is named after D.E. Bowman and Y. Birk, who were the first to identify and characterize a member of this family from soybean (Bowman, 1944; Birk, 1985). The Bowman-Birk inhibitors are double headed and small polypeptides (8-16 kD) typically found in legume seeds. They bind independently to two separate protease molecules such as trypsin and chymotrypsin (Hajela et al., 1999). The Bowman-Birk inhibitor was first identified in 1944 by Bowman from Glycine max and characterized by Birk in 1985. The Bowman-Birk inhibitors are especially rich in cysteine residues and have seven disulfide bonds but devoid of glycine and tryptophan. Because of disulfide bonds these proteins show high stability towards heat, acid and alkali. The inhibitor was extremely heat stable and only 50% inhibitory activity was lost after heating at 100°C for 20 min whereas the protease inhibitor isolated from rice bean (Vigna umbellaia) had a molecular weight at 16.8 kD and lost its activity at 100°C (Maggo et al., 1999). A double-headed protease inhibitor was isolated from Phaseolus mungo having a molecular weight of 14.2 kDa (Hajela et al., 1999).

(iii) Barley trypsin inhibitor

The barley has a lysine rich serine protease inhibitor which is homologous with potato inhibitor I (45% of the amino acids in identical positions). The barley inhibitor is the first
example described by Svendsen et al., (1980) as a protease inhibitor from higher plants in which the structure and reactive site is not stabilized by disulfide bonds.

(iv) Tomato/Potato inhibitor I and II

The inhibitor I having only chymotrypsin inhibitory activity while the Inhibitor II has both trypsin and chymotrypsin inhibitory activity. Two protease inhibitors called inhibitor I and inhibitor II accumulate in tomato and potato leaves wounded by insect attacks or other severe physical damage and are considered to be anti-nutrients produced as a defense response against attacking pests (Green and Ryan, 1972; Ryan, 1980). The two inhibitors from potato tubers have been isolated and characterized (Melville and Ryan, 1973). Plunkett et al., (1982) purified and characterized inhibitor I and II from wounded tomato leaves and compared their properties with two homologous inhibitors, which were isolated from potatoes, but their synthesis and accumulation in tubers was not wound regulated. The homologous of potato PIs I and II have also been found in non-solanaceous plants like alfalfa, broad bean, clover, cucumber, french bean, grape, squash, strawberry, barley and buckwheat (Adawy and Taha, 2001; Ye et al., 2001).

(v) Squash inhibitor

The inhibitors were found in towel gourd (Luffa cylindrica) and consist of 28 and 29 amino acid residues. The sequences had high similarity to other inhibitors of the squash family (Ling et al., 1993). The inhibitor isolated form phloem exudate of pumpkin fruit was shown to be effective against both trypsin and chymotrypsin (Murray and Christellar, 1995). The molecular weight of the protein was 8.1kD. Three full-length cDNA corresponding to one trypsin inhibitor and two chymotrypsin inhibitor have been isolated. These three genes are similar both in their translated and non-translated regions. These inhibitors have been shown to follow the standard mechanism for inhibition.

(vi) Ragil-2/Maize bi-functional inhibitor

The protease inhibitors were isolated from cereals crops. These bi-functional inhibitors have two types of independent reactive sites that inhibit alpha-amylases and proteases. These have been reported in cereal seeds (Shivaraj and Pattabiraman, 1981; Mundy et al., 1983). The double headed inhibitor of alpha-amylase and proteinase K was purified from wheat germ using Cu (II)-streamline-chelating resin. The purified protein had molecular weight of 21 kD (Roy and Gupta, 2000). The endogenous alpha-amylase could be inactivated by heating.

2.2.2 Cysteine protease inhibitors

The members of this family inhibit the activity of cysteine proteases. They are widely distributed in plants, animals and microorganisms (Olivera et al., 2003). These inhibitors are
grouped in four families based on sequence relationship, molecular mass and number of disulphide-bonds number and its arrangements (Turk and Bond, 1999). **Family-1 cystatins (stetin family):** The members of this group have a molecular mass of about 11kD. They are present in cytosol and are devoid of any carbohydrate group and disulphide bonds (Stato et al., 1990). **Family-2 cystatins (cystatin family):** This inhibitor contain two di-sulphide bonds but devoid of any carbohydrate group (Grzonka et al., 2001). All the family inhibitors contain a conserved tripeptide of sequence Phe-Ala-Val near the N-terminus. This sequences are important in binding to target proteases. **Family-3 (Kininogen family):** These inhibitors are glycol proteins and are of three different types as high molecular weight HMW (120 kD), low molecular weight LMW (60-80 kD) and third type with molecular weight of 68 kD has been reported. This family contains a conserved penta-peptide sequence Gln-Val-Ala-Gly involved in the inhibition of cysteine proteinase (Barrett, 1987) and **Family-4 (pytocystatins):** This family includes nearly all the cystein PIs of plants. The cysteine protease inhibitors of plant origin are known as phytocystatins which are 5-87kD and showed similar characteristics found in cystatin subfamilies I and II of mammalian cysteine PIs. Phytocystatins have been identified in a variety of monocot and dicot species, such as maize, rice, potato, soybean and apple (Abe et al., 1992). Majority of phytocystatins contain a single domain, whereas the multi-cystatins with multiple domains are also found in potato tubers, tomato leaves and sunflower seeds. The phytocystatins differ largely from animal cystatins by displaying high inhibitory activity towards insect gut proteases. The rice cysteine proteinase inhibitors are the most studied of all the cysteine PIs, which is proteinaceous in nature (Abe et al., 1987). Serine and cysteine proteinase inhibitors have deleterious effects when fed to lepidoptera and coleoptera respectively.

2.2.3 Aspartic protease inhibitors

Aspartic PIs have been described in sunflower, barley, cardoon flowers and potato tubers (Park et al., 2000). The optimum pH for these PIs is 3-5 and these works best in low pH of midguts of insects like coleoptera, hemiptera etc. Compared to cysteine proteinase, knowledge on the role of aspartic proteases in insect digestion is limited. However, cathepsin D-like proteases were found in six families of Hemiptera order. These proteases are active on low midgut pH (pH 3-5) of many members of Coleoptera and Hemiptera (Houseman and Downe, 1983; Houseman et al., 1987). Cathepsin D (Mares et al., 1989) shares considerable amino acid sequence identity with trypsin inhibitor SBT I from soybean.

2.2.4 Metallo protease inhibitors

They are metallo carboxypeptidase inhibitor family in potato and tomato plants (Graham and Ryan, 1997) and a cathepsin D inhibitor family in potatoes (Keilova and
The cathepsin D inhibitor (27kDa) is unusual as it inhibited trypsin, chymotrypsin and as well as cathepsin D, but did not inhibit aspartyl proteases such as pepsin, rennin or cathepsin E. The inhibitors of the metallo-carboxypeptidase from tomato and potato are small polypeptides (4 kDa) which competitively inhibited a broad spectrum of carboxypeptidases from both animals and micro-organisms, but not the serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen, 1985). The inhibitor is found in tissues of potato tubers where it accumulates during tubers development along with potato inhibitor I and II families of serine proteinase inhibitor. The inhibitor also accumulates in potato leaf tissues along with inhibitor I and II proteins in response to wounding (Graham and Ryan, 1981; Hollander-Czytko et al., 1985). The inhibitors accumulated in the wounded leaf tissues of potato had the capacity to inhibit all the five major digestive enzymes i.e. trypsin, chymotrypsin, elastase, carboxypeptidases A and carboxypeptidases B of higher animals and many insects (Hollander-Czytko et al., 1985). Prophytepsin, a zymogen of barley aspartic proteinase, has 13 amino acids at the N-terminal necessary for inactivation of mature phytepsin (Kervinen et al., 1999).

2.3 Physiological roles of protease inhibitors

Although the physiological role of the proteinase inhibitors in plants is poorly understood despite the fact that they often form an important part of the protein found in the tissues (Richardson, 1977). PIs in legume seeds irreversibly inhibit the action of the animal digestive enzymes and hence considered anti-nutritional (Ryan, 1990). PIs are assumed to regulate endogenous proteinase levels before and during seed germination for storage protein digestion and to control protein turnover (Baumgartner and Chrispeels, 1976). PIs protect the seed reserve from premature hydrolysis, but facilitate hydrolysis of protein for utilization in the germination process (Ussuf et al., 2001).

The PIs accumulate relatively late during seed development but rapidly increase during the desiccation phase, which shows their direct role in protein stabilization. In mungbean and lettuce, expression of trypsin inhibitors is induced by dehydration related stresses such as drought, salinity and abscisic acid (Xu et al., 1996). PIs protect plants from insect attack and microbial infection was established by studying the gene expression in leaves of several species. This has led to intense interest in identifying the PI gene from a wide array of plant sources including barley (Odani et al., 1983), tomato (Lee et al., 1986), rice (Abe et al., 1988), cowpea (Hilder et al., 1987), tobacco (Atkinson et al., 1993), alfalfa (McGurl et al., 1995), sweet potato (Yeh et al., 1997). Recently, a new role for PI in the modulation of apoptosis or programmed cell death was established in soybean. Cysteine proteases play an important role in the regulation of programmed cell death leading to
hypersensitive reaction following pathogen attack. It was suggested that balance between
cysteine proteinase and cysteine proteinase inhibitor activity regulates the programmed cell
death (Solomon et al., 1999). The main roles are as-

1. The PIs regulate endogenous proteinase levels before and during seed germination for
   storage protein digestion and to control protein turnover.
2. The PIs protect plants from insect attack and microbial infection.
3. They play important role in protein stabilization in cell.

2.4 Mechanism of protease inhibitors action

The researchers in this field during 1980s were trying to depict the mechanism of
protease inhibitor-enzyme interactions and that resulted in profound understanding of their
action and regulation in *in-vitro* and *in-vivo* conditions in animals, plants, microbes and
viruses (Huber and Carrel, 1989). They investigate the mechanism of action of the protease
inhibitors. However the mechanism of binding of the plant PIs insect proteases appears to be
similar with all the four classes of inhibitors. The inhibitor binds to the active site on the
enzyme to form a complex with a very low dissociation constant ($10^7$ to $10^4$ M at neutral pH
value), thus effectively blocking the active site. A binding loop on the inhibitor usually
“locked” into conformation by a disulphide bond, projects from the surface of the molecule
and contains a peptide bond cleavable by the enzymes (Walker et al., 1998). Thus inhibitor
directly mimics a normal substrate for the enzyme, where as its normal enzyme activity is
inhibited (Walker et al., 1998). However, ingestion of PIs does not eliminate proteolytic
digestion in the midgut of insect. Instead it results in hyper production of proteolytic
enzymes. This leads to limitation of essential amino acids for protein synthesis and thus,
reduction in growth and development (Broadway and Duffey, 1986a). The specificity of the
inhibitor enzyme interaction is primarily determined by the specificity of proteolysis
determined by the enzyme.

The potential of the PI activity dependent-

i) The structural compatibility of its reactive site with substrate-binding site of targeted
   proteases.
ii) The physiological conditions within the midgut (e.g. temperature; pH) and
iii) The dietary quality (e.g. polyphenyloxidase activity, protein quality and quantity)
   (Broadway and Duffey, 1986a).

The conditions under which digestive processes take place considerably differ
between insects and mammals. Most mammals have an acidic foregut and neutral midgut,
which is the major site of digestion of proteins. The mammalian endoproteases thus function
at pH~7-8. In contrast the midgut of phytophagous coleopterans Larvae have highly acidic
conditions with pH optima of digestive enzymes typically range from 4-5. (Liang et al., 1991). The lepidopteran larvae midgut was found highly alkaline and the digestive proteases have optimal activity at pH 10-11 (Johnston et al., 1991).

The secretion of proteases in insect guts depends upon midgut protein content and not the food volume (Baker et al., 1984). The secretion of food 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 (Applebaum, 1985). The peptidases are secreted into the ectoperitrophic space between the epithelium as a particulate complex (Eguchi et al., 1982) from there the proteases move transversely into the lumen of the gut where degradation of food proteins takes place. Inhibitors 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 (Shukle and Murdock, 1983) as a result of which the insects become weak with stunted growth and ultimately die. For a few PIs, functions other than blocking protease action have been found, such as growth factor activities, receptor clearance singling or involvement in carcinogenesis.

Coleopteran and hemipteran species tend to utilize cysteine proteinase (Murdock et al., 1987) while lepidopteran, hymenopteran, orthopteran and dipteran species mainly use serine proteinases (Wolfson and Murdock, 1987; Ryan, 1990). The effect of class specific inhibitors on the pest digestive enzymes is not always a simple inhibition of proteolytic activity (Taylor et al., 1995). There are often two populations of digestive enzymes in target pests, those that are susceptible to inhibition and those that are resistant (Michaud et al., 1996). Some insects respond to ingestion of plant PIs such as soybean trypsin inhibitor (Broadway and Duffey, 1986a) and oryzacystatin (Michaud et al., 1996) by hyper producing inhibitor-resistant enzymes.

2.5 Regulation of protease inhibitor genes

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 (Koiwa et al., 1997). A signal responsible for the translocation of the wound response includes systemin, abscisic acid (ABA), hydraulic signals and electrical signals (Malone and Alarcon, 1995). These are translocated from the wound site through the xylem or phloem as a consequence of hydraulic dispersal. The wound response pathway begins after wounding when prosystemin (precursor protein of sytemin) releases systemin (an 18-mer peptide), which is translocated throughout the plant where it is perceived by the target tissues and subsequently transduced
by the octadecanoid pathway and results in the expression of a battery of genes including protease inhibitor (Pin) genes. Systemin is an 18-mer peptide that was first isolated from wounded tomato leaves, which strongly induced expression of protease inhibitor (PI) genes. Transgenic plants expressing pro-systemin antisense cDNA exhibited a substantial reduction in systemic induction of PI synthesis, and reduced capacity to resist insect attack (McGurl et al., 1994). Abscisic acid is a candidate as the local and systemic signal molecule for the transduction of the wound response. 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).

The activation of over 20 defensive genes has been shown by systemin in tomato plants in response to herbivorous and pathogenic attacks. The polypeptide activates a lipid-based signal transduction pathway in which linolenic acid is released from plant membranes and converted into an oxilipin-signaling molecule, jasmonic acid (JA) (Ryan, 2000). Expression of wound and JA inducible genes can be positively and negatively regulated by ethylene or salicylic acid, which is the component of the pathogen-induced signaling pathway (Bent, 1996). Plants sometimes specifically forego one type of defense response for another. Salicylic acid (SA) and its methylester (Me-SA) are both defense compounds that potently induce systemic acquired resistance of plants against pathogenic micro-organisms (Hunt et al., 1996). At the same time, SA itself negatively regulates the OD pathway through inhibition of SA biosynthesis and activity (Korth and Dixon, 1997) indicating that SA may suppress the plant defense response through attenuation of the OD pathway, but its methylester positively affects plant defense through another defense mechanisms involving tritrophic plant herbivore interaction (Moura and Ryan, 2001).

By using biochemical genetic approach demonstrated that cyclopentenone precursor of JA, 12-oxophytodienoic acid (OPDA), as a physiological signal eliciting defensive response and resistance in the absence of JA. Also the potential of jasmonate and arachidonate as inducers of partial resistance to the potato beetle in potato was established which suggested the digestive compensation in herbivorous insects by defense related compounds found in plant in response to different stress stimuli (Rivard et al., 2004).

2.6 Isolation of protease inhibitor proteins

The Pis are small proteins generally present in high concentration in storage tissues, contributing up to 10% of total protein content (Habib and Fazili, 2007). They are detectable in leaves in response attack of insect pest and pathogens (Ryan, 1990). The seeds and tubers of Gramineae, Leguminosae and Solanaceae families have natural protease or proteinase inhibitors (Richardson, 1991; Connors et al., 2002).
Baumgartner and Chrispeels, (1976) were homogenized the cotyledons of mung bean (Phaseolus aureus Roxb.) with 25 mM citrate-phosphate (pH 5.7) containing 10mM β-mercaptoethanol and 0.02% sodium azide. The homogenate was centrifuged at 20,000g for 20min and the supernatant used as a source of enzyme. Protein concentration of supernatant was measured quantitatively according to the method of Lowey et al., (1951) using BSA as a standard. Affinity chromatography was performed with a gel consisting of trypsin covalently linked to Sepharose 4B. The gel was equilibrated with 50 mm citrate-phosphate (pH 7.8) containing 100 mm NaCl and packed in a column (1x10cm). Samples were loaded and the proteins were eluted with the starting buffer until elute become free from proteins. The molecular weight of inhibitor was determination by gel filtration chromatography using Sephadex G-100 according to the method of Andrews (1964). The standards used as riboflavin, actinomycin D, Cyt, and pancreatic ribonuclease.

Shoji et al., (1986) extracted PI proteins from defatted wheat germs by shaking with 0.2M acetic acid at 4°C for 15 hours. The extract centrifuged and adjusted pH 7.5 with 1N NaOH of the supernatant. The supernatant was loaded onto a column (5x15cm) to which 1g of bovine trypsin had been coupled by the CNBr method. The column was washed with 0.5M NaCl in 0.01M Tris-HCl containing 0.01M CaCl2 (pH 7.2) until absorbance at 280 nm of the washing became lower than 0.01A. Trypsin inhibitors were eluted by 0.05N HCl containing 0.01 M CaCl2, neutralized by 2M Tris-HCl (pH 8.0), and condensed to a small volume. The affinity- purified fraction was separated by gel filtration on a Sephadex G-75 column (4.5x90cm) equilibrated and developed with 0.025M Tris-HCl (pH 8.0) containing 0.5 M NaCl. Active fraction were pooled separately and lyophilized after dialysis. Active fraction from Sephadex G-75 were dissolved in 0.05M sodium acetate buffer (pH 5.0), and applied to a column of CM-Cellulose (1.0x25cm) equilibrated with the same buffer. Elution was conducted by a linear gradient of concentration from 0 to 0.8 M in the acetate buffer. Active fractions were pooled separately to minimize cross-contamination and lyophilized after extensive dialysis to distilled water.

Domoney et al., (1993) extracted trypsin inhibitor from seed meal of Pisum abyssinicum by shaking with 10 volumes of 50mM sodium borate pH 8.0 for 2h at 4°C. The 40-60% ammonium sulphate fractions were used for further purification of proteins. The protein concentrations were determined by the dye-binding assay of Bradford (1976) using BSA as standard throughout. Proteins were loaded on to a Sephacryl HR S100 column (XK 16/70 column), which was pre-equilibrated in the same buffer at 6°C. Proteins were fractionated (flow rate of 0.5 cm-3 min-1) and fractions of 1.0 cm3 were collected. Samples from alternate fractions were used for assays and for gel analysis. Protein mobility was
checked on SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using 20% (w/v) running gels.

Pichare and Kachole, (1994) isolated PI proteins from pigeonpea defatted seed flours with five volumes of 0.05N HCl for 12h at 5°C. Extracts were treated polyvinylpyrrolidone (final concentration of 2%) to remove phenolics. Extracts were analyzed for trypsin and chymotrypsin inhibitors by SDS-polyacrylamide (12%) gel electrophoresis and 10% native-polyacrylamide gel using Tris-HCl buffer (pH 8.3).

Mulimani and Paramjyothi, (1994) stirred defatted pigeonpea meal with of 50mM phosphate buffer pH 7.6 and centrifuged (1000xg) for 20 minutes. The supernatant was dialyzed against same buffer and then used for inhibitor assay by Casien digestion method of Sumathi and Pattabiraman, (1997).

Prathibha et al., (1995) homogenized the peeled tubers of cassava, yams, aroids and coleus with three volumes of 20mM phosphate buffer pH 7.6. After keeping for 15 minutes the homogenate was centrifuged at 5000 rpm for 20 minutes. The supernatant was used for assay of inhibitory activity after protein estimation. Protein was estimated in inhibitor extracts by the method of Lowry et al., (1951).

Ambekar et al., (1996) were extracted the trypsin inhibitor proteins from defatted flour of pigeonpea with six volumes of distilled water containing 1% PVP (Polyvinylpolypyrrolidone, w/v). Protein was estimated according to Lowry et al., (1951). Trypsin inhibitor assay was performed according to modified method of Kunitz as described by Pichare and Kachole, (1992).

Maggo et al., (1999) prepared the crude protein extracts of rice bean (Vigna umbellata) by stirring defatted flour with ten volumes 0.1M Phosphate buffer (pH 7.5) for 2h at room temperature in a shaking water bath. The suspension was then centrifuged at 10000 x g for 30 min and to the supernatant 85% (NH₄)₂SO₄ was added and the protein allowed precipitating overnight at 4°C. The precipitated protein was collected by centrifugation at 10000 x g for 15 min and the pellet re-suspended in a small volume of phosphate buffer and dialyzed exhaustively for 24h against the same buffer and then subjected to ion exchange chromatography on DEAE-cellulose column (26 x 2.6 cm; flow rate 35ml h⁻¹). The column was first eluted with the same buffer to wash out the unbound proteins. After washing, the bound proteins were eluted with a linear salt gradient of 0.1 to 0.5 M NaCl in phosphate buffer (0.1 M, pH 7.5). Fractions of 5ml each were collected and monitored for protein content at 280nm and also analyzed for trypsin inhibitor activity.

The purity of fraction was checked by native-PAGE using 10% acrylamide gel anionic system of Davis, (1964). The polypeptide composition was determined by SDS-
PAGE. Protein bands were located by staining the gels in 0.25% Coomassie Brilliant Blue R-250 in methanol: acid: water (8:1:20, v/v/v). Molecular weight of the purified trypsin inhibitor was determined by gel filtration through Sephadex G-100 column was calibrated with standard marker proteins viz. Bovine serum albumin (66kD), carbonic anhydrase (29kD) and cytochrome c (12.4kD) while the molecular weight markers used for SDS-PAGE were bovine serum albumin (66kD), egg albumin (45kD), β-lactoglobulin (18.4 kD) and lysozyme (14.4kD). The samples had pronounced antitryptic activity progressive decrease with increase time of heating. The rice bean inhibitor also appeared to have two pH optima one at pH 6.0 and the other at pH 10.0.

According to Hajela et al.,(1999) the seeds of Phaseolus mungo were homogenized with 0.01M phosphate buffer (pH 7.5) with 0.1M NaCl for 10 minutes and then stirred for one hour. The homogenate was centrifuged at 10000xg for 30 minutes and supernatant was heated at 80°C for 20 minutes. The homogenate was centrifuged at 10000xg for 30 minutes. The supernatant was saturated to 80% with ammonium sulphate. The precipitate was dialyzed against same buffer and subjected to ion exchange chromatography on a DEAE column (15cm x2cm). The fractions showing trypsin inhibition were pooled and subjected to gel filtration on Sephadex G-50(50x2cm). The peak fractions showing inhibitory activity were re-chromatograph on the same column. The purity of the preparation was checked by HPLC on Shodex protein KW-803 gel exclusion column. The protein content was determined according to the method described by Lowry et al., (1951) using bovine serum albumin as standard. The molecular weight of trypsin inhibitor a denaturing conditions was measured by SDS-PAGE method. The molecular weight of the native inhibitor was determined by Andrew’s plot between Ve/Vo and log M. The plot fits the equation Ve/Vo=-0.8357 log M+5.1618. The standard molecular weight markers used were ovalbumin, chymotrypsin A, ribonuclease-A and cytochrome-c.

Mulimani et al., (2001) stirred acetone defatted flour of red gram in 2:3 ratio (w/v) with 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% polyvinyl-pyrolidine for 4hrs at 5°C. The extract was centrifuged at 12000 x g at 4°C for 15 minutes and supernatant was used for ammonium sulphate fractination from 0-40%, 40-60% and 60-90% in cold. The precipitate of each fraction was collected by centrifugation at 12000 x g at 4°C for 15 minutes. The obtained pellet was dissolved in the minimum quantity of distilled water and dialyzed extensively, using a membrane with a cut of range of Mr 12400 (Sigma grade), against 10mM Tris HCl buffer (pH 7.6) overnight. Protein concentration was estimated by method of Bradford, (1976) using bovine serum albumin as the standard.
Sudheendra and Mulimani (2002) were defatted the seed flour of various legumes as pigeonpea, chickpea, mungbean and soyabean, by washing with acetone (thrice) and hexane (twice), and air-dried. The defatted flour was stirred with 50ml of 0.1M Sodium phosphate buffer (pH 7.1) for 4h at room temperature (28°C). The mixture was centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was dialyzed with distilled water and used as an inhibitor source. Protein concentration was assayed by method of Bradford (1976).

Haq and Khan (2003) were soaked the seeds of *Cajanus cajan* for overnight in 20mM Tris-HCl buffer (pH 8.2) containing 2mM CaCl₂. Thereafter it was homogenized in a blender for 2-3 minutes at moderate speed. The crude extract was filtered a cheese cloth to remove the coarse residual matter. Then it was centrifuged at 8000 rpm for 15 min at 4°C. The obtained clear supernatant was defatted with 0.1M acetic acid (pH 4.0). After overnight the extract was subsequently centrifuged at 8000 rpm for 45 minutes at 4°C. The supernatant was adjusted to pH 7.0 with liquor ammonia and saturated 50 % with ammonium sulphate. The precipitate was collected by centrifugation at 8000rpm for 45minutes at 4°C. The precipitate was suspended in minimum volume of 2mM CaCl₂-20mM Tris-HCl (pH 8.2) and dialyzed against the same buffer for 20 hrs. The dialyzed sample (10ml) was applied to DEAE cellulose column (2.2 x 9.9cm) equilibrated with 2mM CaCl₂ in 20mM Tris-HCl (pH 8.2) and the bound protein eluted with the same buffer with same buffer containing 0.2M NaCl. The fractions containing inhibitory activities against trypsin and α-chymotrypsin were polled and used as a proteainase inhibitor. Measurement of protein concentration was made by the method of Lowry et al., (1951) using bovine serum albumin as a standard. The purified protein was used for SDS-PAGE on 15% polyacrylamide slab gel under reducing conditions (Laemmli, 1970). Proteins were detected by staining the gel with 0.1% coomassive brilliant blue R-250.

The mature seeds of *Cassia obtusifolia* were ground and extracted with distilled water (1:10, W/V) for 2 hrs under agitation at room temperature (LIO Hai et al., 2008). This extract was centrifuged at 6000xg for 20min and the sediment was submitted to a second extraction with 5 volume of distilled water. The supernatant was combined and utilized as the crude extract. The crude extract was heated at 55°C for 10 min and then centrifuged (600xg, 20minute) to remove coagulated proteins. The supernatant was precipitated with 30%-50% ammonium sulphate. The pellet was dissolved and dialyzed with 100 mM Tris-HCl buffer, pH 8.0 containing 20 mM CaCl₂ and insoluble materials were removed after centrifugation at 10000xg for 10 minutes. The supernatant was loaded in batches onto a trypsin-Sepharose affinity column (1cmx5cm) equilibrated with 100 mM CaCl₂. After washing extensively with 100mM Tris-HCl buffer, pH 8.0 containing 20 mM CaCl₂. The column was first eluted with
the same buffer, containing 0.5mM NaCl additional, and then eluted with distilled water. After the emergence of “non-retained” fractions, trypsin inhibitors were eluted with 50 mM glycine HCl buffer, pH 2.0 and were neutralized with 1.5 mM Tris. The inhibitor fractions were then lyophilized and applied to a Sephadex G-75 column (1cmx80cm) at a rate of 12ml/hour with 50 mM Tris-HCl buffer, pH 8.0. The active fractions were pooled and lyophilized. Protein concentration was determined by Bradford method using bovine serum albumin as a protein standard. SDS-PAGE was performed according to method of Laemmli, (1970).

2.7 Protease inhibitory assay

Kunitz, (1945) studied the toxicity of soybean inhibitors on the complete development of flour beetle, Tribolium confusum, a stored grain pest. Green and Ryan, (1972) investigated the changes occurring in potato and tomato plants upon feeding by adult Colorado potato beetle and their larvae. Shukle and Murdock, (1983) suggested that inhibition of protein digestion alone did not cause the adverse effects of these inhibitors but in addition, caused hyper-production of digestive enzymes and enhanced the loss of sulfur amino acids. The inhibitor shows broad spectrum of activity including suppression of pathogenic nematodes like Globodera tabaccum, G. pallida and Meloidogyne incognita by Cowpea trypsin inhibitor CpTI Williamson and Hussey, (1996). Cysteine protease inhibitors from pearl millet inhibit growth of many pathogenic fungi including Trichoderma reesei (Joshi et al., 1998). Girard et al., (1998) showed the high level of resistance of PI is conferred by proteolytic cleavage in beetle larvae. Tobacco and peas containing multi-domain PI precursor from Nicotiana alata showed increased resistance against Helicoverpa armigera.

Kakade et al., (1969) The trypsin activity was estimated as the by pipetting 0.2ml to 1.0ml of soyabean extract in a test tube in triplicate and adjusted the final volume 1ml with water. In each tube 1ml of trypsin was added and makeup the volume 2ml. The tubes were placed in a water bath at 37°C. To each tube was then added 7ml of BAPA solution, previously warmed to 37°C, and exactly 10 minutes later reaction was terminated by adding 1ml of 30% acetic acid to each tubes. After through mixing the absorbance of each tube was measured at 410nm against the control. One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 A unit at 410 per 10ml of the reaction mixture. Trypsin inhibitor activity is defined as the number of trypsin unit inhibited (TUI).

Domoney et al., (1993) conducted the protease inhibitor assay by pre-incubation of protease and inhibitor samples of Pisum abysinicum at 37°C for 10 minutes or overnight at 15°C before addition of substrate. The inhibitor PMSF was dissolved in absolute alcohol to 0.5M and pre-warmed at 37°C before use. The trypsin or protease also pre-incubated at 37°C for 5 min before addition of substrate under quantitative assay conditions. Assay reactions
containing PMSF and controls (containing absolute alcohol instead of PMSF) were centrifuged before their absorbance was measured.

Mulimani and Paramjyothi, (1994) used defatted meal supernatant of pigeonpea for determination of inhibitor assay by Casein digestion method of Sumathi and Pattabiraman, (1997). One unit of enzyme activity is defined as the amount which releases 10μg of tyrosine h⁻¹ under assay conditions. One unit of inhibitory activity is the amount of the inhibitor that suppresses one unit of proteolytic activity.

Ambekar et al., (1996) performed Trypsin inhibitor assay according to modified method of Kunitz as described by Pichare and Kachole (1992). They conducted the bioassay by feeding Helicoverpa armigera larvae on host and non-host Pls incorporated into an artificial diet (Giri et al., 2003). The basic diet was suplimented with the seeds extract of host and non-host plants in appropriate quantity to give equal TI units (3U/g of feed). The early second instars larvae were reared on this diet and any gain in weight was meticulously recorded on every 2nd day until pupation.

Hajela et al.,(1999) determined Phaseolus mungo protease inhibitor activity as the protease inhibitor was added to 20μg of the enzyme in 200μl of 10mM Tris-HCl buffer (pH 7.5) containing 20mM CaCl₂ and incubated at 37°C for 10 minutes. Residual trypsin activity was measured by adding 1ml of 1mM BApNA (N α-benzoyl-DL-arginine p-nitroanilide) stock and incubated for 10 minutes at 37°C. The reaction was terminated by adding 200μl of 30% acetic acid. The degree of inhibition was determined by measuring the optical density at 410 nm.

Nandeesha and Prasad, (2001) partially purified sababul trypsin inhibitor (STI) was mixed the basic Helicoverpa diet (Veerareddy and Bhattacharya 1990) at three levels, 5,000, 10,000 and 20,000TIU/ml of diet. Five days old larvae of mean weight 0.86 mg (ten each) were fed on control diet and diet with STI. The growth rate in terms of body weight at every three days intervals; number of days required for pupation, pupal weight and % mortality rate of the larvae were recorded.

Mulimani et al., (2001) assayed the proteinase inhibitory activity of red gram by Erlenger et al., (1961) method as incubating the seed extract with 15μg of Trypsin for 15 minutes at RT. To this pre-incubated mixture of 1ml, 1mM, BApNA in Tris HCl buffer pH 7.6 was added and incubated at 37°C for 10 min, and the reaction was stopped by adding 200μl of 30% acetic acid. The liberated p-nitroaniline was measured at 410nm in a spectrophotometer (Extinction coefficient for BApNA is 8800) one proteinase inhibitory unit is defined as the amount of inhibitor that inhibit one unit of proteinase activity.
Sudheendra and Mulimani, (2002) found out the proteinase inhibitory activity of pigeonpea, chickpea, mungbean and soybean by incubating the seed extract with 15µg of trypsin at room temperature (28°C). One ml of 1mM BApNA solution was added and incubated at 37°C for 10 minutes. Reaction was arrested by adding 200µl of 30% acetic acid. The liberated p-nitroaniline was measured at 410nm in a spectrophotometer. One unit of proteinase activity is defined as the amount of enzyme that caused an increase of one optical density (OD) unit. One proteinase inhibitory unit is defined as the amount of inhibitor that inhibits one unit of proteinase activity. Haq and Khan (2003) assayed the trypsin and chymotrypsin activity of Cajanus cajan PI by incubated with fixed amount of trypsin in 1 ml of 2mM CaCl\(_2\)-20mMTris-HCl (pH 8.2) at 37°C for 10 minutes. After incubation 5 ml of BApNA was added and the residual enzyme activity was measured by monitoring at 410 nm.

Shukla et al., (2005) had tested plant lectins and soybean trypsin inhibitor at 0.1% concentration through impregnation into artificial diet. Fifty mg of soybean trypsin inhibitor was dissolved in 5 ml of distilled water and mixed with 45 ml of artificial diet and stirred on a magnetic stirrer. For control treatment 5ml of distilled water was added to 45ml artificial diet. The prepared diet was dispensed in aliquots of five ml into glass vials (2.5cm x 8.0cm) and allowed to cool for 3 hrs. Larvae were released on the artificial diet with camelhair brush. The vials were kept in the insect rearing laboratory at 27- 2°C, 65-5% relative humidity and 12 hrs photoperiod. Larvae weight and mortality were recorded on fifth day after initiation of experiment. The observations were recorded upto pupation and adult emergence. Pupal weights were recorded one day after pupation. The remaining larvae were weighted and then killed in chloroform after 4 hrs starvation and oven dried at 65°C for 72 hrs.

LIAO et al., 2008 determined proteases inhibitor activity of Cassia obtusifolia by using N-abenzoylDL-arginine-p-nitroanilide as the substrate. Inhibitor samples of Cassia obtusifolia were mixed with the trypsin or Pieris rapae proteases in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM CaCl\(_2\) and incubated at 37°C for 10 minutes. To start the reaction, N- α benzoylDL- arginine-p-nitroanilide solution was then added to the mixture. After incubation for 10 min, the reaction was stopped by the addition of 33% (V/V) acetic acid. One unit of inhibitory activity (UI) was defined as the decrease of 0.1A units at 410 nm.

Grethel et al., (2008) assayed proteinase Inhibitory activity of N. alata on SDS-polyacrylamide gel using a modified version of the method of Hou and Lin (1998). The protein samples were dissolved in the sample buffer without either STT or boiling. After electrophoresis, gels were soaked twice in 25% isopropanol in 20mM Tris-HCl (pH 8.0) for 10 min each prior to any additional treatment. The gel was then dipped in 20mM Tris-HCl buffer (pH 8.0) for 30 minutes. Next the gel was placed in a trypsin solution 50 units of
bovine of bovine trypsin (Sigma) in 1 ml of 50mM Tris-HCl pH 8.0mM CaCl₂ for 30 min at 37°C. The gel was rinsed in the same buffer before incubation in a fresh substrate-dye solution and then incubated for 50 min in the dark at 37°C with 80ml of the substrate-dye solution. The substrate-dye solution consisted of 20mg of N-acetyl-DL-phenylanineβ-naphthyl ester (APNE,Sigma) in 8ml of N,N-dimethylformamide that was brought to 80ml with 72 ml of 50mM Tris-HCl (pH 8.0), 50mM CaCl₂ in which 40 mg of tertazotized O-dianisidine was dissolved. The gel was de-stained with 10% acetic acid for 30 minutes The soybean trypsin inhibitor was used as a positive control.

2.8 Effect of temperature and pH on protease inhibitor protein

Prathibha et al., (1995) was subjected inhibitory extract of tubers of cassava, yams, aroids and coleus on various temperatures ranging from 60°C to 100°C. The extracts were heated at different temperatures for 15 minutes and assayed for inhibitory activity against trypsin. Tubers were also peeled and cooked in a pressure cooker for 30 minutes. Cooked tubers were homogenized with 2.0 mM phosphate buffer (pH 7.6), and the inhibitor was prepared by acetone precipitation and also assayed for inhibitory activity.

Maggo et al., (1999) showed that the rice bean (Vigna umbellata) protease inhibitor exposed on different pH (3.0-10.0), it showed two pH optima one at pH 6.0 and the other pH10.0. The result on pH stability showed that rice bean trypsin inhibitor was stable without any decrease in anti-trypsin activity when exposed to a wide range of pH.

Hajela et. al,(1999) studied determined thermal stability on Phaseolus mungo protease inhibitor by incubated at temperature ranging from 40°C-100°C for 10 minutes and for pH stability, the inhibitor was incubated in 0.01 M buffer with pH ranging from 3.0 to 10.0 at room temperature as well as at 70°C for 10 min and the residual inhibitory activity was measured.

According to Haq and Khan, (2003) purified PI sample Cajanus cajan showed thermal stability when incubated at various temperature viz. 25°C, 30°C, 37°C, 56°C and 95°C in a thermostat for 30 minutes. The samples were then adjusted to 37°C and checked for residual inhibitory activity. The effects of pH on the inhibitory potential of C. cajan PI were also checked by pre-incubating the PI sample at the desired pH (2.0-10.0) for 10 minutes and then assaying for residual enzyme activity.

2.9 Characterization of protease inhibitor genes

A number of protease inhibitor genes were isolated from a wide range of crop species. The first gene used for transformation of tobacco was isolated from cowpea, cDNA clones encoding trypsin inhibitor by Hilder et al., (1987). Subsequently these genes have been isolated from many crop plants which include, tomato protease I and II isolated by Johnson
et al., (1989), rice aspartic protease inhibitor from genomic clones (Hashimoto et al., 1992), cysteine protease inhibitor from tomato cDNA clones (Linthorst et al., 1993), a Kunitz type protease inhibitor from potato cDNA and genomic clones (Ishikawa et al., 1994), rice cysteine inhibitor from cDNA clones (Shintani et al., 1995), a 481 bp protease inhibitor II clone isolated from Chinese cabbage flower buds and cysteine protease inhibitor from genomic library of tomato leaves by Johnston et al., (1989).

A large number of protease inhibitor genes isolated from soybean so far and the first being the Bowman-Birk protease inhibitors isolated by Hammond et al., (1984). Jourdrier et al., (1987) isolated a 484 bp cDNA clone encoding soybean inhibitor PI-IV. Mostly the protease inhibitors are products of multi-gene families (Ryan, 1990). A protease inhibitor of 997 bp long has been isolated from cowpea by Lawrence et al., (2001).

The transgenic rice with potato carboxypeptidase inhibitor (PCI) provides resistance against fungal pathogens carboxypeptidase and also insect's carboxypeptidase (Quilis et al., 2007). A full length StPI cDNA protease inhibitor was cloned in diploid potato and studied its amplification. This gene was strongly induced by JA and its mRNA accumulation increased more quickly than that of Rastonia solanacearum (Rs) inoculation. So StPI gene may play a role in potato resistance against Rs (Guang -cun et al., 2007).

Yuemin et al., (2007) identified the full-length cDNA of OCP1 1 from cDNA library of indica rice Minghui 63. The cDNA sequences of OCP1 1 showed 98.5% homology with the OsSCI3. Protein sequences of OCP1 1 showed 27-80% identity with various plant serine-protease inhibitors including the potato inhibitor I family.

Grethel et al., (2008) constructed cDNA library of with the SMART cDNA library construction kit (BD Biosciences, Palo Alto, CA USA) using a poly (A)" RNA from SI N. alata and cloned in TriplEx2™ according to the manufacturer's instructions. S-RNase, HT- and 120K cDNA probes were used to identify and discard some previously identified sequences impudence's implicated in Si. A total of approximately 31000 colonies were screened.

Rice genome contain 17 putative chymotrypsin protease inhibitor (with size from 7.21- 11.9 kDa) gene family. Full-length cDNA encoding for subtilisin-chymotrypsin protease inhibitor obtained from Pusa Basmati 1 (indica) rice seedlings. It has 219 bp long ORF which coding for 72 amino acid long 7.7 kDa subtilisin- chymotrypsin protease inhibitor (CPI) cytoplasmic protein (Amanjot et al., 2009).

2.10 Development of transgenic plants using protease inhibitor genes

A wide range of transgenic Plant has been developed using plant genetic engineering technique as shown in Table-2. A major advantage that genetic manipulation offers over
conventional plant breeding is the ability to improve specific characteristics, including resistance to pests, without risking the loss of existing desirable traits such as palatability, nutritional quality and yield. Since this technique enables the transfer of genes between unrelated taxa, it therefore greatly increases the pool from which desirable agronomic characteristics may be selected. Efforts have been made in the identification and purification of potential candidate proteins, insect bioassay to select those with the desired insecticidal properties, isolation of the encoding gene(s) and incorporation of these into vector constructs which express the protein adequately within the host plant, and in the appropriate plant tissues.

The protease inhibitor of insect digestive enzyme used to develop transgenic plants to control crop pests are designed not to kill the insects that feed, but to retard their development. This is the main difference between this strategy and the chemical pest control that aimed at complete control through pest mortality. Moreover the efficiency of defense proteins can be improved by the inhibitory effects of PIs by preventing their degradation by the target pest proteases (Jongsma and Bolter, 1997). Hilder et al., (1987) produces the first ever transgenic plants using cowpea trypsin inhibitor cDNA clone. These were resistant against herbivorous insects like Heliothis spodoptera, Tribolium sp. etc.

Johnson et al., (1989) transformed tobacco plants with gene coding tomato and potato inhibitory proteins and the plants were resistant to Manduca sexta. Sane et al., (1997) amplified the cowpea genomic DNA and cloned it in a plant expression vector coupled with CaMV35S promoter and NOS terminator and used it for tobacco transformation.

Recently, a protease inhibitor gene isolated from a native variety of cowpea was used to transform pigeonpea through Agrobacterium tumefaciens mediated genetic transformation. Molecular analysis of the in-vitro cultured transgenic pigeonpea plants confirmed the integration and stable expression of the protease inhibitor gene (Lawrence et al., 2001).

The Serine protease inhibitors are most commonly used inhibitor for development of resistant crop plants. The cowpea trypsin inhibitor (CpII) isolated from cowpea and has been mobilized into various crops such as cabbage (Hao and Ao, 1977), tobacco (Hilder et al., 1987; Lee et al., 1999; Ghoshal et al., 2001), oilseed rape, potato, sweet potato, rice (Xu et al., 1996), and strawberry (Graham et al., 1997) resulting in increased resistance in most cases. Transgenic cabbage demonstrated improved resistance to the lepidopteran caterpillars Pieris rapae (L) and Heliothis armigera (Hao, 1977). In tobacco, a 50% reduction in biomass of Spodoptera litura was demonstrated from CpII expressing plants (Sane et al., 1997). This reduction in biomass was also observed in potato on the tomato moth, Lacaonobia oleracea.
(Gatehouse et al., 1997). For the control of brown plant hopper the soybean kunitz trypsin inhibitor gene was expressed in rice plants (Lee et al., 1999). Gut proteases of larvae of rice stem borer were inhibited by the protein extracts from the transgenic rice plants expressing the winged bean trypsin inhibitor (Mochizuki et al., 1999).

The potential role of Mustard trypsin inhibitor MTI-2 was found by studying the interaction between insect pests and plants (Volpicella et al., 2001). The effects of MTI-2 expressed at different levels in transgenic tobacco, arabidopsis and oil seed rape lines was evaluated against three different lepidopteran insect pests (Leo et al., 2001). Nicotiana tabacum plants transformed with the cDNA of barley trypsin inhibitor though expressed protein but the growth of larvae of Spodoptera exigua (Lepidoptera) was not affected when reared on transgenic plants (Lara et al., 2000).

Qing-Yun Bu et al., (2006), isolated both cDNA and a genomic DNA fragment encoding a new potato proteinase inhibitor II were isolated from a diploid potato IVP101 (Solanum phurejia L.) and named PINII-2x. Nucleotide sequencing confirmed that the DNA fragment of PINII-2xwas 580 bp including a 115-bp intron and two exons. The deduced PINII-2x protein contained an intact signal peptide and two active sites. The PINII-2x gene and its deduced PINII-2x protein had 88% and 93% homology with another tetraploid potato proteinase inhibitor II, respectively. Northern blotting analysis indicated that the mRNA of PINII-2x gene was wound induced in potato leaves. Binary vector pNAR301 and pNAR302 were constructed for rice transformation, in which the PINII-2x cDNA was driven, respectively, by rice actin I promoter (ActI) and maize ubiquitin promoter (UbiI). Via an Agrobacterium-mediated method, these two constructs were transferred into japonica rice cv. Xiushui 63. PCR and Southern blotting analysis for transgenic rice revealed the integration of the PINII-2x gene. Insect bioassays using stripe stem borer (Chilo suppressalis Walker) demonstrated that the average weight and body length of larvae in transgenic plants were only nearly 50% and 61% of those of larvae in control plants, respectively.

A defensive role against insect attack has been traditionally attributed to plant protease inhibitors (Quilis et al., 2007). The potato carboxypeptidase inhibitor (PCI) provided resistance to fungal pathogens when expressed in rice as a heterologous protein. It is shown that rice plants constitutively expressing the PCI gene exhibit resistance against the economically important pathogens Magnaporthe oryzae and Fusarium verticillioides. Although pci confers protection against fungal pathogeris in transgenic rice, a significant cost in insect resistance is observed. A full-length cDNA of proteinase inhibitor gene with completed open reading frame of 116 amino acids was cloned from Ralstonia solanacearum.
and provide resistant to potato leaves using the rapid amplification of cDNA ends (RACE) method and designated as StPI (Guang-cun et al., 2007). This gene was strongly induced by JA and its mRNA accumulation increased more quickly than that of Rs-inoculation. The StPI gene may play a role in potato resistance against Rs.

Table 2- Developed transgenic plants containing insecticidal plant genes along with target insect

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant gene</th>
<th>Transgenic</th>
<th>Target insect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cowpea trypsin inhibitor</td>
<td>Tobacco</td>
<td>Heliothis virescens</td>
<td>Hilder et al., 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tobacco</td>
<td>Manduca sexta</td>
<td>Hilder et al., 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rice</td>
<td>Sesamia inferensia</td>
<td>Xu et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potato</td>
<td>Lacosamia oleracea</td>
<td>Gatehouse et al., 1997</td>
</tr>
<tr>
<td>2.</td>
<td>Mustered TI</td>
<td>Arabidopsis, tobacco</td>
<td>Lapidoptera</td>
<td>Jouanin et al., 1998</td>
</tr>
<tr>
<td>3.</td>
<td>Potato PI I</td>
<td>Rice</td>
<td>Sesamia inferensia</td>
<td>Duan et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Potato PI I &amp;</td>
<td>Tobacco</td>
<td>Manduca sexta</td>
<td>Johnson et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Potato PI II</td>
<td>Birch, lettuce</td>
<td>Lapidoptera,</td>
<td>Gatehouse et al., 1993</td>
</tr>
<tr>
<td>4.</td>
<td>Potato chymotrypsin inhibitor</td>
<td>Tobacco</td>
<td>Chrysodeixis eriosoma</td>
<td>McManus et al., 1994</td>
</tr>
<tr>
<td>5.</td>
<td>Sweet potato TI I</td>
<td>Tobacco</td>
<td>Spodoptera litura</td>
<td>Yeh et al., 1997</td>
</tr>
<tr>
<td>6.</td>
<td>Soybean Kunitz inhibitor</td>
<td>Rice</td>
<td>Nilaparvata lugens</td>
<td>Lee et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potato, tobacco</td>
<td>Lapidotera,</td>
<td>Marchetti et al., 1994</td>
</tr>
<tr>
<td>7.</td>
<td>Tobacco PI</td>
<td>Tobacco, pea</td>
<td>Helicoverpa armigera</td>
<td>Charity et al., 1999</td>
</tr>
<tr>
<td>8.</td>
<td>Cabbage Serine PI</td>
<td>Tobacco</td>
<td></td>
<td>Pulliam et al., 2001</td>
</tr>
<tr>
<td>9.</td>
<td>Soybean PI</td>
<td>Sugarcane</td>
<td>Diatraea Saccharalis</td>
<td>Maria et al., 2003</td>
</tr>
<tr>
<td>10.</td>
<td>Maize protease inhibitor</td>
<td>Rice</td>
<td>Chilo suppressalis</td>
<td>Vila et al., 2005</td>
</tr>
<tr>
<td>11.</td>
<td>Potato PI-II &amp;</td>
<td>Tomato</td>
<td>Heliothis obsoleta</td>
<td>Abdeen et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Carboxypeptidase inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Potato PIN-II-2x</td>
<td>Rice Xiushni 63</td>
<td>Chilo suppressalis W</td>
<td>Qing-Yun Bu et al.,</td>
</tr>
</tbody>
</table>

24
<table>
<thead>
<tr>
<th></th>
<th>Plant Family</th>
<th>Organism</th>
<th>Inhibitor (or Protein)</th>
<th>Species/Strain</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Fabaceae</td>
<td>Potato</td>
<td>Carboxypeptidase Inhibitor (PCI)</td>
<td><em>Fusarium verticillioides</em> and <em>Magnaporthe oryzae</em></td>
<td>2006</td>
</tr>
<tr>
<td>14</td>
<td>Fabaceae</td>
<td>Diploid Potato</td>
<td>Potato PI (StPI)</td>
<td><em>Ralstonia solanacearum</em></td>
<td>Guang-cun <em>et al.</em>, 2007</td>
</tr>
<tr>
<td>15</td>
<td>Poaceae</td>
<td>Potato and Tobacco</td>
<td>Buckwheat Serine protease inhibitor (BWI-Ia)</td>
<td><em>Psedomonas syringae</em> and <em>Clavibacter michiganensis</em></td>
<td>Khadeeva <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>16</td>
<td>Poaceae</td>
<td>Tobacco</td>
<td>Tobacco PI</td>
<td><em>Spodoptera litura</em> and <em>Helicoverpa armigera</em></td>
<td>Srinivasan <em>et al.</em>, 2009</td>
</tr>
</tbody>
</table>

The serine proteinase inhibitor genes from the plant families Fabaceae, Solanaceae and Poaceae, were introduced into susceptible crop plants which were targeted mainly against Lepidopteran species, coleopteran and orthopteran pests (Schuler *et al.*, 1988). The cowpea trypsin inhibitor (CpTI) inhibitor was isolated from cowpea, which has been transferred to more than ten other plant species (Lawrence *et al.*, 2001). The serine proteinase inhibitors KTI3, C-II and PI-IV from soybean resulted in up to 100% mortality of first-instar cotton-leaf worms (*Spodoptera littoralis*) when expressed in tobacco. But the resistance levels achieved by the same constructs in potato were much lower, resulting mainly in retarded growth of *S. littoralis* rather than direct mortality. The genes encoding cysteine protease inhibitors are now being used to control coleopteran insects. There have been several studies carried out demonstrating in vitro inhibition of insect digestive proteases by cysteine protease inhibitors.

The gene encoding *oryza* cystatin has been mobilized into potato (Benchekroun *et al.*, 1995) and rice (Vain *et al.*, 1998). Oryza cystatins I and II caused retardation of *Callosobruchus chinensis* (coleoptera) and *Riptortus clavatus* (Hemiptera) (Kuroda *et al.*, 1996). Corn cystatin (CC), a phytocystatin showed a wide inhibitory spectrum against various cystein proteases. Irie *et al.*, (1996) produced transgenic rice plants by introducing cDNA under CaMV 35S promoter. Corn cystatin prepared from transgenic rice plants showed potent inhibitory activity against proteinase that occurs in the gut of the insect pest, *Sitophilus zeamaize*. Genetically modified (GM) potatoes expressing a cysteine protease inhibitor (cystatin) have been developed for the management of plant parasitic nematodes. Khdeeva *et al.*, (2009) developed transgenic tobacco and potato by introducing a single gene encoding the serine protease inhibitor BWI-Ia from buckwheat seeds. It provides sufficient protection against two bacterial phytopathogens, *Psedomonas syringae* pv. tomato and *Clavibacter*.
michiganensis sbsp michiganensis. Srinivasan et al., (2009) confirmed resistance from insect pest as *Spodoptera litura* and *Helicoverpa armiger* in transgenic tobacco by constitutive expression of a trypsin inhibitor gene of tobacco. Luo et al., (2009) produced the transgenic tobacco by *Solanum americanum* protease inhibitor gene, which showed resistant for *Helicoverpa armigera* and *Spodoptera litura*.

The different resistance genes encoding insecticidal proteins isolated from various plant species and used one or more genes in combination. The products of these combinations will be targeted at different biochemical and physiological processes within the insect. These packages of genes will not contain protease inhibitor genes but also lectins, α-amylase inhibitors, or other plants gene 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. At present, screening gene pools without taxonomic constraint can help identifying novel insecticidal determinants, but in future this approach will be augmented by directed in vitro molecular evolution (Koiwa et al., 1998). Given the number of insecticidal 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 (Lawrence and Koundal, 2002).
3. MATERIALS AND METHODS

Materials

3.1 Seeds

The mature seeds of four varieties of pigeonpea viz. Pusa-855, Pusa-33, Pusa, Pusa-987 and Pusa-85 were procured from the Pulse Research laboratory, Division of Genetics, IARI, New Delhi and used for this investigation. For the developing stage seeds, pigeonpea was sown in the National Phytotron Facility, IARI, New Delhi.

3.2 Chemicals

All the chemicals like acrylamide, bisacrylamide, SDS, TEMED, APS, EDTA, agarose, DEAE Cellulose, Biogel P 100 & Sephadex G-50 etc. were of molecular biology grade and obtained from Sigma Chemical Company; St. Louis MO, USA. Antibiotics like ampicillin, kanamycin, and tetracycline, dyes like bromophenol blue and ethidium bromide were of regular grade and purchased from Sigma Chemical Company; St. Louis MO, USA.

Media NZY, Bacto-tryptone, Luria agar (LA) and Luria broth (LB) were procured from Difco laboratories, Detroit, MI, USA. Ammonium sulfate, DEPC etc. were purchased from Merck, Hohenbrun, Germany. Media like tryphtone, Luria agar (LA) and Luria broth (LB) were procured from Difco laboratories, Detroit, MI, USA. Ammonium sulfate, DEPC etc. were purchased from Merck, Hohenbrun, Germany. Chemicals like glacial acetic acid, Tris-base, Folin Ciocalteu’s Phenol Reagent, KOH, mono and dibasic potassium hydrogen phosphate were procured from SRL, Mumbai. The organic solvents like hexane, chloroform, isopropanol, acetone, n-Butanol, 2-Butanol and iso-amyl alcohol etc. were procured from Qualigens, Mumbai and ethanol was obtained from Bengal Chemicals, Kolkata.

3.3 Kits and Enzymes

SMART cDNA construction kit was purchased from M/s Clontech Laboratories, Palo Alto, USA. The Poly (A) + mRNA isolation kit was obtained from Qiagen, Genetic. HexaLabel Plus™ DNA labeling kit, RNase inhibitor and DNase etc. were procured from MBI Fermentas, Lithuania.

3.4 Other Chemicals and Miscellaneous items

Hybond N⁺ nylon membranes and Hyperfilm X-ray sheets were purchased from Amersham International Inc. UK. Developer and Fixer solutions were procured from Kodak Lab chemicals, USA. SDS-PAGE protein markers were obtained from Bangalore Genie Pvt. Ltd., Bangalore, India. Dialysis bags were obtained from Himedia, Mumbai. Plasticware like Eppendorf tubes, micropipette tips and Elisa plates (96 well U-bottoms) were purchased from Tarsons, Kolkata. All the glassware purchased from Schott-Duran, Mumbai. The plastic wares and glass wares were autoclaved at 15-lb/sq inch pressure for 20 minutes before their use for DNA and for RNA the plastic wares and glassware were autoclaved at 15-lb/sq inch pressure for 45 min after treatment of DEPC for over night.
Methods

3.5 Isolation of Protease Inhibitor (PI) proteins from mature seeds of Pigeonpea

3.5.1 Extraction of PI proteins

PI protein was isolated through modified method of Maggo et al., (1999) from defatted flour of mature seeds of four varieties viz. Pusa-855, Pusa-33, Pusa-84 and Pusa-987. Ten grams of defatted flour of different varieties homogenized in shaker with 100ml of 0.1M sodium phosphate buffer (pH 7.6) containing 2% polyvinyl pyrolidone. The homogenates centrifuged at 10,000 rpm for 30 min at RT. The crude extracts were heat denatured at 80°C for 20 min in a shaking water bath and snap cooled on ice bath. The extracts were again centrifuged at 10,000 rpm for 15 minutes at 20°C in RC 5 plus Sorvall centrifuge. The supernatants were subjected to ammonium sulfate fractionation as 0-20%, 20-40%, 40-60%, 60-80% and 80-100%. The obtained precipitates after ammonium sulfate fractionation were dissolved in 2.0 ml of 0.1M sodium phosphate buffer (pH 7.6) and dialyzed extensively against the same buffer. The dialyzed samples were used for estimation of protein and protease inhibitor activity.

3.5.2 Estimation of proteins

The concentration of proteins was determined by using Lowry's (1951) method. The stock solution BSA (10µg/µl) was taken as 1µl, 2µl, 3µl, 4µl, 5µl, 6µl, 7µl, 8µl, 9µl and 10 µl in a series of round bottom test tubes in duplicate. The volumes of all tubes were make-up to 100 µl with water and one another tube was taken as blank which also has 100 µl of distilled water. In each tube 5ml of solution C was added, mixed well by swirling and incubated at room temperature in for 10 minutes. After incubation 0.5ml of 1N Folin Ciocalteau regent was added and again incubated at room temperature in the dark for 30 minutes. The absorbance was taken for all the standards and samples at 660 nm using SAFAS Spectrophotometer. A standard curve (concentration versus absorbance) was plotted. On the standard curve 1µg of BSA gave 0.003 A at 660nm which was used for the estimation of protein concentration of unknown sample. Similarly 20µl-100µl of unknown sample was taken in duplicate along with the blank and took the absorbance at 660nm. By using standard value of BSA, the protein concentration of the unknown sample was estimated.

3.5.3 Enzyme Assay

The modified spectrophotometric Protease Inhibitor assay of Erlanger et al., (1961) and Kakade et al., (1969) used to determine the residual trypsin activity in all samples. Protein estimated samples used for inhibitory assay. Initially 5µl, 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, 70µl, 80µl, 90µl and 100 µl of trypsin solution (1mg/ml) were taken in a round bottom test tube and make up the volume 200µl with distilled water. In each tube 2.4ml of
reaction buffer was added and incubate at room temperature for 20 minutes. After incubation 300μl of BApNA (1mM) solution was added and mixed well. Incubate for 10 minutes and reaction was stopped by adding 100μl of 30% acetic acid and absorbance was taken at 410nm. The standard curve was prepared which is used for the estimation of residual trypsin activity of sample after inhibition. On the standard curve 1μg of trypsin gave the 0.05A which corresponds to 9.59 units i.e. one unit gave 0.005A. One unit of protease is defined as the amount of enzyme that causes an increase of 0.005A. One protease inhibitor unit is defined as the amount of inhibitor that inhibited 1 unit of protease activity. Mixing the equal amount of trypsin and PI protein with in reaction mixture and following the similar protocol as described above, the absorbance was taken of residual trypsin at 410nm and determined the inhibition by residual trypsin activity.

3.6 Purification of PI protein from Pigeonpea

3.6.1 Ion-exchange chromatography

The defatted flour (100gm) of mature seeds of Pusa-33 varieties of Pigeonpea was homogenized in shaking water bath with 1000ml of 0.1 M sodium phosphate buffer (pH 7.6) containing 2% polyvinylpyrrolidone. The supernatants were subjected to ammonium sulfate fractionation as 0-40%, 40-80% and 80-100%. The obtained precipitate of 40-80% was dissolved in 5.0ml of 0.1M sodium phosphate buffer (pH 7.6) and dialyzed extensively against the same buffer. The dialyzed sample of all four varieties used for purification by ion-exchange chromatography after estimation of protein and PI activity.

DEAE-Cellulose is an anion exchanger and used for binding the cationic proteins. Before use the DEAE-Cellulose was swollen overnight in distilled water. The slurry was activated using 0.5M NaOH for two hours then washed with distilled water till the pH reached 7.0. Now the slurry treated with 0.5M HCl for 2 hours and washed with distilled water till the pH became neutral. The activated DEAE-Cellulose used for column packaging (26x2.6cm) and equilibrated with 0.1M sodium phosphate buffer (pH 7.6). The 2.5mg dialyzed protein sample was loaded on the column unbound proteins were washed with 0.1M sodium phosphate buffer (pH 7.6). Bound proteins were eluted using 0.01-0.1M NaCl gradient in 0.1M sodium phosphate buffer (pH 7.6) in 5 ml fractions with the flow rate of 35ml/hr. Eluted fractions were monitored at 280nm for protein and trypsin inhibitor activity in each fraction was determined using BApNA as substrate as mentioned above. The fractions showing activity after ion-exchange Chromatography were pooled, lyophilized and used for heat and pH stability after gel-filtration.
3.6.2 Gel-filtration chromatography

The Biogel-P100 was swollen in distilled water for over-night and packed the column (70x1.6cm). The column was equilibrated with 0.1M sodium phosphate buffer (pH 7.6). The lyophilized fraction showing PI activity was loaded on the column and eluted the proteins using same buffer. The 5ml fractions were collected with the flow rate of 12ml/hr. Each fraction was again monitored at 280nm for protein and at 460nm for PI activity. The samples were lyophilized and used for molecular weight determination after protein estimation.

3.7 Characterization of Pigeonpea Protease Inhibitor (PPI) protein

3.7.1 Polyacrylamide gel electrophoresis (PAGE)

3.7.1.1 Native-polyacrylamide gel electrophoresis

The fraction showing PI activity after ion exchange and gel filtration was resolved on Native- PAGE by the method of Davis, (1964).

(i) Preparation of gel

The glass plates and spacers were thoroughly cleaned with water and air dried then assembled in gel casting apparatus. Resolving (6%) and stacking gel mixture was prepared by adding following ingredients in the same order-

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock acrylamide solution (30%)</td>
<td>8 ml</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>Tris buffer (0.1M)</td>
<td>10 ml</td>
<td>940 μl</td>
</tr>
<tr>
<td>APS (5%)</td>
<td>400 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>24 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Make up the volume with water</td>
<td>40 ml</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

All the ingredients as acrylamide stocks solution, tris buffer, TEMED and distilled water were mixed together in a flask. Then added freshly prepared (5%) APS solution in to it and immediately poured into the gel casting apparatus. A thin layer of distilled water was carefully overlaid and the gel was allowed to stand for 1hrour at room temperature. After resolving gel was polymerized, the overlaid was removed by inverting the apparatus. The stacking gel was prepare and poured over resolving gel. After pouring stacking gel, cleaned comb was inserted into that, without leaving any air-bubble between comb and gel and leave at room temperature for polymerization.
(ii) Electrophoresis

The glass plates along with gel were taken out from casting apparatus and assembled with vertical electrophoresis tank. Electrophoresis tank was then filled with electrode buffer and comb was carefully removed without disturbing the wells. The apparatus was connected to a circulating water bath to maintain the temperature at 10-12 °C of the buffer. The gel was pre-run at 25 mA for 20 minutes before loading the sample. The 50 μg of protein sample was taken and mixed with 6X sample buffer. The sample was then loaded into the well of gel and electrophoresed at constant current of 25 mA. When the tracking dye reached the bottom of the gel, the electrophoresis unit was switched off and gel was peeled off from glass plate with the help of spatula. The gel was kept in staining solution (0.1% Coomassie Brilliant Blue R-250) for 4 hrs. After staining, gel was removed from obtaining solution and kept in de-staining solution. The de-staining solution was kept changing at regular intervals to remove all unbound dye. After complete de-staining, the gel was photographed and finally stored in de-staining solution.

3.7.1.2 SDS-Polyacrylamide gel electrophoresis

The SDS-Polyacrylamide gel electrophoresis was performed using a 15% slab gel according to the method of Laemmli (1970) for molecular weight determination of protein.

(i) Preparation of gel

After cleaning the plates and assembly of PAGE prepared 15% resolving and 4% stacking gel was prepared by adding following ingredients-

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock acryl amide solution (30%)</td>
<td>20 ml</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>Tris buffer (1M)</td>
<td>10 ml</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>400 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>400 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>16 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Make up the volume with water</td>
<td>40 ml</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Both the resolving and stacking gel were prepared as mentioned earlier for native PAGE.

(ii) Electrophoresis

The gel along with glass plates were taken out after polymerization and assembled in a vertical electrophoresis tank. The electrophoresis tank was then filled with electrode buffer and the comb was carefully removed without disturbing the wells. The 100 μl (25 μg) of PPI protein sample was taken and mixed with 6X sample buffer. The sample mix was treated at
100°C for 2 minutes in boiled water and then immediately cooled on ice. The gel was pre-run at 25mA for 20 minutes before loading the sample. The sample was loaded onto the gel along with protein molecular weight markers and electrophoresed at constant current of 25mA till tracking dye reached the bottom of the gel. The gel was removed from the glass plates and stained and destained as mentioned earlier for native PAGE. The molecular weight of PPI protein was confirmed through gel filtration chromatography by using Sephadex G-50 column.

3.7.2 Gel-filtration chromatography

The native molecular weight of purified PPI was determined through gel filtration chromatography on Sephadex G-50 column. A glass column (70x1.6cm) was packed in lab by pre-swelled Sephadex G-50 in 0.1M sodium phosphate buffer (pH 7.6). Blue dextran 2000 was used for determination of the bed volume of the column. The column was calibrated with standard molecular weight markers viz. bovine serum albumin, carbonic anhydrase cytochrome C and aprotinin. The sample (5mg) was loaded on the column and eluted with same buffer with the flow rate of 12 ml/hr. Fractions of 2 ml were collected and monitored for absorbance at 280 nm then for PI assay.

3.8 Effect of temperature on PPI protein

Thermo stability of PPI protein of Pusa-33 varieties was determined according to the protocol of Godbole et al., (1994). The protein was subjected to various temperatures for 30 minutes ranging from ambient to 100°C and under autoclave. The inhibition activity was measured by standard assay as described earlier in Section 3.8.

3.9 Effect of pH on PPI protein

The purified sample of PPI protein was incubated on various pH at 37°C temperature for 30 minutes. The residual trypsin activity was measured by standard assay as reported earlier in Section 3.8.

3.10 Studies on developing seeds of Pigeonpea

3.10.1 Determination of moisture and dry weight content during seed development

The seeds of different stages were collected from tagged flowers from 5th days of flowering (DAF) to maturation by 5 days interval. The seeds of each stage were taken in replicate and kept for 24 hrs in oven at 80°C to dry. The weight of all seeds was taken until the decrease in weight becomes zero after successive incubation. This gave average dry weight of seed of that stage. The moisture content was determined according to standard as-
Fresh weight - Dry weight

\[ \text{Moisture content} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100 \]

3.10.2. Isolation of PI protein from developing seeds

PI proteins were isolated from developing seeds of P-33 of different stages starting from 6\textsuperscript{th} DAF to maturation with 6 days interval by using same method as from mature seed and total proteins were estimated by Lowry's method and PI activity was estimated with the protocol as described earlier in Section 3.8.

3.11 Insect Bioassay

3.11.1. Insects culturing

Insect bioassay was done at Division of Entomology, IARI New Delhi. A test colony of the insects (\textit{Helicoverpa armigera}) was maintained in an insectary at 24°C±2°C, 50-60% relative humidity (RH) and 14 hrs light. The photo phase started at 5:30AM. The rearing procedure followed is from NRI Bulletin 57 (Armes \textit{et al.}, 1992). The eggs of \textit{H. armigera} were incubated in 30 ml transparent cup with unwaxed cardboard lid at 20°C temp until they hatched. The newly hatched larvae were removed from the cup and transferred to chickpea based semi-synthetic diet (Veerareddy and Bhattacharya 1990). On seventh day, the larvae were weighed and transferred to semi-synthetic diet containing three levels of PPI protein.

3.11.2. Bioassay

For the determination of the toxicity of PPI protein on \textit{H. armigera} larval growth and development, the larvae were rear on semi artificial diet supplemented with PPI protein. The purified PPI was mixed in basic diet at three levels of treatments as T-1, T-2, and T-3 respectively (Nandeesham and Prasad 2001). The diet was supplemented with PPI protein as 0.5%, 1.0% and 1.5% in respective treatment and the observations were taken on alternate days from larvae to emergence of adult after pupation.

3.12 Southern hybridization of Pigeonpea genomic DNA with Tomato T III cDNA probe

3.12.1. Isolation of genomic DNA

The genomic DNA was isolated by Dellaporta \textit{et al.}, (1983) from eight days old etiolated seedlings of pigeonpea and grinding them to fine powder in liquid nitrogen to disrupt the cell wall. DNA isolating buffer was pre-heated at 65°C for 20min and 20μl of β-mercaptoethanol was added just before adding the ground material in DNA extraction buffer. After transferring the ground material in the autoclaved Oakridge tube containing buffer incubated at 65°C for 1 hr. Now equal volume (100ml) of chloroform: isooamyl alcohol (24:1) was added to this tube and contents were mixed well by inverting the tube and centrifuged on
11,000 rpm for 20 min at 4°C. The aqueous phase was collected in a fresh autoclaved Oakridge tube and added 0.6 volume (9 ml) of isopropanol then mixed well. Again the solution was centrifuged and the supernatant was discarded. The pellet was washed with 5 ml of 70% ethanol, air dried and suspended in 750 μl of TE buffer.

3.12.2 Purification of genomic DNA

In the 5 ml of genomic DNA samples, CsCl salt (0.90g/ml) of was dissolved completely. The 50 μl of ethidiumbromide (10mg/ml) was added to this solution was transferred to 5 ml Beckman ultra-centrifuged tube with the help of 18 gauge needle, slowly along the wall of tube preventing formation of any air bubble. The tube was sealed by Beckman sealing machine and then centrifuged at 60,000 rpm for 16 hrs at 22°C under vacuum in (model no.11) Hitachi ultra centrifuge. After the ultra centrifugation genomic DNA was seen as a dark pink band in the middle of ultra-centrifuged tube. Genomic DNA was collected with the help of 18 gauge needle in sterile tube. Then 750μl of 2-butanol was added and mixed slowly. The upper aqueous layer was discarded. This process was repeated until the ethidiumbromide was not removed. Then DNA sample was transferred into autoclaved Oakridge tube and added 0.6 volume of isopropanol (1200μl). Mixed well and incubated at room temperature for 30 minutes. Genomic DNA was pelleted down by centrifugation at 11,000 rpm for 30 minutes. The supernatant was decanted and pellet was washed with 70% ethanol. The pellet was air dried and dissolved in TE buffer. The quality and quantity of this DNA was checked on 0.8% gel along with a marker λ/Hind III digest.

3.12.3 Restriction of genomic DNA

After purification the genomic DNA (15μg) was restricted with Eco RI and Bam HI at 37°C overnight. Reactions were stopped by adding 2μl of 0.5 M EDTA (pH 8.0). Added 2μl of 10X Sample dye in each restricted genomic DNA sample and then electrophoresed on 0.8% agarose gel at 25 V for 10 hours using 1X TAE buffer. The gel was documented by gel document system.

3.12.4 Southern blotting

The electrophoresed agarose gel was blotted on nitrocellulose membrane according to the modified protocol of Southern (1975). The agarose was depurinated in 0.25 N HCl until the dye changed the color and left for 10 minutes. The gel was rinsed in distilled water and then transferred in denaturizing solution for 30 minutes at room temperature with shaking. The gel was again rinsed in distilled water and placed in neutralizing solution for 30 minutes at room temperature with shaking. A tray was filled with 20 X SSC buffer. Wick made with Whatman filter paper was saturated with blotting buffer. The gel was carefully placed on the wick and then nylon membrane placed on it leaving no air bubble trapped. Sheets paper
wetted with blotting buffer were put on top of the Hybond membrane. A stack of absorbent paper towels (approximate 5 cm height) was placed on top of the paper. Evaporation was prevented with wrapping the entire tray by a ceiling film. One kilogram weight was placed on top of stack and kept for 16 hours for blotting.

After blotting the membrane was carefully removed and washed with 2 X SSC buffer to remove the extra agarose adhering to membrane. The membrane was air dried and UV-cross linked by UV cross linker for 2 minutes (5000 micro joules/cm²). The UV cross linked membrane used for further analysis.

3.12.5 Preparation of Tomato TI II cDNA probe
3.12.5.1 Isolation of Tomato TI II cDNA clone

The white colonies streaked on fresh LA-amp plates and incubated at 37°C for over night. Next day single colony inoculated in 100ml LB containing ampicillin (100µg/ml) and incubated overnight at 37°C in shaking incubator. Plasmid DNA was isolated by the methods of Stephen et al. (1990). The bacterial culture (1.5ml) was spin down in a micro-centrifuge at 12,000-x g for 2 minutes. The pellet was resuspended in 150µl solution I (50mM Glucose, 25mM Tris-HCl pH. 8.0, 10 mM EDTA with lysozyme @ 5mg/ml). Freshly prepared 300µl solution II (0.2 N NaOH, 1% SDS) was added to the tube, inverted 4-6 times and kept on ice for 5 minutes. At the same time 225 |l of solution III (3M potassium acetate, pH 4.8) was added to the tube, inverted 4-6 times and kept on ice for 20 min again. The sample was centrifuged at maximum speed for 5 min and the clear supernatant was transferred to a fresh tube. Ethanol (675 µl) was added to the supernatant, mixed well and kept at -20°C for 30 min then spin at maximum speed for 10 min in a micro centrifuge. The pellet obtained was washed with chilled 70% ethanol, dried and dissolved in 20µl of TE buffer (pH 8.0). The plasmid was checked on 0.8% agarose gel for quality and quantity. The agarose gel (0.8%) was prepared by boiling and cooling the agarose (0.42 g per 60 ml) in TAE buffer (1x) and added ethedium bromide before pouring the gel. The solidified gel was submerging in TAE buffer (1x) tank. In the restricted plasmid sample 2µl of 10X loading dye containing bromophenol blue-xylene cyanol was added and loaded in to the wells. The samples were run at 60V for 2-3 hours at room temperature.

3.12.5.2 Restriction of Tomato Trypsin Inhibitor II cDNA clone

The plasmid DNA containing Tomato Trypsin Inhibitor II cDNA insert (Fig. 1) was restricted with Hind III and Eco RI in the presence of high salt buffer in a final volume
of 20µl and incubated at 37°C for two hours. The restriction was checked on 0.8% gel and documented by Gel document system.

3.12.5.3 Elution of insert DNA from the gel

The Restricted plasmid DNA was run on a low melting point (LMP) agarose gel (0.8%) at 50 volt for 2-3 hours. The gel was prepared as for the normal agarose except that the gel is allowed to set at low temperature in a refrigerator. The lower band of about 700 bp was the insert DNA and upper band was of vector (pUC19). The insert was eluted by modified protocol of Sambrook et al. (1989) as the insert bands were cut with the help of a sharp scalpel blade under UV light. The cut gel pieces were transferred to sterile micro-centrifuge tubes and heated at 65°C for 5 min in the water bath. Added 50µl of 5M sodium chloride per 500µl of gel melting solution. The micro-centrifuge was vortexed and heated again at 65°C for 5 min in water bath. The equal volume of phenol (pH 8.0, saturated with TE) was added, vortexed and centrifuged at maximum speed for 5 minutes. There were two layers, the upper layer containing the DNA was transferred to a separate micro-centrifuge tube and added double volume of ether, mixed well and centrifuged at maximum speed for 5 minutes. The upper ether layer was removed and remaining ether was allowed to evaporate off at 65°C for 2 min and DNA was precipitated in double volumes of ethanol by incubating at -20°C for overnight. The pellet was washed with 70% ethanol, air dried and dissolved in 20
μl of TE buffer (pH 8.0). The eluted DNA fragment was checked on gel and used for probe preparation after labeling.

3.12.6 Radio-labeling of Tomato TII cDNA probe

The eluted tomato PI cDNA clone inserts were radio-labeled by HexaLabel™ DNA labeling kit. All the required components were added into 1.5 ml micro centrifuge tube:

- DNA template (Tomato TII) - 10 μl (100ng)
- Hexanucleotide in 5X reaction buffer - 10 μl
- Deionized water - 20 μl

All the contents were mixed spin down and then tube incubated in a boiling water bath for 5-10 minutes. After incubation the tube was cooled on ice and added the following components

- Mix C - 3 μl
- [α³²P]-dCTP - 2 μl
- Klenow fragment, exo⁻ (5U) - 1 μl
- Deionized water - 4 μl

All the above contents were mixed, spine down and incubated for 10 minutes at 37°C. After incubation finally 4μl of dNTP mix was added to same tube and incubated at 37°C for 5 minutes. The reaction was stopped by adding 1μl of 0.5 M EDTA (pH 8.0). Now the probe was ready for hybridization.

3.12.7 Southern hybridization

Hybridization of restricted genomic DNA was done by method of Kochert et al., (1989) with slight modification. The cross linked filter were rolled in pair with their DNA side up facing outside and were placed inside a hybridization bottle. In the bottle transfer 25 ml of pre-warmed hybridization solution after adding the 2.5 ml of salmon DNA (10mg/ml stock) in hybridization buffer. The bottle was transferred to hybridization oven for two hours at 65°C. The Excess of pre-hybridization buffer was removed from bottle, so that it contains only 50 μl buffer/cm² of blot. The radiolabelled cDNA probe was denatured by placing in boiling water bath for 10 minutes and chilled on ice immediately. The labeled probe was added in the hybridization solution and cap was tightened properly then hybridization was allowed for 24 hrs at 65°C with continuous shaking.

3.12.8 Washing of blots and autoradiography

After hybridization, the bottle was removed from hybridization oven and hybridization buffer was drained out carefully into a disposable bottle. Remove the un-bound probes from the blots by washing as follows-

1st wash: 2 X SSC+ 0.1% SDS at RT for 15 minutes
2\textsuperscript{nd} wash: 1 X SSC + 0.1\% SDS at 65°C for 10 minutes
3\textsuperscript{rd} wash: 0.5 X SSC + 0.1\% SDS at 65°C for 5 minutes

All the washes of blot were done with continuous shaking in a shaker. The washed filter with proper counts used for radiography. The blot was placed on a support and wrapped with Saran wrap and keep in the photography cassette. The photography X-ray film was placed over the blot in the dark room. The cassette was closed tightly and wrapped in a black paper to prevent light and exposed the film for 24 hrs at -76°C. After incubation the film was developed in Kodak developer at room temperature for 2 minutes, washed in water for 1 minute and fixed in Kodak fixer for 2 minutes. Finally the radiogram washed with water for 4-5 minutes and air-dried at room temperature.

3.13 Constructing cDNA library of Pigeonpea

3.13.1 Isolation of total RNA

Total RNA was isolated from 12 days old developing seeds of pigeonpea by an improved RNA isolation method of Tai \textit{et al.} (1999) with some modifications. One gram of developing (12 days old) seed sample was grinded to fine powder in liquid N\textsubscript{2} through motor & pestle and add the powder in 5 ml of lysis buffer (Ice cold), mixed vigorously. The mixture was incubated for over night at 4°C. Next day lysed tissue added in to a Qiashredder spin column and centrifuged at 500g for 30 minutes at 4°C. Decant the column, debris and supernatant, only the pellet is saved. The pellet was suspended in 1ml of re-suspension buffer and centrifuge at maximum speed (13000g) at RT for 5 minutes. Removed the supernatant in fresh tube and added equal volume of Phenol. Mixed the both phases properly and centrifuged for 5 minutes. The top aqueous phase removed in a clean tube and added equal volume of phenol: Chloroform: Isoamyl alcohol mixed well then centrifuged at max speed for 5 minutes. The aqueous phase again removed in a clean tube. In the aqueous phase 2% pot acetate in 90\% ethanol is added and precipitated RNA by incubate at -20°C for over night. Next day the tube was centrifuged at 1500g for 15 minutes at 4°C, wash RNA pellet with 70\% ethanol, air dried and suspend in 20µl of DEPC water. The total RNA was checked on the denaturing gel.

3.13.1.1 Preparation of denaturing gel

The 1\% denaturing gel was prepared by dissolving 0.25g of agarose in 22.5ml of DEPC treated water. It was cooled to 50-60°C and added 2.5 ml of 10X MOPS buffer. In the solution 0.750 ml of formaldehyde (37\%) was added and mixed properly under fume hood. The solution was cooled and mixed 0.5µl of EtBr properly. The comb was placed in casting tray and pours the gel solution. After one hour the gel will be polymerized.
3.13.1.2 Preparation of RNA sample and electrophoresis

The RNA sample was prepared by adding the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>7 µl</td>
</tr>
<tr>
<td>10X MOPS buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3 µl</td>
</tr>
<tr>
<td>Formamide</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

All the contents were mixed and spin down in tube and incubate at 65°C for ten minutes. The sample was cooled down on ice for five minutes and added 2 µl of loading buffer properly. After polymerization comb was removed from the gel and placed in to 1X MOPS buffer tank for running the RNA sample. Prepared RNA sample (20 µl) was loaded in the well and electrophoresed at 40 Volt for 2 hours and gel is documented by gel documentation system.

3.13.2 Purification of poly (A)^+ RNA

The poly (A)^+ RNA was purified from total RNA by using Qiagen Kit of Genetix. The total RNA was taken in a fresh tube and adjusts the volume 250 µl with RNAs free water, added 250 µl of OOB buffer and mixed well with 45 µl oligotex suspension through pipetting. The sample incubated at 70°C for 3 minutes then removed from the water bath and placed 20-30°C for 10 minutes. The oligotex-RNA complex was palted by centrifugation for 2 minutes at 13000g and carefully removed the supernatant. The oligotex-RNA complex resuspend in to 400 µl of buffer OW2 by pipetting and pipette out on to a spin column then place it in to eppendorf tube and centrifuged at maximum speed for 2 minutes. The columns transferred In to the fresh tube and added 400 µl of buffer OW2 and centrifuge at mix speed then discard the flow through. Again transfer the column to a new RNAs free tube and pipette out 50 µl of hot (70°C) OEB buffer on to the column, mixed well then column was centrifuged for 1 minute at max speed. The obtained mRNA was checked on the 1% gel and used further.

3.13.3 First strand cDNA synthesis

The Smart cDNA library construction kit from Clontech Ltd. Japan synthesized the first strand. The following ingredients were mixed in a sterile tube as-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (A)^+ RNA</td>
<td>6 µl</td>
</tr>
<tr>
<td>3 Smart CDS prime IIA</td>
<td>2 µl</td>
</tr>
<tr>
<td>Smart II A oligonucleotide</td>
<td>2 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mixed all ingredients and incubate for 2 minutes at 72°C. After incubation place the tube on ice and add the following reagents as -
5x first strand buffer - 4μl
DTT - 2μl
dNTPs - 2μl
Powerscript RT - 1.5μl
Total volume with water - 20.0μl

Mixed the ingredients, spin down and incubate at 42°C for 1 hour. Terminate the reaction after 1 hr by placing on ice and store at -20°C for further use.

3.13.4 Long Distance-PCR

Prepare the master mix by adding the following reagents as-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>First strand</td>
<td>5μl</td>
</tr>
<tr>
<td>PCR Grade water</td>
<td>77μl</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>50x dNTPs mix</td>
<td>2μl</td>
</tr>
<tr>
<td>5' PCR Primer II A</td>
<td>4μl</td>
</tr>
<tr>
<td>50x Advantage 2 Poly mix</td>
<td>2μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Mixed all the components by vortexing and centrifuge the tube and place on thermal cycler on the following conditions as 95°C (1 minute), 95°C (15 second), 65°C (30 seconds), 68°C (6 minutes) and kept it hold at 4°C. When 20 cycles completed 5μl of the product was checked on (1.1%) gel with 5μl of 1 kb DNA marker.

3.13.5 cDNA Polishing

Mix the 50 μl of amplified ds cDNA with 2 μl of Proteinase K (20μg/μl) in a sterile centrifuge tube and incubated at 45°C for 1 hour. After incubation the tube briefly spine and heated at 90°C for 5 minutes to inactivate the Proteinase K. Chilled the tube on ice, added 3μl of T4 DNA polymerase (15 Units) and incubated the tube at 16°C for 30 minutes. The tube was heated at 72 °C for 10 minutes and mixed 27.5μl of 4M ammonium acetate and 210 μl of 95% ethanol thoroughly. The tube Spin immediately at 1400 rpm for 20 minutes at RT the supernatant removed and the pellet washed with 80% ethanol. The pellet air dried and resuspended in 100 μl of deionized water.

3.13.6 cDNA size fractionation with CHROMA SPIN-400 column

The CHROMA SPIN-400 column was inverted several times to completely resuspension of the gel matrix completely. Storage buffer was drained out through the column by gravity flow (flow rate was approximately 1 drop/ 40-60 sec). When the storage buffer stopped dripping out, 700 μl of column buffer was added carefully to the top of column and allowed it to drain out. When column buffer stopped dripping out, 100 μl of cDNA and
xylene cyanol dye (10mg/ml) was applied to top of matrix and allowed the sample to be fully absorbed into the surface of matrix. Sixteen 1.5 ml tubes were labeled and arranged in a rack. 600 µl of column buffer was added to the top of column and immediately begun collecting single-drop fractions (approximately 30µl per tube) in tubes #1-16. From each eluted fraction 3.0µl was electrophoresed on a 1.1% agarose gel along with 1 Kb DNA ladder and gel was run at 150 V for 10 minutes. The peak of fractions was determined by visualizing the intensity of the bands under UV light.

The tube #3-4 fractions were pooled together and added the following reagents as 1/10 volume of 3M sodium acetate (pH 4.8), 1.3 µl of Glycogen (20 mg/ml) and 2.5 volume of 95% ethanol (Chilled). All the reagents were mixed by gently rocking and the tubes were placed overnight at -20°C. Next day the samples were centrifuge at 14,000 rpm for 20 minutes at 4°C temp and the pellet was air dried and resuspended in 6 µl of deionized water. All the size fractionated cDNA samples (1µl each) were checked on the 1% agarose gel.

3.13.7 Ligation of cDNA with pGEM-T easy Vector

Keep all contents on ice and add one by one in sterile tube as follows-

| Insert (cDNA) | - | 4.0µl |
| Buffer (10x) | - | 10.0µl (1x) |
| Ligase | - | 1.0µl (3.5U) |
| pGEM-T easy vector | - | 0.5µl (500ng) |
| Water | - | 4.5 µl |
| Total volume | - | 20µl |

Mix all content and spin down and incubated at 4°C for over night for ligation in water bath. Next day 30µl of water was added to make the volume 50µl. The ligated product was kept at 70°C for 10 minutes to inactivate the enzyme. The sample removed and cools down then added 500µl of n-butanol. Spin the tube at 12,000 pm for 10 minutes and the pellet was dried in laminar air flow to remove the smell trace of n-butanol. The pellet was dissolved in 10 µl of water by keeping 37°C for 1 hour at 1000 rpm in a shaker.

3.13.8 Electroporation

Take 40 µl of super charge (EZ-10) cells and cleaned ligated product in a cuvette. Mix well inside the cuvette but there should not be any bubble. Give the pulse of 1700 mill volt and immediately added 1ml of LB broth and incubate at 37°C in dry bath shaker for one hour. After one hour incubation transformed cells were pelleted down and suspended in 100µl LB medium. The cDNA library was spread over fresh LA plate supplemented with ampicillin, X-Gal and IPTG and incubated at 37°C for over night. Next day the plates were checked B/W colonies. The white transformed colonies of cDNA library were picked up on the fresh girded
LA plates supplemented with ampicillin, X-Gal and IPTG in duplication. One plate labeled as master plate and other for colony lift. All the girded plates were kept at 37°C for over-night. On next day after getting proper sized colonies all the plates were place at 4 °C for further use.

3.13.9 Colony Hybridization

3.13.9.1 Preparation of colony blots

After making the grid on fresh LA plate, white colonies were inoculated with the pointed side of autoclaved toothpick. Next day cDNA library colonies were immobilized on the Hybond N+ nylon membrane by carefully placing the membrane on to the agar surface. Marked the membrane and agar using a sterile needle to ensure correct orientation of the colonies. Hybond N+ membrane was removed after two minutes and place colonies up side on sterile filter paper for 15-20 minutes in laminar air flow and then place colony up side on a pad of absorbent filter paper soaked in denaturing solution for 7 minutes. The member was picked up with the help of forceps and placed side-up on a pad of absorbent filter paper soaked in neutralizing solution and lease for 3 minutes. Washed the member in 2X SSC for 2 minutes and transferred for air dry colony side up and the nucleic acid fixed by UV-Cross linkers.

3.13.9.2 Pre-hybridization

The cross linked filter were rolled in pair with their DNA side up facing out side. These were transferred to hybridization tube (bottle) and add 25ml of pre-hybridization buffer was added.

Pre-hybridization buffer (100ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%SDS</td>
<td>35ml</td>
</tr>
<tr>
<td>1M Phosphate buffer</td>
<td>50ml</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>2ml</td>
</tr>
<tr>
<td>Water</td>
<td>13ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>100ml</td>
</tr>
</tbody>
</table>

After adding 2.5 ml of the salmon DNA (10mg/ml) in hybridization buffer transfer the tube in hybridization oven for two hours at 65°C.

3.13.9.3 Hybridization with Radio-labeled Tomato TII cDNA

Colony hybridization was done according to the protocol of Kochert et al., (1989) with slight modification. Excess of pre-hybridization solution was removed from bottle, so that it contains only 50 µl buffer/cm² of blot. Radiolabelled Tomato TII cDNA probe obtained from cDNA clone as previous section was denatured by placing in boiling water bath for 10 minutes, and chilled on ice immediately. The probe was added in the pre-
hybridization solution and the bottle cap was tightened properly. For hybridization bottles were transferred to hybridization oven and allowed to proceed for 24 hrs at 65°C with shaking.

3.14 Library screening

3.14.1 Primary screening

After hybridization, the bottle was removed from oven and drained out hybridization buffer carefully into a disposable bottle. Removed the free nucleotides and un-bound probes, by washing as follows:

1\textsuperscript{st} wash: 2 X SSC+ 0.1% SDS at RT for 15 minutes
2\textsuperscript{nd} wash: 1 X SSC+ 0.1% SDS at 65°C for 10 minutes
3\textsuperscript{rd} wash: 0.5X SSC+0.1% SDS at 65°C for 5 minutes

All washings except first were done with continuous shaking in a shaker at 65°C. The washed blots were radio-graphed after checking counts as described earlier in southern hybridization. By fluorescent light illuminator the punched marked on filter were aligned with the spot on radiogram. The master plate was superimposed over it and inoculated the marked colonies on the fresh plate.

3.14.2 Secondary screening

For the secondary screening, each of the selected colonies was picked from the master plate and inoculated on the fresh LA amp plate containing ampicillin. After over night incubation at 37°C the colonies were appeared, then lifted on nylon membranes and membranes were processed as described earlier. The processed membranes were dried and cross-linked and used for hybridization. Filters were hybridized overnight at 65°C. The hybridized filters were washed to remove unbound probe and autoradiograph as described previously. The positive clones were picked and used for tertiary screening.

3.14.3 Tertiary screening

The selected positive colonies were inoculated on girded LA amp plates and incubated at 37°C for overnight. The colonies were lifted on nylon membrane hybridized and auto-radiographed as described earlier.

3.15 Restriction of positive clones

After tertiary screening the positive clones were inoculated on the fresh LB amp and grown for overnight. The plasmid were isolated and restricted by EcoRI for three hrs and run on 1% agarose gel, stained with ethidium bromide and photographed.

3.16 Sequencing

The recombinant clone, which has higher size insert, was sequenced using vector specific T7 and SP6 primers. The sequencing was carried out commercially.
3.17 Sequence analysis

BLAST (Basic Local Alignment Search Tool) and Expsy bio-informatics software tool used for analyzing gene ORF, protein data and its nature.
4. RESULTS AND DISCUSSIONS

4.1 Screening of Pigeonpea varieties for Protease Inhibitor (PI) protein

PI proteins were isolated from four varieties viz. Pusa-855, Pusa-33, Pusa-987 and Pusa-84 of pigeonpea through modified method of Maggo et al., (1999). The protein concentration of crude samples was estimated through Lowry (1951) method by using BSA as a standard. On the standard curve 1μg of BSA gave 0.003 A at 660 nm wavelength. In-vitro estimation of PI activity was done by modified methods of Erlanger et al., (1961); Kakade et al., (1969). The standard trypsin units were 9590U/mg or 9.59 U/μg. The 20μg of trypsin gave 1.0 A, so one unit will gave 0.005A. One unit of protease is defined as the amount of enzyme which will increase 0.005A at 660nm. One protease inhibitor unit (PIU) was defined as the amount of inhibitor that inhibited one unit of protease activity. The protease inhibitor activity was expressed as trypsin unit inhibited/mg of protein as shown in Table-3 and Fig.2. Among the four varieties of Pigeonpea, Pusa-33 had the highest protease inhibitor activity in 40-80% fractions as and shown in Table-3, which was used for further purification of PI protein.

4.2 Purification of PI protein from Pigeonpea

PI protein was purified from 100 gm of defatted flour of variety Pusa-33. The crude of 100 gm defatted flour had 19,790 mg of total proteins and 69,660 units of PI activity. The specific activity was found 352 U/mg as shown in Table-4. The crude was extracted, heat denatured at 80°C for 20 minutes and centrifuged, as described by Hajela et al., (1999). The supernatant has 11,020 mg of proteins and 61,050 units of PI activity (Table-4). The supernatant after heat denaturation (AHD) was subjected to 40-80% ammonium sulphate fractionation. After dialysis protein and PI activity was estimated. It had the 6,960 mg of protein with specific activity of 860 U/mg. The dialyzed sample used for purification by ion exchange chromatography and gel filtration.

4.2.1 Ion exchange and Gel-filtration of PI protein

DEAE-Cellulose is an anion exchanger, used for purification of PI protein. It will bind all the cationic protein and rest all anionic proteins washed out through buffer. The bound proteins were eluted using 0.01-0.1M NaCl gradient. Eluted fractions were monitored at 280nm for protein and 410nm for trypsin inhibitor activity. The fraction number 5 to 13 showing PI activity after ion-exchange chromatography (Fig.3) were pooled, lyophilized and used for gel filtration after estimation of protein. The lyophilized sample had 282 mg of proteins and specific activity as 1,010 units/mg (Table-4).
Table 3- Protease Inhibitor activity (U/mg) in four varieties of the Pigeonpea

<table>
<thead>
<tr>
<th>Fractions</th>
<th>P-855</th>
<th>P-33</th>
<th>P-971</th>
<th>P-84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>140</td>
<td>352</td>
<td>143</td>
<td>195</td>
</tr>
<tr>
<td>AHD</td>
<td>320</td>
<td>554</td>
<td>301</td>
<td>275</td>
</tr>
<tr>
<td>0-20%</td>
<td>146</td>
<td>207</td>
<td>130</td>
<td>186</td>
</tr>
<tr>
<td>20-40%</td>
<td>358</td>
<td>410</td>
<td>255</td>
<td>250</td>
</tr>
<tr>
<td>40-60%</td>
<td>320</td>
<td>620</td>
<td>343</td>
<td>140</td>
</tr>
<tr>
<td>60-80%</td>
<td>325</td>
<td>510</td>
<td>356</td>
<td>340</td>
</tr>
<tr>
<td>80-100%</td>
<td>125</td>
<td>130</td>
<td>108</td>
<td>115</td>
</tr>
</tbody>
</table>

Table 4- Purification profile of Pigeonpea Protease Inhibitor protein

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>PI activity (U/mg)</th>
<th>Total PI units</th>
<th>Recovery of Protein (%)</th>
<th>Recovery of PI activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>850</td>
<td>19790(100%)</td>
<td>352</td>
<td>6966080</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ADH</td>
<td>720</td>
<td>11020</td>
<td>554</td>
<td>6105080</td>
<td>55.68</td>
<td>87.64</td>
</tr>
<tr>
<td>40-80%</td>
<td>35</td>
<td>6960</td>
<td>860</td>
<td>5985600</td>
<td>35.16</td>
<td>85.92</td>
</tr>
<tr>
<td>DEAE</td>
<td>140</td>
<td>282</td>
<td>1010</td>
<td>2848200</td>
<td>1.42</td>
<td>40.88</td>
</tr>
<tr>
<td>Gel-filtration</td>
<td>28</td>
<td>124 (0.62%)</td>
<td>1234</td>
<td>153052</td>
<td>0.062</td>
<td>2.19</td>
</tr>
</tbody>
</table>
The lyophilized sample was used for gel-filtration chromatography. The samples were collected from Biogel P-100 column. Each fraction was again monitored at 280 nm for protein and 410 nm for PI activity. The positive fractions 8 to 14 as shown in Fig. 4 were lyophilized and again determined the protein concentration and PI activity. The purified sample had 124 mg of protein and total PI activity as 1, 53,052 units as shown in Table-4. The specific activity was 1234 U/mg. The Pusa-33 variety of pigeonpea had 19.7% of total protein out of which 0.62% PI protein (Table-4). The protein content of commonly grown pigeonpea cultivars ranged between 17.9 to 24.3 g/100 g (Salunkhe et al., 1986) for whole grain samples and between 21.1 to 28.1 g/100 g for split seed. Similarly the protease inhibitors were purified through ion exchange and gel-filtration chromatography by Baumgartner and Chrispeels (1976) from mung bean (Phaseolus aureus Roxb.); Hajela et al., (1999) from Phaseolus mungo; Sanjay Maggo et al., (1999) from rice bean (Vigna umbellata) and from Cassia obtusifolia by (LIO Hai et al., 2008).

4.3 Characterization of Pigeonpea Protease Inhibitor (PPI) protein

4.3.1 SDS-PAGE and Gel-filtration

As shown in Fig. 5 the purified PPI protein was resolved on SDS-PAGE (Laemlli, 1970) as approximately 26 kD band. The molecular weight was also checked through gel-filtration chromatography after calibration with standard molecular weight markers on Sephadex G-50 column. The PPI protein showed Ve/Vo of 1.31. On standard curve the log molecular weight was calculated by extrapolating the value on Y-axis that was 4.397 and the antilog of it was 24950. Hence the molecular weight of PPI protein is found 24.95 kD (Fig. 6).

Similarly Baumgartner and Chrispeels (1976) determined the molecular weight of mung bean (Phaseolus aureus Roxb.) inhibitor by gel-filtration chromatography using Sephadex G-100. The standards used as riboflavin, actinomycin D, Cytchrom, and pancreatic ribonuclease. The molecular weight of bean (Vigna umbellata) purified trypsin inhibitor was determined by Maggo et al., (1999) through gel-filtration through Sephadex G-100 column after calibrated with standard marker proteins viz. Bovine serum albumin (66 kD), carbonic anhydrase (29 kD) and cytochrome c (12.4 kD) while the molecular weight markers used for SDS-PAGE were bovine serum albumin (66 kD), egg albumin (45 kD), β-lactoglobulin (18.4 kD) and lysozyme (14.4 kD)

The molecular weight of Phaseolus mungo trypsin inhibitor in denaturing conditions was measured by SDS-PAGE method. The molecular weight of the native inhibitor was determined by Andrew's plot between Ve/Vo and log M. The plot fits the equation Ve/Vo = -
0.8357 log M + 5.1618. The standard molecular weight markers used were ovalbumin, chymotrypsin-A, ribonuclease-A and cytochrome-C (Hajela et al., 1999).

4.3.2 Effect of temperature on purified PPI protein

The purified PPI protein showed the temperature effect. The PPI protein retains its activity up to the temperature 90°C as 85% but it becomes inactive on 100°C and on autoclaving within 5 minutes (Table-5). Because PPI become inactive on boiling and autoclave therefore it will be inactive on cooking and will not harm to the human health on consumption. The similar results was earlier reported in pigeonpea by Mulimani and Paramjyothi, (1994); Ilaq and Khan, (2003) that PI protein showed approximately 85% residual PPI activity after 30 minutes heating at 95°C and it activity completely lost upon boiling the crude extract at 100°C prior to ammonium sulphate fractionation. Nandeesha and Thurtha Prasad (2001) observed that partially purified Subabul (Leucaena leucocephala) seeds trypsin inhibitor possesses only 50% and 20% activity on 90°C and 100°C respectively. The PPI reported in jackfruit seeds is stable up to 100°C (Annapurna et al., 1991) and rice bean protease inhibitor stable up to 100°C (Maggo et al., 1999). Whereas the PI of Phaseolus vulgaris less stable to temperature. It becomes completely inactive within 20 minutes exposure of 100°C. The Phaseolus mungo protease inhibitor was found to be extremely heat stable. On incubation at 100°C for 20 minutes it lost only 50% activity when the time was increased as 60 minutes then it inactivated and lost 80% activity but on when autoclave for 20 minutes, it became completely inactivated (Hajela et al., 1999).

4.3.3 Effect of pH on purified PPI protein

The purified PPI protein also showed the pH stability. When incubated on various pH at RT for 30 minutes. The PPI protein significantly retains its activity on the pH range from 7.0-10.0 (Fig.7). This indicated that PPI also quite stable on alkaline pH as shown in the table-6. These results were supported by Godebole et al., (1994) that trypsin inhibitor of pigeonpea seeds retained its full activity between pH 7.0-10.0 but when exposed to acidic pH 3.0-5.0, only showed 20% of activity. Similar observations were found by Mulimani and Paramjyothi, (1994) that the PI was significantly inhibited the trypsin activity in the pH range 6.0-10.0. Nandeesha and Thurtha Prasad, (2001) observed that partially purified Subabul (Leucaena leucocephala) seeds trypsin inhibitor also stable over wide range of pH from 3.0-12.0 at RT. The rice bean protease inhibitor remains active in alkaline medium as on pH range from 6.0-10.0 but not on acidic pH (Maggo et al., 1999). These findings suggested that PI was also quit stable to alkaline pH. The mid gut of lepidopteran larvae is highly alkaline and digestive proteases have the optimal activity at pH 10-11. Because PPI remain active in
Table 5- Effect of temperature on purified Pigeonpea Protease Inhibitor protein

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>PI Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient (RT)</td>
<td>91</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>40</td>
<td>89</td>
</tr>
<tr>
<td>50</td>
<td>92</td>
</tr>
<tr>
<td>60</td>
<td>91</td>
</tr>
<tr>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>121</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6- Effect of pH on purified Pigeonpea Protease Inhibitor protein

<table>
<thead>
<tr>
<th>pH</th>
<th>PI Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>8</td>
<td>93</td>
</tr>
<tr>
<td>9</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>11</td>
<td>62</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
</tr>
</tbody>
</table>
alkaline pH so it will remains active in the mid gut of lepidopteran larvae and will affect the growth and development of these insect pest.

4.4 Feeding trail of PPI on *Helicoverpa armigera*

The feeding trails were conducted for the estimation of *in-vitro* effect of purified PPI protein toxicity on the growth and development of *Helicoverpa armigera*. The purified PPI protein was incorporated into an artificial diet (Veerareddy and Bhattacharya 1990). There were three treatments as T1, T2 and T3, each treatment contains 0.5% (6,170U/ml), 1.0% (12,340U/ml) and 1.5% (18,510U/ml) of PPI protein respectively. The basic diet was supplemented with appropriate quantity of PPI protein to give equal units of inhibition. The purified PI protein influenced the growth and development of *H. armigera*. The PPI significantly reduced the mean larval weight and mortality as shown in Table-7. All the untreated (control) larvae stopped feeding after eighteen days and entered into pupal stage. The larvae of the three treatments, viz. T-1, T-2 and T-3 were extended their pupation up to 21, 28 and 30 days respectively to complete the larval stages. The extended larval period was 3, 9 and 12 days in three treatments respectively (Table-7). The body weight was also adversely influenced by the treatments. The weight of the larvae before pupation in control was 470.70 mg where as it was reduced to 328.0mg in T-1, 287.0mg in T-2 and 220.2mg in T-3 (Fig.8). The pupal weight was also drastically reduced in T-1, T-2 and T-3, as 239.80mg, 194.40mg and 104.00mg respectively in comparison to control pupal weight (304.40mg). The larval mortality was dose dependent with highest mortality was 46% in T3 treatment when the diet was mixed with 1.5% (12,340U/ml) PPI protein as shown in Table-7 and Fig.9. The molting of the insect larvae also affected by PPI protein and stopped so larvae die-off (Fig.10A). The adults emerge from the treated pupa were observed that they failed to mate and lay eggs. The adults did not survive more then one week. Some adults were even deformed and have crippled wings as shown in Fig.10B. The result of the study showed that PPI retarded the growth and development of *H. armigera* larvae. The fecal output was also reduced up to 30-80%. The results of feeding trail suggest that PPI has a good potential for protection of crop plants against *H. armigera*. The protease inhibitor was interfering within the normal proteolysis and cause starvation of the larvae (Johnston *et al.*, 1991). The protease inhibitor leads to decline in feeding behavior of the insect, resulting in a decrease in growth causing death in several days (Pulliam *et al.*, 2001). Similarly Nandeeshha and Theerta (2001) partially purified sababul trypsin inhibitor (STI) was mixed the basic *Helicoverpa* diet (Veerareddy and Bhattacharya 1990) at three levels, 5,000, 10,000 and 20,000 TIU/ml of diet. STI showed significantly reduced the mean larval weight with mortality to an extent of
Table 7- Effect of Pigeonpea Protease Inhibitor protein on growth parameter of *Helicoverpa armigera*

<table>
<thead>
<tr>
<th>Age of larvae (days)</th>
<th>Control wt. (mg.)</th>
<th>T-1 wt. (mg.)</th>
<th>T-2 wt. (mg.)</th>
<th>T-3 wt. (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>25.80 (15)</td>
<td>25.40 (15)</td>
<td>25.80 (15)</td>
<td>25.60 (15)</td>
</tr>
<tr>
<td>9</td>
<td>59.10 (15)</td>
<td>44.30 (15)</td>
<td>38.40 (15)</td>
<td>34.20 (15)</td>
</tr>
<tr>
<td>11</td>
<td>182.10 (15)</td>
<td>120.10 (15)</td>
<td>111.00 (15)</td>
<td>98.10 (15)</td>
</tr>
<tr>
<td>13</td>
<td>250.15 (15)</td>
<td>204.10 (14)</td>
<td>151.10 (13)</td>
<td>130.20 (11)</td>
</tr>
<tr>
<td>15</td>
<td>320.50 (15)</td>
<td>251.10 (14)</td>
<td>199.20 (12)</td>
<td>157.10 (10)</td>
</tr>
<tr>
<td>18</td>
<td>470.70 (15)</td>
<td>311.00 (13)</td>
<td>272.30 (11)</td>
<td>212.50 (9)</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>328.00 (13)</td>
<td>278.00 (11)</td>
<td>211.20 (9)</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>-</td>
<td>280.00 (10)</td>
<td>218.20 (8)</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
<td>-</td>
<td>287.00 (10)</td>
<td>219.50 (8)</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>220.20 (8)</td>
</tr>
<tr>
<td>Pupal wt.</td>
<td>304.40 (15)</td>
<td>239.80 (13)</td>
<td>164.40 (10)</td>
<td>104.00 (8)</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0%</td>
<td>13%</td>
<td>33%</td>
<td>46%</td>
</tr>
<tr>
<td>Extended Larvae growth</td>
<td>Normal 3days</td>
<td>10days</td>
<td>12days</td>
<td></td>
</tr>
</tbody>
</table>
40%. The larval duration was extended by 5, 10 and 12 days in T1, T2 and T3 treatments respectively.

Gatehouse (1998) also observed prolongation in larval duration, growth retardation and mortality of *Helicoverpa armigera* and *Lacanodation oleracea* by soybean Kunitz trypsin inhibitor and soybean Bowman-Birk trypsin-chymotrypsin inhibitor. Shukla *et al.*, (2005) tested plant lectins and soybean trypsin inhibitor at 0.1% concentration through impregnation into artificial diet. Larval survival rate was 49% in artificial diet impregnated with soybean trypsin inhibitor compared to control diet as 90%. Lower pupation and adult emergences was also observed as 50%. The soybean trypsin inhibitor can be considered for development of transgenic plant for the management of *H. armigera*.

The Mediterranean flour moth, *Anagasta kuehniell* is one of the most important insect pests of grains reported worldwide which feed on stored grains and products of rice, rye, corn and wheat. *Plathymenia foliolosa* trypsin inhibitor (PFTI) was purified and was tested for insect growth regulatory effect. The survival and mass of *A. kuehniella* larvae on seeds containing 0.7% PFTI was about 56%, while 66.1% reduction in the average mass of the larvae was observed. The Results indicate that PFTI possesses a toxic effect against *A. kuehniella* larvae. (Vanessa da Silveira *et al.*, 2009).

4.5 Biomass and PI activity of developing seeds
4.5.1 Moisture content during seed development

Pre weighted developing seeds were kept in oven at 80°C for drying and weight after 24 hrs. The weight was taken until the decrease in weight becomes zero after successive incubation. The dry weight of seeds increased continuously up to maturation. The rate was slower from 5 to 15DAF days but rate become faster from 15 to 35DAF as shown in Table-8 and Fig.11. During the development in the initial stage cell division occur in the embryo with little synthesis of storage reserve food material. In the latter phases cell division decreased cotyledons form in the seed. In the end there is increase in synthetic activity and dry weight of the seed as reported by Gatehouse *et al.*, (1984).

4.5.2 PI activity in developing seeds

The PI protein was isolated from development seeds and check for inhibitory activity. In early stage the inhibitory activity was lower and increased up to maturation as shown in Fig.12. The PI activity was low in 12DAF but it increased 18DAF. This indicated that mRNA for PI protein was more in this stage which translated into protein and given more inhibitory activity. So 12DAF stage will be rich in mRNA of this gene, so this stage can be used for
Table 8- Study on biomass of developing seeds of Pigeonpea

<table>
<thead>
<tr>
<th>Days after flowering (DAF)</th>
<th>Fresh wt. (mg)</th>
<th>Dry wt. (mg)</th>
<th>Moisture %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>40</td>
<td>10</td>
<td>83.30</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>39</td>
<td>80.50</td>
</tr>
<tr>
<td>15</td>
<td>450</td>
<td>109</td>
<td>75.70</td>
</tr>
<tr>
<td>20</td>
<td>580</td>
<td>210</td>
<td>63.79</td>
</tr>
<tr>
<td>25</td>
<td>600</td>
<td>290</td>
<td>51.60</td>
</tr>
<tr>
<td>30</td>
<td>400</td>
<td>331</td>
<td>17.25</td>
</tr>
<tr>
<td>35</td>
<td>380</td>
<td>350</td>
<td>7.89</td>
</tr>
<tr>
<td>Mature</td>
<td>360</td>
<td>335</td>
<td>6.99</td>
</tr>
</tbody>
</table>
isolation of mRNA for construction of cDNA library. This cDNA library will be abundance for the PI genes. The similar results also have been reported by Godbole et al., (1994).

4.6 Southern hybridization

The genomic DNA was isolated from eight days old dark grown seedling by using method of Dellaporta et al., (1983). The isolated DNA was purified by CsCl₂ gradient method. Its purity and quantity was determined by taking its A at 260 nm and 280 nm. Gel electrophoresis on a 0.7% agarose gel also confirmed it quality and quantity (Fig.13A). The quantity DNA was found to be 1μg/μl. The purified DNA was restricted separately with Bam HI, Eco RI and Hind III respectively (Fig.13B). The digested samples were electrophoresed and blotted on Hybond N⁺ membrane. The crossed linked membrane was hybridized with radio-labeled Tomato Trypsin Inhibitor II cDNA as heterologous probe available in the lab which previously obtained from C.A. Ryan laboratory Washington State University, Pullman, USA. The isolation and preparation of probe has done as described earlier in material and method. The non-homologous and unbound probes were washed from the Hybond N⁺ membrane with the stringency of 0.5 X SSC + 0.1% SDS at 65°C for 5 minutes. After sufficient washing of Hybond N⁺ membrane, X-Ray film was exposed. The autoradiogram showed the presence of more than one copy of protease inhibitor gene in the Pigeonpea (Fig.13C).

4.7 Construction of cDNA library of Pigeonpea

4.7.1 Isolation of total RNA and poly (A⁺)RNA from developing seeds

Total RNA was isolated from 12 days old developing seeds of pigeonpea (Pusa-33) by various established protocols as GTC method, Trizol (Gibco-BRL), Trireagent (Ambion), RNaseasy plant mini kit (Qiagen), One step RNA isolation kit (Ambion), Hot phenol method, Lithium chloride method, Urea-LiCl₂ method etc. All the protocols gave degraded total RNA, which was not good for library construction. Finally good quality total RNA was isolated according to Tai et al., (2004) with some modification as 2% PVP in lysis buffer and reduced the time of incubation as four hours instead of over night. The total RNA gave two distinct bands (28S & 18S) on formaldehyde agarose gel (Fig.14A). The mRNA was isolated from the oligotex suspension (Qiagen). The 5mg of total RNA gave approximately 100μg of poly (A⁺)RNA.

4.7.2 Synthesis of cDNA

The first strand was synthesized from mRNA by the cDNA construction kit from Clontech Ltd. Japan. The single stranded cDNA was amplified by LD-PCR in to double stranded cDNA. The cDNA was checked on 1.0% agarose gel along with 1kb DNA marker
The size of double stranded cDNA range from 0.5-5.0kb as shown in Fig.14B. The cDNA was size fractionated by CHROMA SPIN-400 column and select only 0.5-2.0 kb fragments (Fig.15). The size fractionate cDNA of 0.5-2.0kb was ligated with pGEMT-easy vector (Fig.16). The ligated product was electroporated in to electro-competent cell (Super charge-EZ 10). The electroporated cells were incubated for one hour at 37°C then pelleted down and suspended in 100μl of supernatant. The suspended cells were spread on LA plate supplemented with ampicillin, X-gal and IPTG in four dilutions. The plates were incubated at 37°C for over night. Next day blue-white colonies were appeared on all the plates as shown in Fig.17. In case of recombinant cells β-galactosidase gene of pGEMT easy was disrupted so these cells produced white colonies instead of blue colony. In non-recombinants the β-galactosidase gene remains functional and able to hydrolyze X-gal which produced blue colony.

4.7.3 Screening of cDNA library

The cDNA library was constructed in pGEM-T easy plasmid vector which gave the blue white colony screening. The transformed white colonies were inoculated on the two girded LA plates supplemented with ampicillin, X-gal and IPTG (one master plate and one for colony lift) by the pointed side of autoclave tooth pick and incubated at 37°C for over night. For primary screening of cDNA library the blots were prepare from colony lift plate by immobilizing colonies on the Hybond N+ nylon membrane as described in materials and methods. The blots were hybridized with Tomato Trypsin Inhibitor II cDNA probes and autoradiographed on photography film. The black spots identified as positive clones as shown in Fig.18A. After primary screening, 34 positive clones were identified and their corresponding colonies were inoculated on fresh plate. In the positive clones some clones will be false due to non-specific binding. So the secondary screening was done to find out the real clones. The secondary screening was performed and autoradiographed as described earlier in materials and methods. The secondary screening did not gave strong singles for all the clones of primary screening however some gave less intense signals as shown in Fig.18B autoradiogram. Only fourteen clones gave strong signals which were marked and corresponding colonies were picked up on fresh LA plate for tertiary screening. Finally secondary screening clones were hybridized with tomato TI probes and autoradiographed as described in materials and methods. As shown in Fig.17C after tertiary screening only six positive clones showed strong signals.

4.7.4 Isolation of insert DNA and sequencing

The six positive clones were denoted as PPI-1 to PPI-6. The plasmid DNA was isolated from the putative positive clones by plasmid isolation kit and restricted with EcoRI. The size of
clones inserts range from 500-800 bp approximately. The sixth clone has large size insert of approximately 800bp as shown in Fig.19. The larger size insert clone was sequenced commercially.

4.7.5 Sequence analysis

The insert of sixth clone was sequenced by Sanger dideoxy method from Cromas Biotech Pvt. Ltd., New Delhi which gave 785 bp sequences. The sequence was analyzed through bioinformatics tools available on National Center for Biotechnology Information. Initially the vector sequences were found out on both ends of gene which was removed as shown in Fig.20&21. The PPI gene sequence was used for BLAST homology and confirmed 93% homology with *Cicer arietinum* trypsin inhibitor 1 gene (Fig.22,23&24). On the ORF finding analysis it showed largest open reading frame of 693 bp which has start and stop codon (Fig.25&26). The ORF was started from 19-771 nucleotides as shown in Fig.26. The ORF has 37.21% G+C and 62.79% A+T content. This gave protein product of 230 amino acids (Fig.27). The theoretically molecular weight was calculated as 24848 Dalton (24.84 kD). The Cn3D view of Kunitz-type inhibitor deduced from amino acid sequence is illustrated in Fig.28. The amino acid sequences analysed and showed 100% homology with soybean trypsin inhibitor (STI) (Fig.29&30). It is of Kunitz type inhibitor and belongs to the STI super family of Protease inhibitors. The members of this family are mostly active against serine proteases but may also inhibit other proteases (Laing and McManus, 2002; Ritonja et al., 1990). These inhibitors are canonical and form a tight complex with the target protease which dissociates very slowly (Ritonja et al., 1990). These inhibitors usually have molecular mass of 18-22kD and two disulphide bonds with one reactive site. The protease inhibitors are widely reported in legumes, cereals and in solanaceae species (Ishikawa et al., 1994; Laskowsk and Kato, 1980).

Pulliam et al., (2001) constructed cDNA library of cabbage (*Brassica oleracea* L. cv. Superpack) in Lambda Zap II with mRNA purified from the young leaves of mature cabbage plants. The library was screened with antibodies produced in rabbits against affinity-purified cabbage PI. Several positive clones were identified. One 809 bp clone was plaque-purified and sequenced using terminator cycle sequencing. The 809 *Brassica oleracea* proteinase inhibitor (Bopi) coding region was isolated as a EcoRI-XhoI restriction fragment then subcloned into pBlurscript II SK (Stratgene,USA). The full-length cDNA of OCPI 1 identified from cDNA library of *indica* rice Minghui 63 (Yuemin et al., 2007). The cDNA sequences of OCPI 1 showed 98.5% homology with the OsSCI13. Protein sequences of OCPI
showed 27-80% identity with various plant serine-protease inhibitors including the potato inhibitor I family.

Similarly Greathel et al., (2008) constructed cDNA library with the SMART cDNA library construction kit (BD Biosciences, Palo Alto, CA, USA) using poly (A)+ RNA of S. \textit{Nicoliana alala} pistils and cloned according to the manufacturer’s instructions. A total of approximately 31,000 colonies were screened with SRNasa, HT-B and 130K probes. After screening one major sequence was identified this designated as NaStEP. \textit{NaStEP} was most similar to Kunitz-type protease inhibitors.

Amanjot et al., (2009) obtained full-length cDNA encoding for subtilisin-chymotrypsin protease inhibitor from Pusa Basmati 1 (indica) rice seedlings. It has 219 bp long ORF which coding for 72 amino acid long 7.7kDa subtilisin-chymotrypsin protease inhibitor (CPI) cytoplasmic protein.

The PI genes have been particularly useful in development of transgenic plants resistant to insect pests. These defensive genes can be expressed through wound-inducible or constitutive promoters of the host (Bolter, 1993). Recently, protease inhibitors have used to increase resistant against viruses and insect pests (Ussuf et al., 2001). Bean α-amylase Inhibitor \textit{I} transgenic peas provides complete protection from pea weevil under field conditions (Roger et al., 2000). Several transgenic plants expressing PIs have been produced, and these are found to be more resistant against insect pests (Habeeb and Fazili, 2007). Srinivasan et al., (2009) confirmed resistance from insect pest as \textit{Spodoptera litura} and \textit{Helicoverpa armiger} in transgenic tobacco by constitutive expression of a trypsin inhibitor gene of tobacco.

The majority of protease inhibitor studied in the plant kingdom. Plant PIs are well known to play potent defensive role against predators and pathogens. In addition many plant PIs have been shown to act as defensive compound against insect by direct assay or by expression in transgenic crop plant. The role and mechanism of action for most of these have been or are being studied in detailed and their respective genes have been isolated. These genes have been used for the development of transgenic crop plant to be incorporated in integrated pest management programme. With the development of insect and pest resistant transgenic crop plant the proteinase inhibitor genes will make a promising contribution toward maximizing yield and minimizing losses due to insects and pests.

So, the isolated Pigeonpea protease inhibitor gene can be mobilizes in to susceptible crop plants by genetic transformation for development of transgenic crop plant. These crop plants will be resistant to insect pest and help in enhancing crop productivity by minimizing the losses of insect pests.
Fig. 2 Protease Inhibitor activity (U/mg) in four varieties of Pigeonpea

Fig. 3 Elution profile of PI protein of Pigeonpea on DEAE-cellulose column chromatography
Fig. 4 Elution profile of PI protein of Pigeonpea on Gel-filtration chromatography

Fig. 5 Purified Pigeonpea Protease Inhibitor protein resolved on SDS-PAGE
Lane 1. Marker Lane, 2. Purified PI Protein
Fig. 6 Molecular Weight determination of purified Pigeonpea Protease Inhibitor protein on Gel-filtration

Fig. 7 Effect of pH on purified Pigeonpea Protease Inhibitor protein of Pigeonpea
Fig. 8 Effect of Pigeonpea Protease Inhibitor protein on larval weight of *Helicoverpa armigera*

Fig. 9 Effect of Pigeonpea Protease Inhibitor protein on mortality of *Helicoverpa armigera*
Fig. 10 Effect of Pigeonpea Protease Inhibitor protein on molting of *Helicoverpa armigera* larvae and adult

Fig. 11 Study on biomass of developing seeds of Pigeonpea
Fig. 12 PI activity in developing seeds of Pigeonpea

Fig. 13 Agarose gel electrophoresis and Southern hybridization of restricted Pigeonpea genomic DNA

A- Purified genomic DNA of Pigeonpea with marker
B- Restricted genomic DNA
Lane-1 Eco RI, Lane-2 Bam HI, Lane-3 Hinb DIII
C- Autoradiogram
Fig. 12 PI activity in developing seeds of Pigeonpea

Fig. 13 Agarose gel electrophoresis and Southern hybridization of restricted Pigeonpea genomic DNA
A- Purified genomic DNA of Pigeonpea with marker
B- Restricted genomic DNA
Lane-1 Eco RI, Lane-2 Bam HI, Lane-3 Hinb DIII
C- Autoradiogram
Fig. 14 Total RNA from developing seeds of Pigeonpea and LD-PCR
A- Lane M- RNA Marker
   Lane 1, 2, 3- total RNA
B- LD-PCR Lane M- Marker 1 Kb ladder
   Lane 1, 2, 3- LD-PCR product

Fig. 15 Size Fractionation of LD-PCR with 1 Kb ladder
   Lane M- Marker 1 Kb ladder
   Lane 1, 2, 3, 4- Fractionated LD-PCR product
Fig. 16 pGEM-T Easy vector

Fig. 17 Blue-White colony screening of cDNA library
Fig. 18 Screening of Pigeonpea cDNA library with radio-labeled Tomato TIII cDNA probe
A - Primary screening of cDNA library
B - Secondary screening of cDNA library
C - Tertiary screening of cDNA library

Fig. 19 Restriction digestion of positive clones (1-6) with Eco RI
Lane M - Marker 1 Kb ladder
Lane 1, 2, 3, 4, 5, 6 - Positive clones
**Fig. 20** Distribution of vector matches on query sequence

```
GAATGGGCCCGGCCTCGCATGCTCCCCGGCCGCCATGGCGGCGGCGGG
GAATTCCGATTTCTCCTCTATTTTGCAATTCTAAACCACCTCTTTTATTTGCATTCT
CCAAATAACATGCTATTGAGCAGTTTGATGATACAATGTGGTACCCCTTA
TCCCTGCTGTGATGAACTACACTACATTCCACAGGCAAGTGATAACCCCTAAAACCTG
GAGGACTAACCCCTAAACAAAAATTAGTGATGCAGAGTGTCCTGTAACCTGTC
CTACAAAAATAATGCAGCAGAAGGTTTATCCCAGGTTAAAATTCACCTATACGGGA
AGTAATAACACTGTGAATAAATATCTTTGACTAATATCCTGACTTTGAAAATGAGT
TCACAAAGAAGCCAAATTGCGTTGAATCATCAAAATGGATATTCTTTGTTG
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TTGGTTTGAAGAATACTCAATGGCAGAATTTTTAATTGTAAGACATGCTTCTGG
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TTGGATTGGAATACCTTTTGTGATGAGGAGGTTGGAATGAGACTACAAGTTTTTAA
AATCA
CTAGTGAATTCCGCGCCGCCCTGCAAGTGACCAATATGGGAGACAGCTCC
AACGGCGTTGATGCAATAGCTGA
```

Fig. 21 Gene sequence of Pigeonpea Protease Inhibitor
The vector sequences are shown red on both side of gene sequence
Fig. 22 BLAST similarity of PPI sequence with other protease inhibitors sequence

![BLAST similarity](image)

**Table 1**: BLAST scores for PPI sequence

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Coverage</th>
<th>E-value</th>
<th>Mass Ident</th>
<th>Links</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ278262.1</td>
<td>Cicer arietinum mRNA for trypsin protein inhibitor 1 (tpi1 gene), clone CePPI-1</td>
<td>1200</td>
<td>1200</td>
<td>61%</td>
<td>0.0</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>AC122730.27</td>
<td>Medicago truncatula chromosome 6 clone mbr2-3605, complete seq</td>
<td>171</td>
<td>171</td>
<td>61%</td>
<td>5e-39</td>
<td>75%</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 23 PPI gene sequence alignment with *Cicer arietinum* mRNA protease inhibitor 1 gene sequence

**Alignment**

![Gene alignment](image)

**Description**

Legend for links to other resources:
- UnGene
- GEO
- Gene Structure
- Map Viewer

**Table 2**: Sequence alignments

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Alignment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ278262.1</td>
<td>Cicer arietinum mRNA for trypsin protein inhibitor 1 (tpi1 gene), clone CePPI-1</td>
<td>102</td>
</tr>
<tr>
<td>Cicer 25</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Celer 85</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td>Celer 145</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>Celer 185</td>
<td>222</td>
<td></td>
</tr>
<tr>
<td>Celer 205</td>
<td>282</td>
<td></td>
</tr>
<tr>
<td>Celer 2083</td>
<td>284</td>
<td></td>
</tr>
<tr>
<td>Celer 265</td>
<td>342</td>
<td></td>
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<tr>
<td>Celer 325</td>
<td>384</td>
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<tr>
<td>Celer 403</td>
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<td>Celer 385</td>
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<td>Celer 453</td>
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<tr>
<td>Celer 523</td>
<td>522</td>
<td></td>
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<tr>
<td>Celer 505</td>
<td>582</td>
<td></td>
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<tr>
<td>Celer 583</td>
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<td></td>
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<tr>
<td>Celer 643</td>
<td>624</td>
<td></td>
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<tr>
<td>Celer 625</td>
<td>678</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 24 PPI sequence alignment with other legumes crop PI sequence

The identical sequences in three legumes are red, two legumes are blue and sequences which are different from others are shown black.
Fig. 25 ORF of Pigeonpea Protease Inhibitor gene on BLAST

Fig. 26 ORF sequence of Pigeonpea Protease Inhibitor gene
The start codon is blue and the stop codon is pink
MLPGRHGGRGEGFDLLFAILTNPLFAFSNNNNAIEQVLD
TNNGNPLIPGDEYYPFASDNPKTGGLTLNKISDAECPV
TVLQNNATRGLPVKFTLSGSNNTNNTLNDLTLDIEF
TTKPNCVESSKWHVFVDFTPGGCVGIGGEPENHLLGLEI
LNGKFLIVRHASGYVYRFGLCDVSGDCGLLLGGLNTFDS
REGGSRLILTIIFNSYNVVFVDVASVKGSGRIMPLKGGFD
ASV Stop

Fig. 27 PPI gene sequence deduced amino acids sequence

Fig. 28 Cn3D view of PPI gene sequence deduced amino acids sequence
Fig. 29 BLAST for amino acid sequence homology with other protease inhibitors

Score = 437 bits (1123), Expect = 4e-121, Method: Compositional matrix adjust.

Identities = 217/217 (100%), Positives = 217/217 (100%), Gaps = 0/217 (0%)