# अरहर लेक्टिन जीन की *इन विट्रो* अभिव्यक्ति एवं इसका अभिलक्षणन

# *In Vitro* Expression of Pigeonpea Lectin Gene and its Characterization

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# *In Vitro* Expression of Pigeonpea Lectin Gene and its Characterization

**By**

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# CERTIFICATE

This is to certify that the thesis entitled "***In Vitro* Expression of Pigeonpea Lectin Gene and its Characterization**" submitted to the Faculty of the Post-Graduate School, **Indian Agricultural Research Institute, New Delhi**, in partial fulfilment of **Master of Science** in **Molecular Biology and Biotechnology**, embodies the results of *bona fide* research work carried out by **Mr. SUDHIR KUMAR**, under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma. The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

Date: 03/07/2014
Place: New Delhi

**(Dr. Rekha Kansal)**
Chairperson
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# *Abbreviations*

- **APS:** Ammonium Persulfate
- **ASAL :** *Allium sativum* leaf lectin
- **bp :** base pair
- **EDTA :** Ethylene Diamine Tetra acetic Acid
- **GNA :** *Galanthus nivalis* Agglutinin
- **IPTG** : Isopropyl β-D-1-thiogalactopyranoside
- **KDa :** Kilo Dalton
- **LA :** Luria Agar
- **LB :** Luria Broth
- **mM** : Milli Molar
- **ºC :** Degree Celsius
- **PPL :** Pigeonpea Lectin
- **PVDF :** Polyvinylidene fluoride
- **RT :** Room Temperature
- **SDS-PAGE :** Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
- **TAE :** Tris-Acetate-EDTA
- **TBS :** Tris-Buffered Saline
- **TEMED :** Tetramethylethylenediamine
- **TSS** : Transformation and Storage Solution
- **SSC :** Saline sodium citrate
- **ml :** Milliliter
- **µg :** Microgram ($10^{-6}$ g)
- **µl :** Microliter ($10^{-6}$ l)
- **ng :** Nanogram ($10^{-9}$ g)

# *1. Introduction*

Worldwide use of insecticides and pesticides has led to the harmful effects on environment, aquatic ecosystem, non-target organism, human health and increased insect resistance. The development of resistance to insecticides has necessitated the application of higher dosages of the pesticides or increased number of applications. This not only increased the cost of pest control, but has also resulted in insecticidal hazards and pollution to the environment. Recent efforts have been made to reduce broad spectrum of toxicant added to the environment as it has become necessary to balance agricultural needs with environmental and health issues when using insecticides. Therefore, the use of natural entomotoxic metabolites have increased because of the growing need to obtain better food quality and protection of environment (Jaber *et al*., 2008).

Efforts were made to obtain insect resistant plants by introducing *Bt* gene. However, the codon bias and low expression of *Bt* genes, shifted the attention towards isolation of insect resistance genes of plant origin. Currently, the inhibitors of the digestive enzymes (protease and amylase) and lectins are in limelight to confer resistance against various insect pests. Lectin genes of plant origin have an added advantage, as when transferred, they are correctly translated and processed in the plant host. Moreover, most of lectin genes are devoid of introns and therefore, both genomic as well as cDNA clones can be mobilized for developing transgenic plants.

Most plants contain one or more carbohydrate binding proteins termed as lectins. They are heterogeneous group of proteins having a protective function against a wide array of organisms viz. insects, bacteria, fungi, virus etc. They reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran (*Spodoptera litura, Helicoverpa armigera* etc.) and homopteran (aphids, plant hoppers etc.) insect pests, but have no effect on human metabolism (Boulter *et al*., 1993). The harmful effects of lectins on biological

parameters of insects includes loss in weight, mortality, feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation. Realizing their importance, lectin genes have been isolated and characterized from various plant species, e.g. snowdrop (Peumans and Van Damne, 1995), garlic (Smeets *et al*., 1997), pea (Gatehouse and Gatehouse, 1998), cowpea (Datta *et al*., 2000), chickpea (Qureshi *et al.,* 2006), Onion (Hossain *et al.,* 2006), mungbean (Anshuman *et al.,* 2010), mothbean (Singh *et al.,* 2010) and pigeonpea (Accession number JN561784.1).

Lectins are highly specific for binding to oligosaccharides, hence if specific carbohydrate is present on the surface of tissue, it can bind to them. The ingested lectin bound to the midgut tract causes disruption of the epithelial cells including elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut leading to the complete closure of the lumen (Majumder *et al.,* 2006, Karimi *et al*., 2010).

GNA, (the mannose specific Lectin from snowdrop (*Galanthus nivalis*) *w*as found to be insecticidal in artificial diets against the peach potato aphid (*Myzus persicae*). The high efficacy of the mannose binding lectin, *Allium sativum* lectin (ASAL) on *Lipaphis erysimi* using artificial diet bioassay also showed a correlation between binding of the toxin to the gut epithelial membrane and toxicity to the insect (Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004). The Galβ1-3GalNAc-ser/thr-binding lectin from *Sclerotium rolfsii* Sacc., a soil-borne plant pathogen has two distinct carbohydrate-binding sites, a primary and a secondary and elicits the structural determinants of the molecular recognition for carbohydrate binding. Immunolocalization and expression studies on SRL led to the identification of putative endogenous glycolsphingolipid receptor recognizing and interacting with SRL during the functional role of development and morphogenesis of *Sclerotium rolfsii* (Swamy *et al*., 2004). Both the crystal structure and the amino acid sequence were reported to explain structural basis of carbohydrate recognition (Leonidas *et al*., 2007).

In the present study the efficacy of the isolated pigeon pea lectin gene needs to be tested by expressing in an expression vector and *In Vitro* insect bioassay. Then the gene coding for pigeonpea lectin will be used to develop transgenic crop plants resistant

to lepidopteran and homopteran insect pests. Exploitation of these insect resistance genes for developing resistant plant varieties will provide better option for plant protection. Therefore, the present study was undertaken with the following objective:

"Cloning and Expression of Pigeonpea Lectin Gene in an Expression Vector and its Characterization"

# *2. Background*

Crop losses due to insect pests are a significant factor in limiting food production. Worldwide use of chemical insecticides and pesticides has increased the cost of pest control, resulted in insecticidal hazards to biological organisms, pollution to the environment and increasing insect resistance. Therefore, the challenge today is to achieve higher and stable crop production with safe and eco-friendly strategies. Plants accumulate a set of defense proteins including lectins, proteinase inhibitors, amylase inhibitors etc., of which lectins are the plant proteins that reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran and homopteran insect pests.

## 2.1 Plant Resistance Genes

Few plant resistance (R) genes and their homologues associated with insect resistance have been reported. The nematode resistance gene *Mi-1.2* gene from *Lycopersicon peruvianum* confers resistance to the potato aphid, *Macrosiphum euphorbiae* and belongs to the nucleotide-binding leucine-rich repeat (NB-LRR) family of resistance genes. *Mi-1.2* gene mediates resistance against both aphids and nematodes, organisms belonging to distantly related phyla (Rossi *et al.*, 1998). The virus aphid transmission (*Vat*) gene from *Cucumis melo* controls resistance to the cotton aphid, *Aphis gossypii*. *Vat* putatively encodes a cytoplasmic protein with NB-LRR characteristics (Klingler *et al.,* 2005). *Mi-1.2* and *Vat* both are single dominant R genes but exhibit differential mechanism of inhibition to aphids. Resistance conferred by Mi-1.2 gene results in aphid starvation and desiccation while that of *Vat* gene retards growth of aphid population by decreasing fitness and fecundity. Recent studies into the differential expression of *Pto* and *Pti1*-like kinase genes in wheat plants resistant to the Russian wheat aphid, *Diuraphis noxia* provide evidence of the involvement of the *Pto* class of resistance genes in aphid resistance (Boyko *et al.*, 2006). *Pto* was reported to be

cytoplasmic serine/threonine kinase which is involved in both elicitor recognition and ser/thr phosphorylation (Afzal *et al*., 2008).

**2.2 Lectins**

Lectins have been defined as the carbohydrate-binding proteins (or glycoproteins) of non-immune origin that agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al*., 1980). The use of carbohydrate-binding activity rather than haemagglutination activity is considered as the functional criterion of lectins. Later with the observation that some lectins contain a second type of binding site that interacts with non-carbohydrate ligands, lectins were redefined as carbohydrate-binding proteins other than antibodies or enzymes (Barondes, 1988). This definition was updated with the new insights obtained from the molecular cloning of lectin and lectin-related protein coding genes. Because some plant enzymes (like Type2 ribosome inactivating proteins [RIP] and the class I chitinases) being fusion proteins built up of a carbohydrate-binding domain tandemly arrayed with a catalytic domain, definition of lectins cannot exclude them. On the other hand, observation that some proteins that are related to lectins, but lack carbohydrate-binding domain insisted on including functionality as a criterion. Now the only prerequisite for a protein to be named as lectin is the presence of at least one non-catalytic domain that binds reversibly to a specific carbohydrate (Peumans and Van Damme, 1995).

**2.3 Classification of Lectins**

Based on the overall structure and carbohydrate-binding sites, lectins are distinguished into merolectins, hololectins, chimerolectins and superlectins. Merolectins consist of a single carbohydrate-binding domain (monovalent), hence cannot precipitate glycoconjugates or agglutinate the cells. Hololectins also are built exclusively of carbohydrate binding domains but contain two or more such domains that are either identical or homologous and bind either the same or structurally similar sugar(s). Majority of all known plant lectins are hololectins and behave as haemagglutinins. Chimerolectins are fusion proteins possessing a carbohydrate-binding domain tandemly arrayed with an unrelated domain which has catalytic activity that can bind to carbohydrates. Depending on the number of carbohydrate-binding sites, chimerolectin

could be like merolectin or hololectin. Superlectins, like hololectins, consist exclusively of two carbohydrate-binding domains. However, unlike the hololectins, the carbohydrate-binding domains of the superlectins recognize structurally unrelated sugars. Therefore, superlectins can also be considered as a special type of chimerolectin composed of two tandemly arrayed structurally and functionally different carbohydrate-binding domains.

Several attempts were made to subdivide plant lectins based on carbohydrate-binding specificity into mannose-, mannose/glucose-, mannose/maltose-, Gal/GalNAc-, GlcNac/ (GlcNAc)n-, fucose and sialic acid-binding lectins (Goldstein and Poretz, 1986; Van Damme *et al*., 1998). Analysis of the available plant lectin sequences identify seven families of evolutionarily related proteins. Four of these families, namely, the Legume lectins, the Monocot mannose-binding lectins, the Chitin-binding lectins, and the type 2 RIP, comprise numerous members. In contrast to the above four families, the Jacalin related lectins, the Amaranthin lectin family, and the Cucurbitaceae phloem lectins are at this moment only small protein families.

**2.4 Biological Activities of Plant Lectins**

Lectins recognize diverse sugar structures (mono- or oligosaccharides) and hence mediate a variety of biological processes (Nathan and halina, 2004) (Table 2.1) such as cell-cell (Fig. 2.1) and host-pathogen interaction and innate immune response (Vijayan and Chandra, 1999). They also have a major role in plant defense; protection of plants from phytopathogens (Chrispeels and Raikhel, 1991) and insects (Peumans and Van Damme, 1995).

**2.5 Plant Defense**

Many plant lectins are able to bind simple sugars such as glucose, mannose or galactose, which are not common or totally absent in plants. For instance, chitin binding plant lectins recognize a carbohydrate that is a typical constituent of the cell wall of fungi and the exoskeleton of invertebrates. A circumstantial argument in favor of a defense role of plant lectins is their marked stability under a wide range of pH, heat and

Table 2:1 Functions of Lectins:

| Lectin | Role in |
|---|---|
| Microorganisms | |
| Amoeba | Infection |
| Bacteria | Infection |
| Influenza virus | Infection |
| Plants | |
| Various | Defense |
| Legumes | Symbiosis with nitrogen-fixing bacteria |
| Animals | |
| Calnexin, calreticulin, ERGIC-53 | Control of glycoprotein biosynthesis |
| Collectins | Innate immunity |
| Dectin-1 | Innate immunity |
| Galectins | Regulation of cell growth and apoptosis; regulation of the cell cycle; modulation of cell–cell and cell–substratum interactions |
| Macrophage mannose receptor | Innate immunity; clearance of sulfated glycoprotein hormones |
| Man-6-P receptors | Targeting of lysosomal enzymes |
| L-selectin | Lymphocyte homing |
| E- and P-selectins | Leukocyte trafficking to sites of inflammation |
| Siglecs | Cell-cell interactions in the immune and neural system |
| Spermadhesin | Sperm-egg interaction |

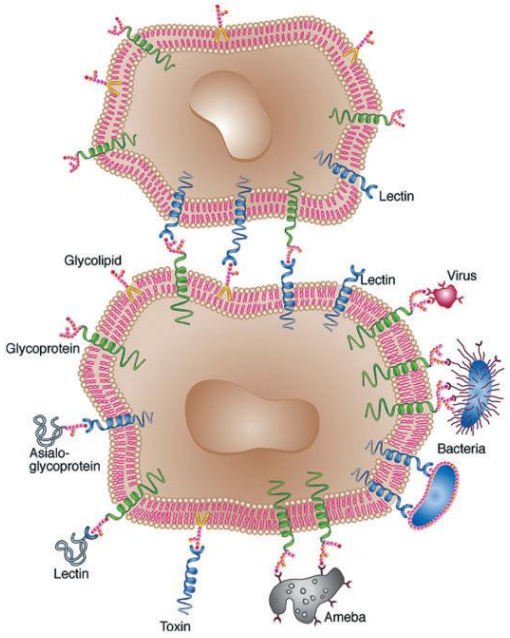



**Fig. 2.1 Cell Surface Lectin-Carbohydrate Interactions:** In some cases, cell surface lectins bind particular glycoproteins, whereas in other cases the carbohydrates of cell surface glycoproteins or glycolipids serve as sites of attachment for biologically active molecules that themselves are lectins (e.g. galectins)

exposure to proteases. The preferential association of lectins with those parts of the plant such as resting storage organs and seeds that are most susceptible to attack by pests is also an argument for a protective role. However, the direct evidence that lectins play a role in plant defense was obtained using purified protein in artificial diets or by using transgenic plants (Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004).

### 2.5.1 Antiviral Activity

Except type 2 RIPs no other plant lectins have been reported to inhibit viral infection, replication, or systemic spread because of the absence of glycans on plant viruses. Several plant lectins are potent inhibitors *in vitro* of animal and human viruses, which have glycoproteins in their virions (Balzarini *et al.*, 1992). Some plant lectins

have an indirect antiviral role, as the presence of insecticidal lectins may prevent or reduce the spread of insect transmitted viral diseases.

### 2.5.2 Antibacterial Activity

Sequeira and Graham (1977) showed that the cell wall of bacteria not only avoid any interaction between the glycoconjugates on their membrane and carbohydrate-binding proteins but also prevents these proteins from penetrating the cytoplasm. The plant lectins cannot alter the structure and/or permeability of the membrane or disturb the normal intracellular processes of invading microbes. Lectins play a role in the plant's defense against bacteria, through an indirect mechanism that is based on interactions with cell wall carbohydrates or extracellular glycans. The potato lectin (which is considered as a cell wall protein) immobilized avirulent strains of *Pseudomonas solanacearum* in the cell wall. Virulent strains were not recognized by the lectin, which escaped attachment to the cell wall, and therefore were able to multiply and spread over the plant. Another indirect defense mechanism is the blocking of the movements of normally motile bacteria at the air-water interface by the thorn apple *(Datura stramonium)* seed lectin (Broekaert and Peumans, 1986). By counteracting the chemotactic movement of soil bacteria toward the germinating seed, the lectin may prevent invasion of the seedling roots by potentially harmful bacteria. Since recent studies of the binding of plant lectins to bacterial cell wall peptidoglycans indicated that several legume seed lectins strongly interact with muramic acid, N-acetylmuramic acid, and muramyl dipeptide, the involvement of lectins in the plant's defense against microbes have been underestimated (Ayouba *et al.*, 1994).

### 2.5.3 Antifungal Activity

Plant lectins cannot bind to glycoconjugates on the fungal membranes or penetrate the cytoplasm of the cells because of the presence of a thick and rigid cell wall. However, indirect effects based on the binding of lectins to carbohydrates exposed on the surface of the fungal cell wall are possible. By virtue of their specificity, chitin-binding lectins seemed likely to have a role in the plant's defense against fungi (and insects). *In vitro* studies, demonstrating that wheat germ agglutinins (WGA) inhibited

spore germination and hyphal growth of *Trichoderma viride,* strongly supported the hypothesis of the antifungal role of the chitin-binding plant lectins (Schlumbaum *et al.*, 1986).

**2.5.4 Anti-Insect Activity**

The epithelial cells along the digestive tract of phytophagous insects are directly exposed to the contents of the diet and, therefore, are possible target sites for plant defense proteins. Since glycoproteins are major constituents of these membranes, the lumenal side of the gut is literally covered with potential binding sites for dietary lectins. The binding of a lectin to a glycoprotein receptor provokes a local or systemic deleterious effect; the insect may be repelled, retarded growth, or even killed. *Phaseolus vulgaris* lectin (PHA) was the first lectin to which anti-insect properties were ascribed on the basis of its deleterious effect on the larvae of bruchid beetle *Callosobruchus maculatus* (cowpea weevil). More recent experiments indicated that the lectins from wheat germ, potato tuber, and seeds from peanut, thorn apple *(Datura strmonium),* and osage orange *(Maclurap omifera)* had an inhibitory effect on the development of larvae of the cowpea weevil. However, only WGA was active at a physiological concentration (Murdock *et al.*, 1990). WGA and the *Baukinia purpurea* seed lectin were lethal to neonate *Ostrinia nubilalis* larvae at fairly low concentrations. Similarly, the pokeweed *(Phytolacca americana)* lectin killed larvae of the Southern corn rootworm, whereas several other lectins, including WGA, inhibited larval growth. Therefore, both maize insects appear to be much more sensitive to dietary lectins than the seed predator cowpea weevil. Feeding trials with purified lectins from *Galanthus nivalis* (Fitches *et al*., (2001a) and garlic *Allium sativum* (Chakraborti *et al*., 2009) indicated that they are moderately active against chewing insects, such as the cowpea weevil and the tobacco horn worm *(Spodoptera littoralis).* The snowdrop lectin showed a high toxicity toward sucking insects not only in tests with artificial diets but also in experiments with transgenic plants (Hilder *et al.*, 1995). Lectin binds to the glycan receptors present on the surface lining of the insect gut and interfere with the formation and integrity of the peritrophic membrane of the mid-gut (Pusztai and Bardocz, 1996).

Sauvion (1996) showed that ConA altered the amino acid metabolism of the pea aphid *Acyrthosiphon pisum*. Fitches *et al.* (1997) fed GNA and ConA (2% of total protein) in a semi-artificial diet to *L. oleracea* caterpillars and assessed lectin binding in insects, GNA showed binding to specific regions on the brush border membrane villi and peritrophic matrix proteins, whereas ConA showed more prominent binding to gut tissues. Furthermore, both lectins also altered gut enzyme activity. Protein concentrations and activities of some digestive enzymes in the insect gut also increased. The changes inflicted by ConA-binding to gut surface receptors resulted in changes in metabolism and cell function in the epithelium, which, through a feedback mechanism, led to altered feeding behavior. Although it is very likely that their toxicity is based on a specific binding to glycoconjugates somewhere in the gut of the insect, the exact mechanism of action of plant lectins is not known.

**2.6 Physiological Effects of Lectins on Insects**

Plant lectins have severe effects on fecundity, growth and development of an insect. Screenings of a wide range of lectins demonstrated that the *Galanthus nivalis* agglutinin (GNA) has the highest anti-insect potential (Rahbe *et al.*, 1995). Subsequently, the effects of other GNA-related lectins were also investigated. However, non-mannose-specific lectins were also shown to possess insecticidal activity. Taking into account the diversity of glycan structures in insects, it is likely that lectins with different specificities interact with different glycoproteins or glycan structures and thus interfere with different physiological processes in the insects.

**2.7 Mode of Action of Plant Lectins**

The luminal side of the midgut epithelium of many insects is lined up with a physical barrier called the PM (Peritrophic Matrix) that is secreted by certain epithelium cells. This PM consists of a chitineous grid-like network held together by chitin-binding glycoproteins such as peritrophins. The PM-associated glycoproteins contain many glycan structures that fill the interstitial spaces creating a molecular sieve (Hegedus *et al*., 2009). Since both the chitin fibrils and many glycoproteins are present in the PM, this midgut structure is an obvious target for lectins. Indeed, studies that analyzed the

effect of plant lectins on the ultrastructural organization of the insect midgut have shown clear abnormalities in the formation of a functional PM and disruption of microvilli structures.

**2.8 Toxicity of Plant Lectins**

Plant lectins affect various biological parameters of insects including larval weight decrease, mortality, feeding inhibition, delay in total developmental duration, adult emergence and fecundity on the first and second generation (Powell *et al*., 1993; Habibi *et al*., 1993).Plant lectin either directly or indirectly cause profound morphological and physiological modifications in the insect intestine. In insects lectins bound to the midgut epithelium and caused disruption of the epithelial cells including elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut which led to complete closure of the lumen, and impaired nutrient assimilation. Among plant Lectins, *Galanthus nivalis* (snowdrop) agglutinin (GNA) (Fitches *et al*., (2001a). *Allium sativum* (garlic) leaf and bulb Lectin (ASAL) (Chakraborti *et al*., 2009), Concanavalin A (Con A), *Phaseolus vulgaris* lectin (PHA) (Fitches *et al*., (2001b) are more investigated and delivered successfully via artifitial diet and expressed in a range of crops (Fitches *et al.,* 2002). The high efficacy of the mannose binding lectin, ASAL on *Lipaphis erysimi* using artificial diet bioassay showed a correlation between binding of the toxin to the gut epithelial membrane and toxicity to the insect (Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004).

**2.9 Cloning and Expression of Lectin Genes**

Expression of plant lectins in heterologous systems offers a means of producing proteins of defined amino acid sequence for various purposes. Almost all the known lectin genes are known to be intron-free and hence the molecular weight of the protein can be predicted by prokaryotic expression. In these cases, DNA or cDNA coding for lectin is cloned into expression vector with His tag and then transformed to *E. coli.* After induction with IPTG, inclusion bodies from *E. coli* were solubilized and lectin was purified and characterized by SDS-PAGE. Initial attempts on cloning and expressing in *E. coli* included lectin genes from *Bauhinia purpurea* (Kusui *et al.*, 1991), *Erythrina*

*corallodendron* (Arango *et al.*, 1992), *Dolichos biflorus* (Chao and Etzler, 1994), lima bean (Jordan and Goldstein, 1994), *Galanthus nivalis* (Longstaff *et al.*, 1998), *Canavalia brasiliensis* (Nogueira *et al.*, 2002), *Pinellia ternate* (Lin *et al.*, 2003), *Phlebodium aureum* (Tateno *et al.*, 2003) and *Polyporus squamosus* (a mushroom) (Tateno *et al.*, 2004), recently a new lectin like protein was identified and cloned in tea against sap sucking insect pests (Bandyopadhyay *et al*., 2014). Most widely cloned lectins belong to mannose-binding types of monocots, where lectins have been cloned from seven families of angiosperms including Amaryllidaceae, Araceae, Alliaceae, Orchidaceae, Liliaceae, Iridaceae and Bromeliaceae (Van Damme *et al.*, 1991; Van Damme *et al.*, 1994; Van Damme *et al.*, 1996; Smeets *et al.*, 1997; Van Damme *et al.*, 2000; Neuteboom *et al.*, 2002; Chai *et al.*, 2003; Fei *et al.*, 2003; Kai *et al.*, 2003; Yao *et al.*, 2003; Zhao *et al.*, 2003), among which lectins from Amaryllidaceae species have been extensively studied and well documented (Van Damme *et al.*, 1992; Van Damme *et al.*, 1998; Kai *et al.*, 2003; Pang *et al.*, 2003; Zhao *et al.*, 2003). For gene cloning, conserved region [MQ(G/E/Y/D)D(C/G)NL] of plant lectins (Kai *et al.*, 2003; Pang *et al.*, 2003; Zhao *et al.*, 2003) has been used for initial amplification of partial genes followed by RACE (Rapid Amplification of cDNA Ends) (Frohan *et al.*, 1988). Mannose-binding lectins also exist in gymnosperms such as Taxaceae, where a novel lectin gene from *Taxus media* was isolated (Kai *et al.*, 2004). Primer specific to mannose-binding domain (QDNVY) has also been used to amplify and clone lectin gene from *Typhonium divaricatum* (L.) (Luo *et al.*, 2007). In another approach of cloning lectin genes, degenerate primers were designed based on complete or partial amino acid sequence. N terminal amino acid sequence based gene specific primers in combination with 3' RACE primer were used to clone lectin genes from *Viscum album* (Eck *et al.*, 1999) *Dolichos lablab* (Colucci *et al.*, 1999) and *Lycoris aurea* (Liu *et al.*, 2007). Full length genes were cloned by 5' RACE by designing primers based on 3' RACE sequence.

Many lectins are glycosylated, and the presence or absence of the carbohydrate side chains can have significant effects on the functional and physical properties of the expressed proteins. *E. coli* is also incapable of glycosylating eukaryotic proteins expressed in this host. Few reports indicate that plant lectins are non-glycosylated in *E. coli* (Hoffman and Donaldson, 1987; Rodriguez-Arango *et al.*, 1992; Zhu *et al.*, 1996),

and form insoluble inclusion bodies (Arango *et al.*, 1992; Adar *et al.*, 1997; Longstaff *et al.*, 1998).

Expression of lectins in *Saccharomyces cerevisiae* has not been widely employed mainly because it accumulates lectin mainly in the vacuole (Tague and Chrispeels, 1987), due to the presence of `cryptic' vacuolar targeting determinants (Von Schaewen and Chrispeels, 1993), and only about 1% is secreted. In addition, a significant proportion of lectin expressed in this host was not correctly processed; approximately half of the lectin accumulating in the vacuole appeared to contain the uncleaved signal peptide. The only report of the correct processing and secretion of a plant lectin in *Saccharomyces* is that of WGA which was secreted and exhibited sugar binding activity, however, yields of protein were relatively low, of the order of 200 μg/l (Nagahora *et al.*, 1992). Considering the problems associated with expression in *Saccharomyces*, methylotrophic yeast *Pichia pastoris* has been used successfully especially for the secretion of heterologous proteins (Raemaekers *et al.*, 1999; Huang *et al.*, 2005; Lannoo *et al.*, 2007).

**2.10 Transgenic Plants with Lectins**

Lectins have been successfully employed for developing transgenics in various crops. Genetic engineering of lectins in important crops is an effective and viable strategy to reduce survival, development and fecundity of sap-sucking insects and thereby can reduce crop damage. Based on the toxicity effects of lectins against insects the Genes encoding *Allium sativum* (garlic) leaf and bulb Lectin (ASA), *Galanthus nivalis* agglutinin (Allsop and McGhie, 1996), have been introduced in various crops like rice, sugarcane, potato, wheat etc. to reduce predation by phloem feeding insects such as aphids. Expression of wheat germ agglutinin (WGA) in *Brassica juncea* has shown reduction in mustard aphid, *Lipaphis erysimi* survival (Kanrar *et al*., 2002). In vitro studies demonstrated that *Allium sativum* Leaf Agglutinin (ASAL) was very effective against the mustard aphid, *Lipaphis erysimi*, and the red cotton bug, *Dysdercus cingulatus* (Bandhyopadhyay *et al*., 2001; Roy *et al*., 2002). Expression of ASAL in tobacco and Indian mustard also offered protection against *Myzus nicotianae*, *M. persicae* and *Lipaphis erysimi* (Dutta *et al*., 2005; Hossain *et al*., 2006; Sadeghi *et al*.,

2007). The developed transgenic plants expressing lectins showed significant entomotoxic effects as evidenced by insect bioassays under controlled conditions. The protein Tarin 1, from *Colocasia esculenta*, was expressed in tobacco. Bioassays were done on plants expressing Tarin 1 at different levels using *Spodoptera frugiperda* larvae, various bacteria and fungi and the root-knot nematode *Meloidogyne javanica*. It was found that *S. frugiperda* larvae fed on transformed plants had retarded growth and lower pupation, and higher mortality rate than larvae fed on control plants. Also, Tarin 1 was found to inhibit the growth *in vitro* of *Pseudomonas syringae* pv. tomato. For *Meloidogyne javanica*, both relative replication and root damage were greater in control plants than in transformed plants (Leal-Bertioli *et al.,* 2003). During last two decades numerous reports have indicated insecticidal activity of plant lectins against many insect pests belonging to the orders of Lepidoptera, Coleoptera, Diptera and Homoptera (Boulter *et al.*, 1990; Gatehouse *et al.,* 1995; Hilder *et al.,* 1995; Sharma *et al.*, 2004). The first demonstration was that of enhanced resistance of transgenic tobacco plants expressing glucose/mannose-binding lectin from pea (*Pisum sativum*). Bioassay of transgenic tobacco against *Helicoverpa virescens* showed significant control (Boulter *et al.*, 1990). Greater insecticidal activity was shown by chitin binding lectin, WGA and common bean (PHA) lectin. WGA has been expressed in transgenic maize demonstrating inhibition of larvae of European corn borer and *Diabrotica* sp. (Maddock *et al.*, 1991). GNA has been the most widely used lectin for developing transgenic plants. It was introduced into tobacco, which showed enhanced resistance to the peach potato aphid (*M. persicae*) as well as tuber moth larvae (Gatehouse *et al.*, 1997). GNA expressed in transgenic rice plants conferred resistance to rice brown plant hopper (BPH) *Nilaparvata lugens* (Powell *et al.*, 1998; Rao *et al.,* 1998). Its expression was driven by a phloem specific promoter of rice sucrose synthase (RSs1) gene. Histochemical and immunochemical assays demonstrated that the expression of both GUS (β-glucuronidase) and GNA was restricted to phloem tissue, and was not observed in any other tissues. This phloem-specific expression pattern was consistent in stem, leaf and root, and in different transgenic plants. In addition, GNA was detected by immunological assay in the honeydew produced by peach potato aphids (*Myzus persicae*) feeding on RSs1-GNA transgenic tobacco plants. This provided direct

evidence that GNA was not only expressed in the phloem tissue, but was also present in the phloem sap of transgenic tobacco plants. Three insecticidal genes encoding Cry1Ac and Cry2A and GNA were transformed to rice by particle bombardment (Maqbool *et al.*, 2001). Transgenic plants expressed Cry1Ac, Cry2A and GNA at different levels, either singly or in combination. The transgenes showed stable transmission and expression. Transgenic rice plants showed significantly higher resistance to leaf roller (*Cnaphalocrocis medinalis*), yellow stem borer (*Scirpophaga incertulas*) and brown plant hopper (*Nilaparvata lugens*). This approach maximizes the utility of gene transfer technology to introduce combinations of genes whose products disrupt different biochemical or physiological processes in the same insect, providing a multi-mechanism defense. Recently, a novel short, lepidopteran-selective toxin, ButaIT, from the red scorpion (*Mesobuthu stamulus*) was fused N-terminally to a GNA polypeptide (ButaIT/GNA). Recombinant protein produced in *Pichia pastoris* was purified (Trung *et al.,* 2006). Recombinant ButaIT/GNA was acutely toxic when injected into larvae of tomato moth (*Lacanobia oleracea*), causing slow paralysis, and leading to mortality or decreased growth. ButaIT/GNA was chronically toxic when fed to *L. oleracea* larvae, causing decreased survival and weight gain under conditions where GNA alone was effectively non-toxic. Intact ButaIT/GNA was detected in larval haemolymph from insects fed the fusion protein orally, demonstrating transport of the linked polypeptide across the gut. ButaIT/GNA was significantly more toxic than GNA alone when fed to the homopteran *Nilaparvata lugens* (rice brown plant hopper) in liquid artificial diet.

The snowdrop lectin (GNA) and garlic lectins (ASAL) have a detrimental effect on plant hoppers. GNA has been expressed in diverse crop plants (Nagadhara *et al*., 2003, 2004; Couty *et al*., 2001) to confer protection against different sucking pests. Similarly, expression of garlic lectin (ASAL) in rice (Saha *et al*., 2006; Yarasi *et al*., 2008; Chandrasekhar *et al*., 2014) and cotton (Vajhala *et al*., 2013) conveyed marked resistance against homopteran and lepidopteran pests. ASAL and GNA pyramided rice lines showed higher degree of resistance against sap sucking insect families in comparison to those that express individual genes (Bharathi *et al*., 2011).

# 3. Materials and Methods

The research work was carried out at National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute Campus, New Delhi. The details of the research materials and methods adopted in the present investigation are described in this chapter.

## 3.1 Materials

### 3.1.1 Bacterial Culture

For cloning, the culture of Pigeonpea lectin gene cloned in pGEMTEasy vector transformed into DH5α was used. Lectin gene cloned in pGEMTEasy vector was streaked on LA plate supplemented with ampicillin (100 µg/ml). *E. coli* BL21 DE3 pLysS strain was grown on LA plate supplemented with chloramphenicol (34µg/ml).

### 3.1.2 Chemicals

The general chemicals like Tris buffer, acetic acid, hydrochloric acid, NaCl, EDTA, magnesium chloride, glycerol, ethanol, glacial acetic acid, methanol and Coommassie Brilliant Blue R-250 were purchased from M/s Sisco Research Laboratory, Mumbai and were of molecular biology grade. Agarose and Ethidium bromide were purchased from M\s Geneaid. The antibiotics ampicillin, kanamycin and chloramphenicol were procured from Sigma Chemical Company, St. Louis MO, USA. LA and LB were procured from M/s Himedia, Mumbai. Acrylamide and bis-acrylamide were purchased from Amresco. APS and TEMED were purchased from M/s G Biosciences.

### 3.1.3 Kits and Enzymes

Plasmid DNA isolation kit was procured from M/s Macharey-Nagel. 1Kb DNA Ladder was procured from M/s Takara. Phusion DNA polymerase, Taq DNA polymerase, dNTPs, 10 x Taq buffers, 6x loading dye were purchased from M/s Biolabs. pENTR-D-TOPO cloning kit, Destination vector and LR clonase were purchased from

M/s Invitrogen life Technologies. Primers for lectin gene amplification were purchased from M/s BIO-Engineering chemical company, St. Louis MO, USA. PVDF membrane and Western Breeze kit for protein analysis was procured from M/s Invitrogen life Technologies.

### 3.1.4 Other Materials

Microfuge tubes, PCR tubes, Micro tips and sterile petri plates were purchased from M/s Tarsons, Kolkata.

## 3.2 Methods

### 3.2.1 Cloning

#### 3.2.1.1 Preparation of Media

4.0 g LA was weighed and dissolved in 100 ml of distilled water. The media was autoclaved at 121 °C, 15 lbs pressure for 20 minutes and allowed to cool to about 50 °C. The antibiotic Ampicillin (100 µg/ml) and Kanamycin (50 µg/ml) was added to the media, poured on to sterile Petri plates and allowed to solidify.

2.0 g of Luria broth (LB) powder was dissolved in 100 ml of distilled water. 10 ml of LB media was dispensed into 10 culture tubes and autoclaved.

#### 3.2.1.2 Streaking of Culture

Inoculum of plasmid containing lectin gene in pGEMTEasy vector was streaked across one side of the LA plates containing Ampicillin (100 µg/ml) and protein expression host *E. coli* BL21 DE3 pLysS cells was streaked across the plate containing chloramphenicol (34 µg/ml) with sterile transfer loop. The loop was sterilized again by flaming, cooled and passed once through the first streak. This process was repeated once again. Plate was incubated at 37 °C for 16 hrs. Single colonies were obtained and stored at 4 °C for further use.

#### 3.2.1.3 Isolation of Plasmid DNA

Plasmid DNA of lectin gene cloned in pGEMTEasy vector was isolated and purified using Macherey-Nagel plasmid isolation kit based on alkaline lysis method. Lectin gene cloned in pGEMTEasy vector was inoculated in 10 ml of LB medium containing Ampicillin (100µg/ml). The medium was incubated overnight at 37 °C in an incubator shaker at 200 rpm for bacterial growth. Overnight grown culture was transferred into sterile centrifuge tubes and centrifuged for 10 minutes at 7000 rpm at RT for the harvesting of bacterial cells. The Pellet was suspended in 100 µl of solution P1 containing RNAse A and vortexed to remove any cell clumps formed. The resuspended pellet was transferred to microfuge tube, 100 µl of P2 buffer was added to the above mixture at room temperature and mixed gently by inverting the tube 8-10 times and was allowed to stand for 2 minutes until lysate became clear. After the lysis, 200 µl of chilled P3 buffer was added and mixed gently by inverting the tube 8-10 times. After the neutralization process, the solution was centrifuged at 15000 rpm for 15 minutes. The supernatant was transferred to a filter column tube and the pellet was discarded. The column along with the collection tube was centrifuged and discarded the flow through. After binding of DNA to binding column 400 µl of DNA wash buffer containing ethanol was added in the center of the column and centrifuged for 30-60 seconds and the flow through was discarded again. Washing with 500 µl DNA wash buffer was repeated and flow through was discarded. The column was centrifuged for 1 minute again to dry the column. Finally the DNA was eluted in 50 µl of elution buffer. 2 µl of the DNA was analyzed on 1% Agarose gel for the quality and quantity and rest of the DNA was kept at -20 °C till further use.

### 3.2.1.4 Agarose Gel Electrophoresis

**Casting of Gel**

For preparation of 1% agarose gel, 0.30 g agarose was weighed and transferred to a 150 ml conical flask, containing 30 ml of 1X TAE buffer and heated till agarose dissolved completely. The solution was allowed to cool down to 45 °C and then 1 µl of ethidium bromide (10 mg/ml) was added. The gel was poured into casting tray. The

comb was placed in the tray for the formation of wells. The gel was allowed to solidify, removed the comb and transferred the tray to the gel electrophoresis tank.

**Preparation of Loading Samples and Electrophoresis**

To 2µl of the plasmid DNA sample, 6µl sterile water and 2 µl of 6X loading dye (containing 0.25% (W/V) xylene Cyanol and 0.25% (W/V) bromophenol blue) was added. The contents were mixed, spun down and loaded into the wells. The 1 Kb DNA ladder (5 µl, 0.1 µg/µl) was also loaded in a separate well and electrophoresed at 45 V for 2 hour using 1X TAE buffer as running buffer at room temperature. The gel was viewed under UV trans-illuminator and photographed using gel documentation system (Syngene).

**3.2.1.5 PCR of the Lectin Gene with Phusion DNA Polymerase**

Isolated plasmid DNA of pigeonpea lectin gene was used for the PCR amplification using high fidelity Phusion DNA polymerase. Each cycle of PCR included three main steps i.e. template denaturation, primer annealing and primer extension. Polymerase chain reaction (PCR) amplification was performed using gene specific primers. Forward and reverse primers were designed from the ORF region of pigeonpea lectin cDNA with the addition of CACC at the 5' end of the forward primer.

Forward primer: 5' CACCATGGCTTCTCTTCAAACCCAAA 3'

Reverse primer: 5' TGCATCTGCAGCTTGCTTAGAACTCGA 3'

The PCR reaction was carried out with Phusion DNA polymerase enzyme mix along with supplied high fidelity Phusion buffer with it. The reaction mix was prepared as per Table 3.1. The PCR was carried out for 35 cycles. Given below are the thermo cyclic conditions for the PCR reactions (Table 3.2). The master mix for three reactions was prepared and distributed equally into 3 microfuge tubes (25 µl each). The tubes were transferred to PCR machine and amplification was performed as per conditions given in Table 3.2. The tubes were stored at 4 °C till further use. The PCR product (5µl) was loaded on the 1% agarose gel and the electrophoresis was performed at 80 V for 2 hrs

and observed on UV Trans-illuminator and a picture of the gel was taken using the gel documentation system.

**Table: 3.1 Preparation of PCR Master Mix**

| Components | Standard Reaction (25 µl) |
|---|---|
| Phusion H.F buffer (5X) | 5.00 |
| dNTPs (40 mM) | 0.25 |
| Forward primer (10 µM) | 1.00 |
| Reverse primer (10 µM) | 1.00 |
| Phusion DNA Polymerase (1 U/µl) | 0.50 |
| Plasmid DNA (100 ng/µl ) | 1.00 |
| Distilled water ($H_2O$) | 16.25 |

### 3.2.1.6 Ligation

The Phusion amplified pigeonpea lectin gene was ligated in pENTR-D-TOPO vector. The ligation reaction was prepared as per the manufacturer's instructions (Invitrogen's Gateway cloning kit, Catalog no. K6300-01) (Table 3.3).

**Table 3.2: Thermo Cyclic Conditions for Phusion DNA Polymerase PCR Reaction**

| Temperature (°C) | Time |
|---|---|
| 98 | 30 sec |
| 98 | 30 sec |
| 60 | 1.0 min |
| 72 | 30 sec |
| 72 | 10 min |
| 4 | ∞ |

**Table: 3.3: Ligation of PCR products with pENTR-D-TOPO Vector**

| Components | Volume (µl) |
|---|---|
| PCR product (5 ng/µl) | 1.0 |
| pENTR D-TOPO vector | 1.0 |
| Salt solution | 1.0 |
| Distilled water ($H_2O$) | 3.0 |
| Total volume | 6.0 |

The ligation mixture was kept at 22 °C for 15 minutes and transformed into the supplied competent cells.

### 3.2.1.7 Transformation

The ligated product (3 µl) was added to the chemically competent *E. coli* cells (single shot TOP10 strain provided in the Invitrogen's gateway cloning kit). The tubes were thawed on ice for 30 min. Then the cells were subjected to heat shock for 45 seconds at 42 °C and immediately chilled on the ice bath for 5 minutes. 250 µl of prewarmed SOC medium was added to the cells and kept on incubator shaker at 37 °C at 200 rpm for 1 hour. The transformed cells were spread evenly on the LA plates supplemented with kanamycin (50 µg/ml). The plates were incubated at 37 °C for 16-18 hours. Next day the plates were checked for appearance of transformed colonies.

### 3.2.1.8 Screening of Transformants

The transformed colonies obtained after overnight incubation at 37 °C were picked and colony PCR was performed using Taq DNA polymerase.

### 3.2.1.9 Colony PCR for the Transformants

Well separated single colony was streaked on the LA plate supplemented with kanamycin (50 µg/ml) in the form of a grid. The plates were incubated at 37 °C for overnight. From each plate single colony was picked and used in place of template DNA for colony PCR reaction. PCR amplification was performed using gene specific primers

and Taq DNA polymerase enzyme along with supplied Taq buffer with it. The reaction mix was prepared as per the Table 3.4.

**Table: 3.4 Components for the Colony PCR**

| Components | Volume (µl) |
|---|---|
| Taq buffer (10X) | 5.0 |
| dNTPs (10 mM) | 1.0 |
| Forward primer (10 µM) | 1.0 |
| Reverse primer (10µM) | 1.0 |
| Taq DNA polymerase (1U/ µl) | 0.50 |
| Colony | - |
| Sterile water | 41.5 |

The master mix was prepared for 6 colonies and distributed equally into 6 microfuge tubes (50 µl each). To each tube the colony was added from the plate. The tubes were transferred to PCR and amplification was performed under the conditions given in Table 3.5. The final extension was done at 72 °C for 10 min and the tubes were stored at 4 °C.

The PCR product was loaded on the 1% Agarose gel and the electrophoresis was performed at 80 Volts for 2 hrs and observed on UV Trans-illuminator and the gel picture was taken using the gel documentation system.

### 3.2.2 Isolation of Plasmid DNA from Positive Transformants.

Pigeonpea lectin gene cloned in pENTR D-TOPO vector was inoculated in 10 ml of LB medium supplemented with kanamycin (50 µg/ml) and incubated overnight at 37 °C, 200 rpm for bacterial growth. Overnight grown culture was used for isolation of plasmid DNA using Macherey-Nagel plasmid isolation kit as in 3.2.1.3. Finally the DNA was eluted in 50 µl elution buffer and isolated DNA was analyzed on 1% Agarose gel to check the quality and quantity.

**Table 3.5: Thermo Cyclic Conditions for Colony PCR**

| Temperature (°C) | Time |
|---|---|
| 98 | 3 Min |
| 94 | 1 Min |
| 60 | 45 Sec |
| 72 | 1 Min |
| | 35 cycles |
| 72 | 10 Min |
| 4 | ∞ |

### 3.2.3 Transformation in Destination Vector

#### 3.2.3.1 LR Recombination Reaction

The LR recombination reaction mix was prepared as given in the Table 3.6 and incubated at 25 ºC for 90 min. 2 µl of the proteinase K solution was added and vortexed and incubated again at 37 ºC for 10 min. The chemically competent *E. coli* cells (one shot TOP10) were thawed on ice for 5 min. The recombined reaction mix was added to the cells and incubated for 30 min on ice. The cells were given a heat shock for 45 seconds at 42 °C and immediately chilled on ice bath for 5 minutes. 450 µl of pre-warmed SOC medium was added to the cells and incubated at 37 °C, 200 rpm for 1 hour in an incubator shaker. The transformed cells were given a short spin and spread evenly on the LA plates supplemented with kanamycin (50µg/ml). The plates were incubated at 37 °C for 16-18 hours. Next day the plates were checked for appearance of transformed colonies. Single colonies were selected and streaked on the LA plate with kanamycin. The colony PCR was done to confirm the presence of the lectin gene. The plasmid DNA of the positive colony was isolated and used to transform host *E. coli* BL21 DE3 pLysS cells.

### 3.2.4 Transformation in *E. coli* BL21 DE3 pLysS Cells

#### 3.2.4.1 Preparation of Competent BL21 DE3 pLysS Cells

The *E. coli* BL21 DE3 pLysS colony was inoculated in 5 ml LB medium supplemented with chloramphenicol (34µg/ml) and was incubated overnight at 37 ºC and 200 rpm. 1 ml of overnight grown culture was reinoculated in 25 ml of LB medium without antibiotics and incubated for 2 hrs at 37 ºC and 200 rpm. The culture was kept on ice for 10 min and pelleted down at 4 ºC, 5 min and 3000 rpm. The supernatant was discarded and the pellet was suspended in 2.5 ml of TSS solution. 100 µl of the prepared competent cells were stored at -80 ºC till further use.

**Table 3.6 LR Recombination Reaction**

| Components | Volume (µl) |
|---|---|
| Entry clone (300 ng) | 5 |
| Destination vector | 2 |
| LR clonase reaction buffer (5X) | 4 |
| LR clonase enzyme mix | 4 |
| TE buffer | 5 |
| Total | 20 |

### 3.2.4.2 Transformation of *E. coli* BL21 DE3 pLysS Cells

Plasmid DNA was isolated from the selected positive colony and transformed into the prepared competent BL21 DE3 pLysS cells. 10 µl plasmid DNA (100ng), 20µl 5X KCM, and 70 µl SDW were added to 100 µl competent cells and kept on ice for 20 min. The cells were given heat shock for 5 min at 37 ºC. 900 µl of LB medium was added and incubated for 2 hrs at 37 ºC and 200 rpm. The culture was centrifuged at 1000 rpm for 2 min and spread on LA plates supplemented with kanamycin (50µg/ml) and chloramphenicol (34µg/ml). The plates were incubated overnight at 37 ºC. Next day the plates were checked for appearance of transformed colonies. Single colonies were selected and streaked on the LA $Kan^+$ plate. The colony PCR was done to confirm the presence of the lectin gene. The lectin protein expressing colony was selected after colony blotting.

### 3.2.5 Colony Blotting of the Transformed Colonies

A nitrocellulose filter membrane was placed in close contact with the colonies on the surface of the LA plate for 10 min at RT. The filter (colony side up) was transferred to a fresh plate containing kanamycin (50 µg/ml) and IPTG (1.0 mM). The plates were incubated for 4 hrs at 37 ºC for induction of protein expression.

The filters were incubated sequentially for 10 min on a sheet of 3 MM Whattman filter paper soaked in SDS solution (10%), 5 min in denaturing solution, 5 min in neutralization solution (two times) and for 15 min in the 2X SSC solution. The filter was washed twice with TBS buffer for 10 min each. The membrane was incubated in 10 ml of blocking solution for 1 hr at 12 rpm and the membrane was rinsed twice with 20 ml of water. The membrane was incubated with 10 ml of primary antibody solution for 1 hr. The antibody solution was decanted and the membrane was washed thrice, with 20 ml of prepared antibody wash. The membrane was incubated in 10 ml of secondary antibody solution for 30 min and washed thrice with 20 ml of antibody wash solution. The membrane was rinsed twice with 20 ml of water. Now the membrane was incubated in 5 ml of chromogenic substrate until purple dots develop on the membrane and finally rinsed twice with 20 ml of water. The membrane was dried on a filter paper by a stream of slightly warm air or under an infrared lamp. The positive (purple colored) colonies were then selected from the master plate and streaked on a LA plate with kanamycin (50 µg/ml) for protein expression studies.

## 3.3 Protein Expression and Analysis

### 3.3.1 Induction of Protein Expression

The transformed BL21 colonies were inoculated in fresh LB medium (10 ml) supplemented with kanamycin (50µg/ml) and chloramphenicol (34µg/ml) and was incubated overnight at 37 ºC and 200 rpm. 1.0 ml of the overnight grown culture was inoculated into seven culture tubes containing each containing 5.0 ml of fresh LB medium and incubated for 2 hrs at 37 ºC and 200 rpm. Now different concentration of IPTG (0.25, 0.50, 1.0, 1.5, 2.0, 2.5 mM) was added to the culture tubes for induction of protein expression and one was left uninduced. 2ml samples were collected at different time intervals (3, 4, 5 hrs.) and stored at 4 ºC. The cultures were pelleted down at 7000

rpm for 5 min and the supernatant was discarded. The pellet was dissolved in 80 µl sample loading buffer, vortexed to suspend and boiled for 5 min in a boiling water bath before loading on the SDS PAGE gel.

### 3.3.2 SDS-Polyacrylamide Gel Electrophoresis

The polyacrylamide gel electrophoresis in the presence of Sodium dodecyl sulphate was performed according to the method of Laemmli (1970) using AE6120 Atto slab gel casting apparatus (Atto Corporation, Tokyo, Japan).

#### 3.3.2.1 Preparation of SDS PAGE Gel

Glass plates and spacers were thoroughly cleaned, dried and assembled in gel casting apparatus. 12% resolving and 5% stacking gel was prepared by adding the following ingredients as in the Table 3.7.

Stock acrylamide solution, Tris buffer, SDS, APS, TEMED and distilled water were mixed. Freshly prepared APS solution was added, mixed by gentle swirling and immediately poured into the gel mould. A thin layer of iso-butanol was carefully overlaid to prevent the acrylamide solution coming in contact with air. The gel was allowed to polymerize for 30-45 min at room temperature. The overlaid iso-butanol and water of polymerization accumulated were removed by inverting the apparatus. The stacking gel was layered over the resolving gel and clean comb inserted without leaving any air bubble. The gel was kept at RT for polymerization.

#### 3.3.2.2 Electrophoresis

After polymerization of stacking gel, the gel along with glass plates were taken out, assembled in a vertical electrophoresis tank. The tank was filled with electrode buffer and the comb was carefully removed without disturbing the wells. The gel was pre run at 20 mA for 30 min, prior to loading the samples. Protein samples were boiled, centrifuged and 15-20 µl was loaded onto the gel along with the protein marker (6 µl) and electrophoresed at constant current of 40 mA till the tracking dye reached the bottom of the gel.

**Table3.7: Composition of SDS-Polyacrylamide Gel**

| Solutions | Resolving Gel (ml) | Stacking Gel (ml) |
|---|---|---|
| Acrylamide solution (30%) | 20 | 3.32 |
| Tris buffer (1.5M pH 8.8) | 12.5 | - |
| Tris buffer (0.5M pH 6.8) | - | 2.52 |
| SDS (10%) | 0.5 | 0.2 |
| APS (10%) | 0.5 | 0.2 |
| TEMED | 0.020 | 0.020 |
| SDW | 16.5 | 13.6 |
| Total | 50 | 20 |

After the run was complete, the electrophoresis unit was switched off and the gel was peeled out from glass plate with the help of spatula. The gel was kept in a staining solution (0.1% Coomassie Brilliant Blue R-250) for 1 hr. After staining, the gel was removed and kept in a destaining solution that was replaced at regular intervals to remove all unbound dye. The destained gel was photographed and stored in destaining solution.

### 3.3.3 Western Blotting

The sample which showed the band of interest on SDS-PAGE was further used for western blotting. In order to prepare the trans blot, the PVDF membrane was soaked in methanol for 1 min. and then it was placed in the “sandwich” chamber with 2 fibre pads and 2 filter papers all soaking in transfer buffer. The sandwich was set as black side down. The “sandwich” was like black side, fiber pad, filter paper, gel, PVDF membrane, filter paper, fiber pad, and red side. After each layer any air bubbles which may have formed was rolled out. The Trans blot was run at 4°C using an ice pack with transfer buffer. The unit was run at 90 V for 100 minutes. The membrane was removed from the sandwich and was washed twice for 10 min with TBS buffer. Then the membrane was kept in 10 ml of blocking solution and incubated for 1 hr at 12 rpm. Blocking solution was discarded and the membrane was rinsed twice with 20 ml of water. The membrane

was then incubated with 10 ml of primary antibody solution for 1 hr. The antibody solution was decanted and the membrane was washed thrice, with 20 ml of prepared antibody wash. The membrane was then incubated in 10 ml of secondary antibody solution for 30 min. The membrane was then washed thrice with 20 ml of antibody wash solution. The membrane was rinsed twice with 20 ml of water. Now the membrane was incubated in 5 ml of chromogenic substrate until colored dots develop on the membrane. The membrane was rinsed twice with 20 ml of water. The membrane was dried on a filter paper by a stream of slightly warm air or under an infrared lamp. The western blot membrane was then scanned and the image was saved.

# *4. RESEARCH PAPER*

## In vitro Expression of Pigeonpea Lectin Gene and its Characterization

### 4.1 Abstract

Globally, the insect pests are causing economic losses to the agricultural production. The worldwide use of chemical insecticides and pesticides to control these insect pests has increased the cost of pest control, resulted in insecticidal hazards to biological organisms, pollution to the environment and increasing insect resistance. Therefore, the challenge today is to achieve higher and stable crop production with safe and eco-friendly strategies. Plants accumulate a set of defense proteins including lectins, proteinase inhibitors, amylase inhibitors etc. Lectins reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran and homopteran insect pests. The harmful effects of lectins on biological parameters of insects include loss in weight, mortality, feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation. Realizing their importance, lectin gene have been isolated and characterized from pigeon pea. The isolated pigeonpea lectin (PPL) gene (~825 bp) was first cloned in pENTR-D-TOPO vector, subcloned into an expression vector (Gateway Destination vector pET300/NT-DEST) and transformed into BL21 DE3 pLysS competent cells of *E. coli* for protein expression studies. The PPL gene expression studies were carried out at different temperatures, IPTG concentrations and time intervals. The expression was maximum at 2.0 and 2.50 mM IPTG concentration at 37ºC for 5 hrs. The size of the protein was found to be around ~30 KDa. The expression was confirmed by SDS-PAGE and western blotting. Thus, transferring these defense genes under the control of tissue specific promoters will be an effective tool for sustainable insect pest management programme.

**Keywords:** Cloning, Expression, Insecticides, IPTG, Lectin, Transformation etc.

## 4.2 Introduction

To meet the basic demand of food for this increasing population, quintals of insecticides and pesticides are being dumped in the crop lands to control the crop damage caused due to insect pests and to increase the food production. This in turn, has led to the harmful effects on environment, aquatic ecosystem, non-target organism, human health and increased insect resistance. Therefore, recent efforts have been made to reduce broad spectrum of toxicant added to the environment as it has become necessary to balance agricultural needs with environmental and health issues when using insecticides. Therefore, the use of natural entomotoxic metabolites have increased because of the growing need to obtain better food quality and protection of environment (Jaber *et al*., 2008).

Efforts were made to obtain insect resistant plants by introducing *Bt* gene. However, the codon bias and low expression of *Bt* genes, shifted the attention towards isolation of insect resistance genes of plant origin. Currently, the inhibitors of the digestive enzymes (protease and amylase) and lectins are in limelight to confer resistance against various insect pests. Lectin genes of plant origin have an added advantage, as when transferred, they are correctly translated and processed in the plant host. Moreover, most of lectin genes are devoid of introns and therefore, both genomic as well as cDNA clones can be mobilized for developing transgenic plants.

Most plants contain one or more carbohydrate binding proteins termed as lectins. They are heterogeneous group of proteins having a protective function against a wide array of organisms viz. insects, bacteria, fungi, virus etc. They reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran (*Spodoptera litura, Helicoverpa armigera* etc.) and homopteran (aphids, plant hoppers etc.) insect pests but have no effect on human metabolism (Boulter *et al*., 1993). The harmful effects of lectins on biological parameters of insects include loss in weight, mortality, feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation. Realizing their importance, lectin genes have been isolated and characterized

from various plant species, e.g. snowdrop (Peumans and Van Damne, 1995), garlic (Smeets *et al*., 1997), pea(Gatehouse and Gatehouse, 1998), cowpea (Datta *et al.,* 2000), chickpea (Qureshi *et al.,* 2006), Onion (Hossain *et al.,* 2006), mungbean (Anshuman *et al.,* 2010),moth bean (Singh *et al.,* 2010) and pigeon pea (Accession number JN561784.1).

Lectins are highly specific for binding to oligosaccharides, hence if specific carbohydrate is present on the surface of tissue, it can bind to them. The ingested lectin bound to the midgut tract causes disruption of the epithelial cells including elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut leading to the complete closure of the lumen (Majumder *et al.,* 2006, Karimi *et al*., 2010).

GNA, (the mannose specific Lectin from snowdrop (*Galanthus nivalis*) *w*as found to be insecticidal in artificial diets against the peach potato aphid (*Myzus persicae*). The high efficacy of the mannose binding lectin, ASAL on *Lipaphis erysimi* using artificial diet bioassay also showed a correlation between binding of the toxin to the gut epithelial membrane and toxicity to the insect (Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004). The Galβ1-3GalNAc-ser/thr-binding lectin from *Sclerotium rolfsii* Sacc., a soil-borne plant pathogen has two distinct carbohydrate-binding sites, a primary and a secondary and elicits the structural determinants of the molecular recognition for carbohydrate binding. Both the crystal structure and the amino acid sequence were reported to explain structural basis of carbohydrate recognition (Leonidas *et al.*, 2007).

The efficacy of the isolated pigeon pea lectin (PPL) gene needs to be tested by expressing in an expression vector and *In Vitro* insect bioassay. Then the gene coding for pigeon pea lectin will be used to develop transgenic crop plants resistant to lepidopteran and homopteran insect pests. Exploitation of these insect resistance genes for developing resistant plant varieties will provide better option for plant protection. Therefore, the present study was undertaken with the following objective: “Cloning and Expression of Pigeonpea Lectin Gene in an Expression Vector and its Characterization”

## 4.3 Materials and Methods

The research work was carried out at NRCPB, IARI campus, New Delhi. For cloning, the culture of Pigeonpea lectin gene cloned in pGEMTEasy vector transformed into DH5α was used. Plasmid DNA of lectin gene cloned in pGEMTEasy vector was isolated and purified using Macherey-Nagel plasmid isolation kit based on alkaline lysis method.

### 4.3.1 Cloning PPL Gene in pENTR-D-TOPO Vector

Isolated plasmid DNA of pigeonpea lectin gene was used for the PCR amplification using high fidelity Phusion DNA polymerase. PCR amplification was performed using gene specific primers. Forward and reverse primers were designed from the ORF region of pigeonpea lectin cDNA with the addition of CACC at the 5' end of the forward primer.

Forward primer: 5' CACCATGGCTTCTCTTCAAACCCAAA 3'

Reverse primer: 5' TGCATCTGCAGCTTGCTTAGAACTCGA 3'

The amplified PPL gene was ligated in pENTR-D-TOPO vector. The ligation reaction was prepared as per the manufacturer's instructions (Invitrogen's Gateway cloning kit, Catalog number K6300-01). The ligation mixture was kept at 22 °C for 15 minutes and transformed into the supplied competent cells (one shot TOP10). The transformed colonies obtained after overnight incubation at 37 °C were picked and colony PCR was performed using Taq DNA polymerase.

### 4.3.2 Sub-cloning PPL Gene in pET300/NT-DEST Vector

The LR recombination reaction was set up by mixing together the Entry clone, Destination vector, LR clonase enzyme mix and buffer as per the manufacturer's manual. The recombined reaction mix was transformed into one shot TOP10 cells. The positive colonies were confirmed by colony PCR. The plasmid DNA was isolated using Macherey-Nagel plasmid isolation kit based on alkaline lysis method.

### 4.3.3 Transformation in *E. coli* BL21 DE3 pLysS Cells

The *E. coli* BL21 DE3 pLysS competent cells were prepared using the TSS buffer. The isolated plasmid DNA was then used to transform the competent host cells using the 5 X KCM solution.

### 4.3.4 Colony Blotting

The transformed colonies were further confirmed for PPL protein expression. A nitrocellulose filter membrane was placed in close contact with the colonies on the surface of the LA plate for 10 min at RT. The filter (colony side up) was transferred to a fresh plate containing kanamycin (50 µg/ml) and IPTG (1.0 mM). The plates were incubated for 4 hrs at 37 ºC for induction of protein expression. The filters were incubated sequentially for 10 min on a sheet of 3MM Whattman filter paper soaked in SDS solution (10%), 5 min in denaturing solution, 5 min in neutralization solution (two times) and for 15 min in the 2X SSC solution. The membrane was washed and treated with blocking solution, again washed and treated with primary and secondary antibody solution. The protein expressing colonies appeared purple colored on exposure to chromogenic substrate.

### 4.3.5 Protein Expression and Analysis

The transformed BL21 DE3 pLysS cells were used for induction of protein expression. The expression studies were carried out at 30 ºC and 37 ºC at 3, 4 and 5 hrs interval with IPTG concentrations of 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mM. The culture were collected, centrifuged, pellet was dissolved in sample loading buffer and loaded on SDS polyacrylamide gel (12% resolving and 5% stacking). The sample was resolved at 40 mA for 3-4 hrs, gel was separated from the glass apparatus, stained for 1 hr and then destained till the background gets clear.

Western blotting was done using His-Tag specific antibodies and the colored bands developed on exposure to chromogenic substrate specific to the enzyme linked to the antibody.

## 4.4 Results

This section deals with the outcome of the experiments conducted for cloning of pigeonpea lectin (PPL) gene in an expression vector and its characterization.

### 4.4.1 Cloning of Lectin Gene in pENTR D-TOPO Vector

#### 4.4.1.1 Streaking of Culture

The *E.coli* strain DH5α containing PPL gene cloned in pGEMTEasy vector was streaked on LA plates containing ampicillin (50 µg/ml). The plates were incubated at 37 °C. Next day the single colonies were obtained on the plates (**Fig. 4.1**)

#### 4.4.1.2 Isolation of PPL Plasmid DNA

The plasmid DNA from E. coli strain DH5α containing PPL gene was isolated and purified from 10 ml of overnight grown *E.coli* culture by alkaline lyses method (Birnboim and Dolly, 1979) using Macherey-Nagel plasmid isolation kit. The isolated DNA was electrophoresed on 1% agarose gel to check for its quality and quantity. Quantitatively about 4 mg of plasmid DNA was obtained per 10 ml of overnight grown culture. The plasmid DNA was free of impurities and RNA as shown in gel (**Fig. 4.2**)

#### 4.4.1.3 PCR of the PPL Gene using Phusion DNA Polymerase

PCR of the isolated lectin gene was performed using high fidelity phusion DNA polymerase and the gene specific primers. The forward primer had CACC at its 5'end. The amplified product of was run on 1% agarose gel at 45 V for 3 hour. After the completion of electrophoresis the gel was viewed under UV light and showed a single sharp bright band at approximately 825 base pairs (**Fig. 4.3**). The PCR product was used for ligation.

#### 4.4.1.4 Ligation and Transformation

The PPL amplicon was ligated into pENTR D-TOPO vector using salt solution and incubated at 22 °C for 15 minutes and transformed into TOP10 competent cells. The cells was spread on LA plates supplemented with Kanamycin and were incubated at 37

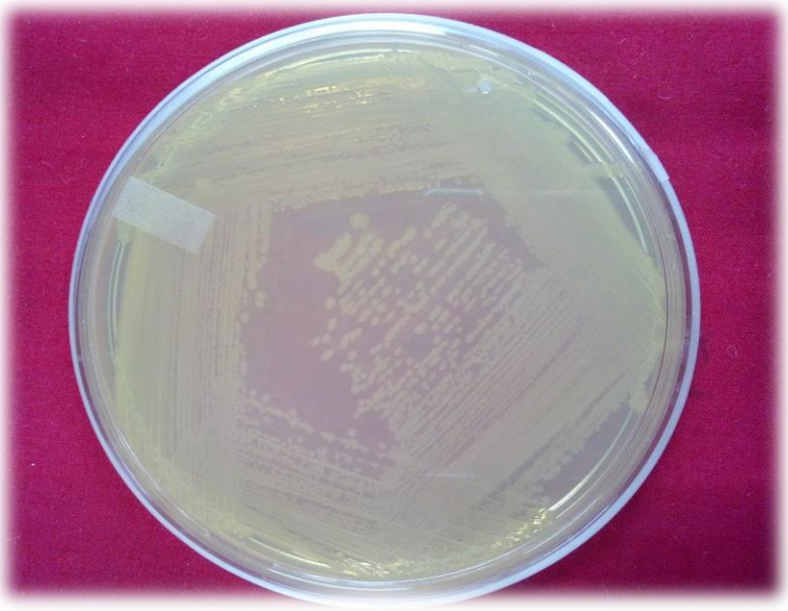


**Fig. 4.1: Streaking of PPL gene cloned in pGEMTEasy vector on LA plate supplemented with ampicillin**

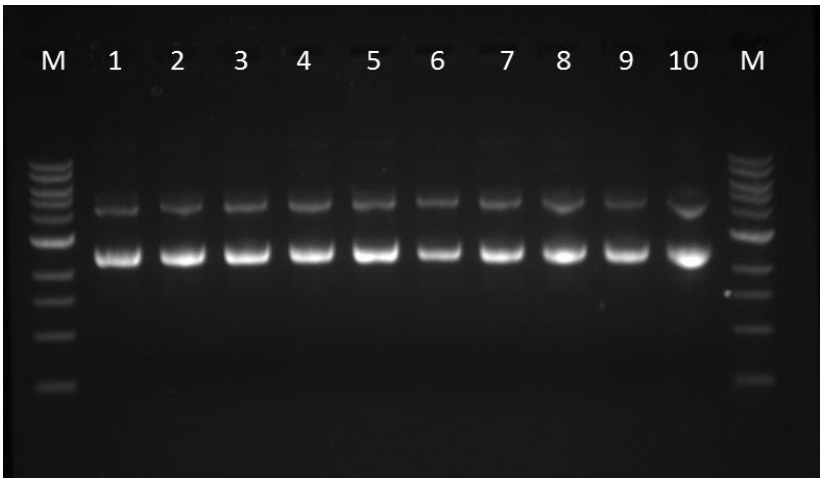



**Fig. 4.2: Agarose gel electrophoresis of isolated plasmid DNA of PPL gene cloned in pGEMTEasy vector**

**Lane M: 1 Kb DNA ladder**

**Lane 1-10: plasmid DNA of lectin gene in pGEMTEasy vector**

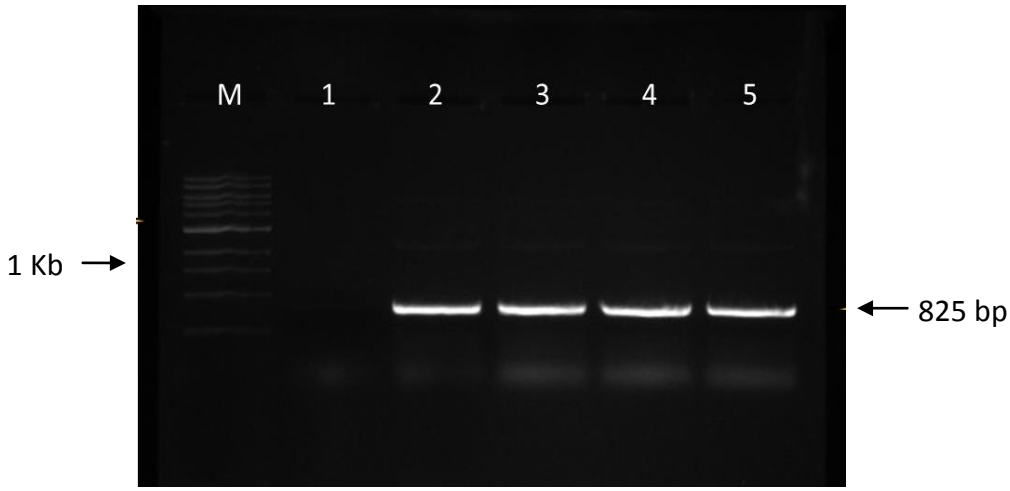



**Fig. 4.3 : Agarose gel electrophoresis of PCR amplified PPL gene using Phusion DNA polymerase**

**Lane M: 1Kb DNA Ladder**

**Lane 1: Negative control**

**Lane 2-5: PCR amplified PPL gene**

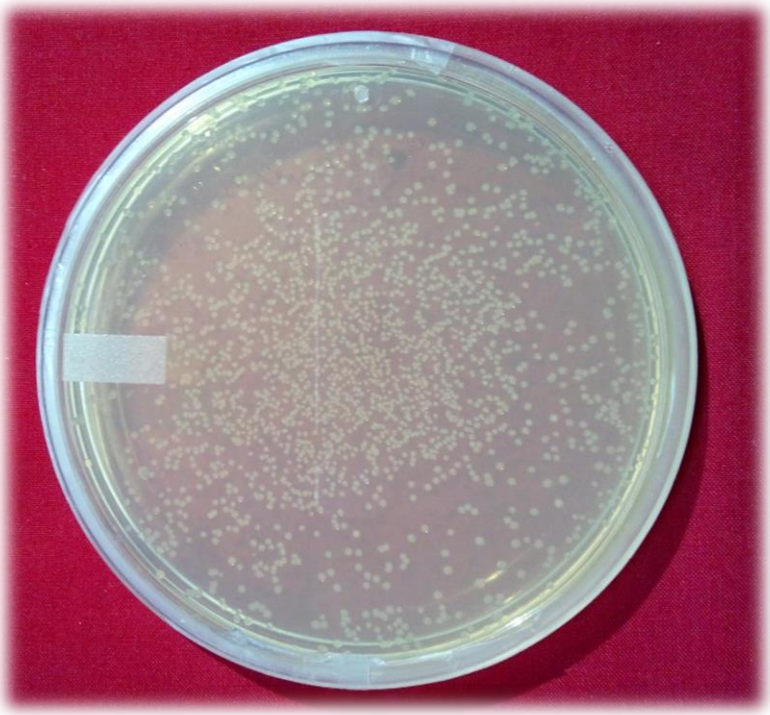


**Fig. 4.4: Transformed colonies of PPL gene cloned in pENTR D-TOPO vector**

°C overnight. Next day, the colonies appeared on the plates (**Fig. 4.4**). The transformed colonies were picked and streaked on fresh LA plate with kanamycin. (**Fig. 4.5**)

#### 4.4.1.5 Colony PCR for Transformants

Colony PCR was performed to confirm the transformants. A single transformed colony was picked as a template for amplification. The amplified product of colony PCR was run on 1% agarose gel at 45 V for 3 hour. The PCR product of 825 bp was observed on the gel (**Fig. 4.6**). The positive colonies were used for the isolation of plasmid DNA.

### 4.4.2 Cloning of Lectin Gene in a Gateway Destination Vector

Gateway cloning technology provides a rapid and highly efficient route to protein expression, functional analysis and cloning/subcloning of DNA segments. It is a very simple and reliable technology to clone a gene in any destination vector based on the site specific recombination reactions. The efficiency of cloning is >99%.

#### 4.4.2.1 Isolation of Plasmid DNA from Positive Transformants

The plasmid DNA containing PPL gene in pENTR D-TOPO vector was isolated using Macherey-Nagel plasmid isolation kit. The DNA was eluted in 50 µl of TE buffer. The isolated DNA was electrophoresed on 1% agarose gel to check for its quality and quantity and photographed using gel documentation system (**Fig. 4.7**). Quantitatively about 4 mg of plasmid DNA was obtained per 10 ml of overnight grown culture.

#### 4.4.2.2 Transformation in Destination Vector

The LR recombination reaction mix was prepared as per manufacturers' instructions and incubated at 25 ºC for 90 min. 2 µl of proteinase K solution was added to the reaction mix and incubated at 37 ºC for 10 min. The transformation was done using one shot TOP10 chemically competent *E. coli* cells and spread evenly on LA plates with kanamycin and incubated at 37 ºC for 16-18 hrs. Next day the transformed colonies grew on the plate (**Fig 4.8**).

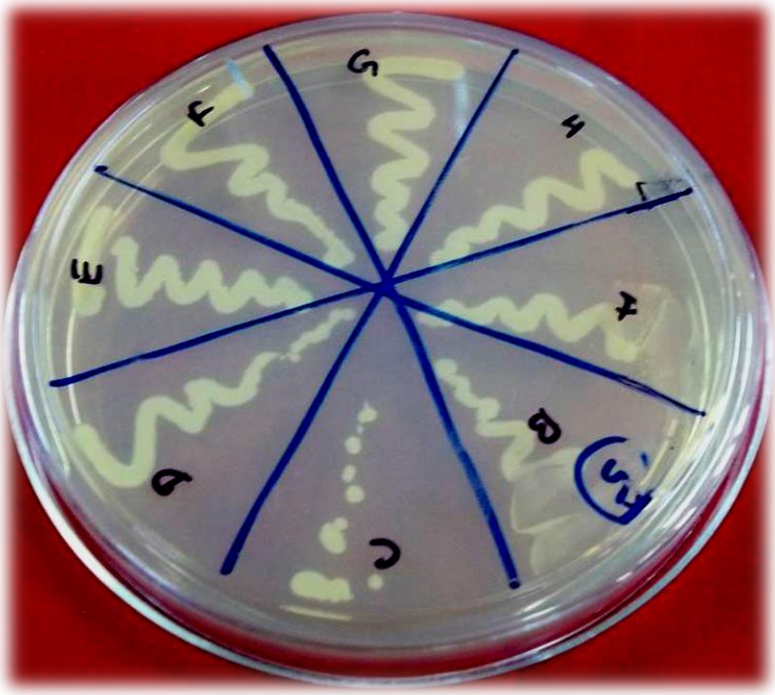



**Fig. 4.5: Streaking of selected transformed pENTR D-TOPO vector colonies on kanamycin supplemented LA plate**

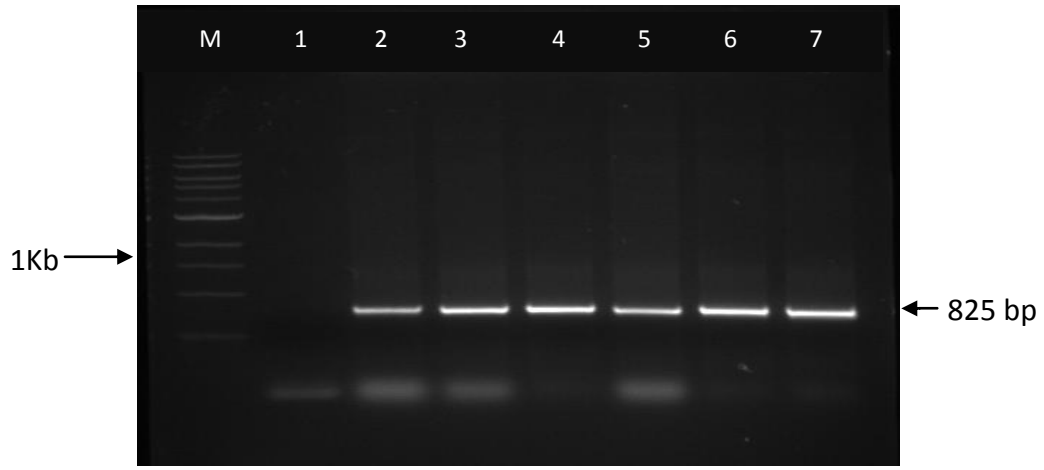



**Fig. 4.6: Agarose gel electrophoresis of Colony PCR amplified PPL gene cloned in pENTR D-TOPO vector**

**Lane M: 1 Kb DNA Ladder**

**Lane 1: Negative control**

**Lane 2-7: PCR amplified PPL gene**

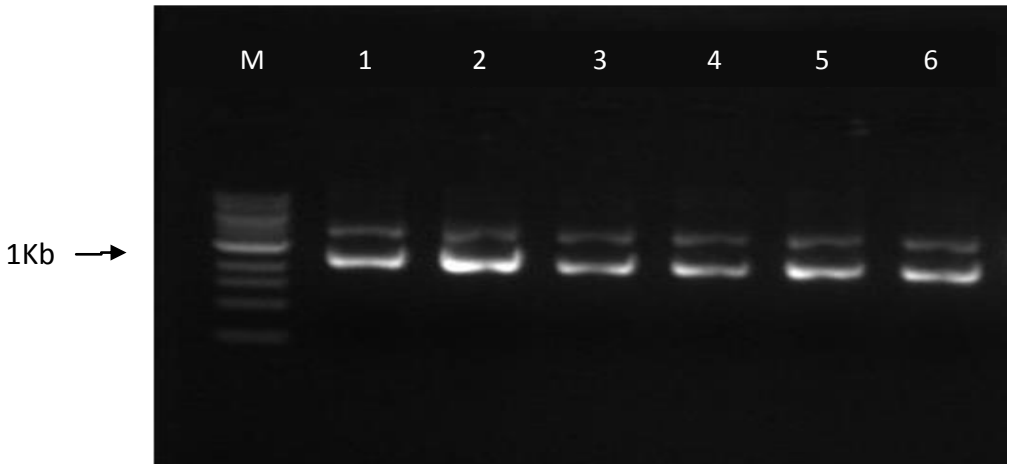



**Fig. 4.7: Agarose gel electrophoresis of plasmid DNA isolated from positive transformants of pENTR D-TOPO vector containing PPL gene**

**Lane M: 1 Kb Ladder.**

**Lane 1-6: Plasmids of pENTR D-TOPO vector with PPL**

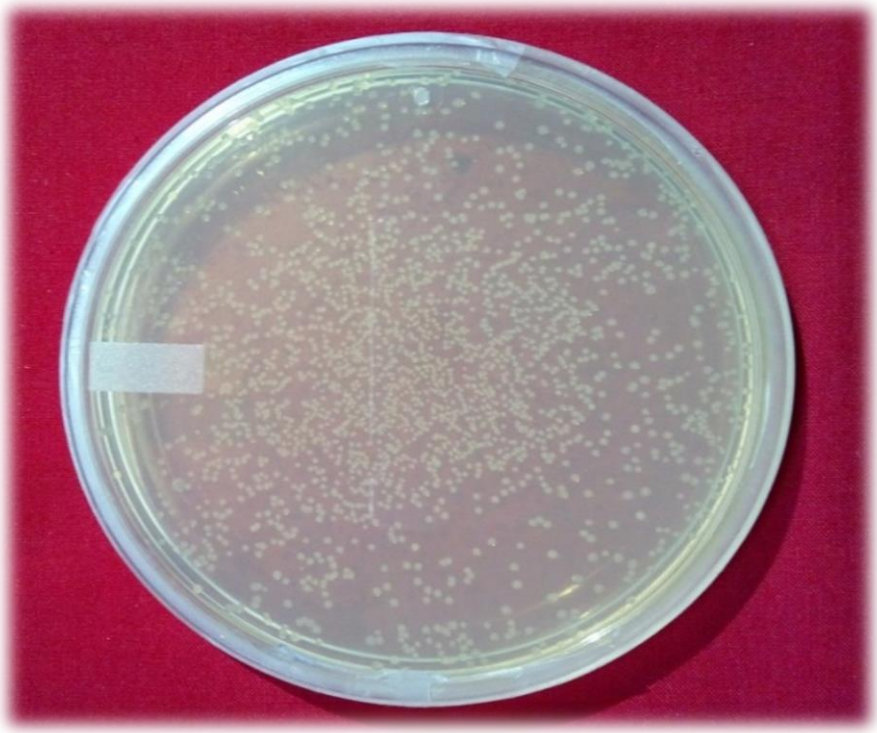


**Fig. 4.8: Transformed colonies of Destination vector containing PPL in chemically competent cells**

Well separated single colonies were streaked on the fresh plate (**Fig. 4.9**) and confirmed by colony PCR (**Fig. 4.10**) for the presence of the PPL gene. Lectin protein expressing colonies were selected after colony blotting.

### 4.4.3 Transformation in *E. coli* BL21 DE3 pLysS Cells

Plasmid DNA of PPL gene cloned in Destination vector was isolated from the selected positive colonies and transformed into competent BL21 DE3 pLysS cells. The transformed cells were spread on LA plates with kanamycin (50 µg/ml), chloramphenicol (34 µg/ml) and incubated overnight at 37 ºC. Colonies were observed on the plate (**Fig. 4.11**) and single colonies were streaked (**Fig. 4.12**) and confirmed for the presence of PPL gene by colony PCR (**Fig. 4.13**).

### 4.4.4 Colony Blotting of the Transformed Colonies

The colonies were transferred onto the nitrocellulose membrane at RT. The filter (colony side up) was transferred to a fresh plate containing kanamycin (50 µg/ml) and IPTG (1.0 mM). The plates were incubated for 4 hrs at 37 ºC for induction of protein expression. The membrane was treated with primary and secondary antibodies. Many purple colored colonies were observed on the membrane on addition of the chromogenic substrate (**Fig. 4.14**). The positive (purple colored) colonies were then selected from the master plate and streaked on a LA plate with kanamycin (50 µg/ml).

### 4.4.5 Protein Expression and Analysis

#### 4.4.5.1 Induction of Protein Expression

The transformed BL21 colonies were inoculated in fresh LB medium and different concentrations of IPTG (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mM) were added for induction of protein expression at different time intervals (3, 4, 5 hrs.). The cells were lysed and the supernatant was used for SDS PAGE. After staining and destaining of the gel was photographed in the gel documentation system. The protein was expressed even at lower concentrations of IPTG and time period (3 hrs.) (**Fig. 4.15**), but the expression was maximum at 2.0 and 2.50 mM IPTG concentration after 5 hrs (**Fig. 4.16**).

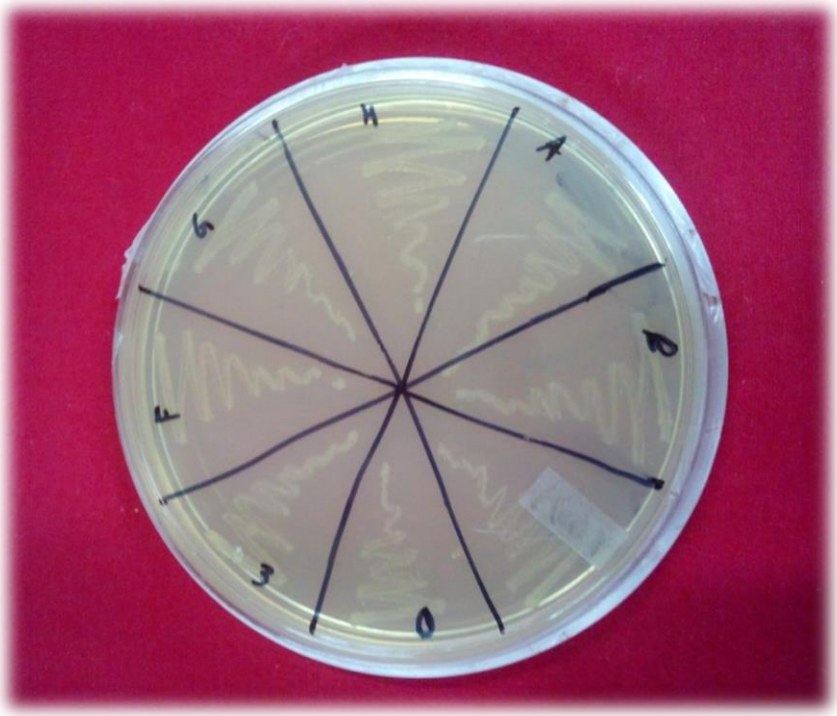


**Fig. 4.9: Streaking of selected PPL transformed Destination vector colonies on the kanamycin supplemented LA plate**

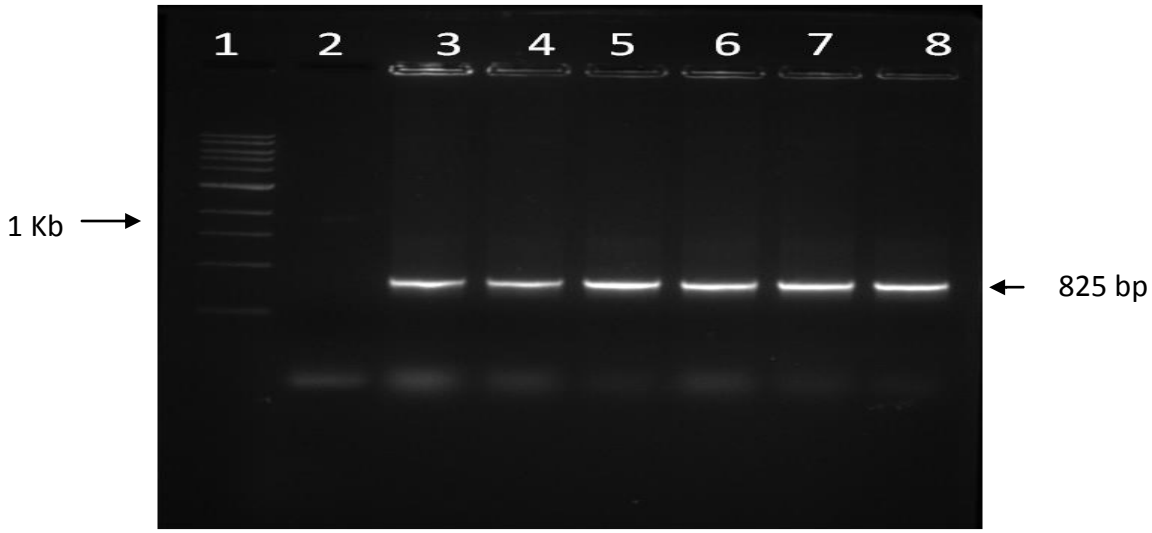



**Fig. 4.10: Agarose gel electrophoresis of colony PCR amplified (colony PCR) PPL gene cloned in Destination vector**

**Lane 1: Ladder**

**Lane 2: Negative control**

**Lane 3-8: PCR amplified lectin gene**

**Fig.4.11: PPL transformed colonies of *E. coli* BL21 DE3 pLysS cells**


**Fig.4.12: Streaking of PPL transformed colonies of BL21 DE3 pLysS cells on LA plate supplemented with kanamycin**

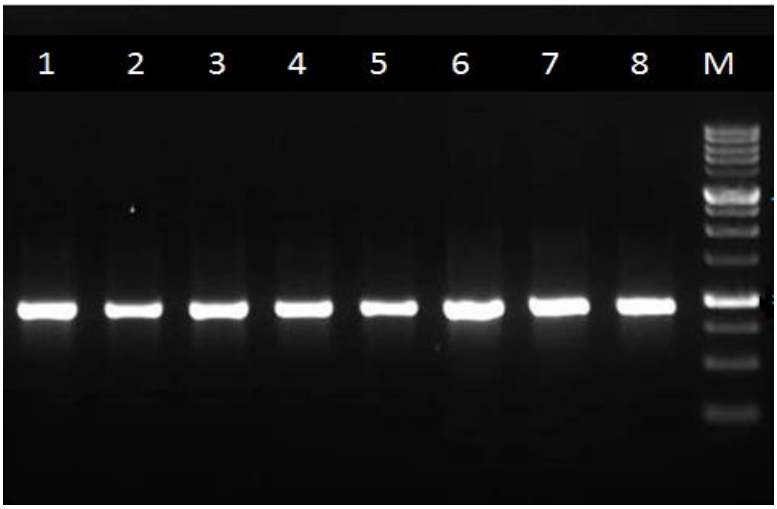



**Fig. 4.13: Colony PCR of the transformed Destination vector colonies in BL21 host cells**

**Lane M: 1 Kb DNA ladder**

**Lane 1-8: amplified DNA of PPL gene**

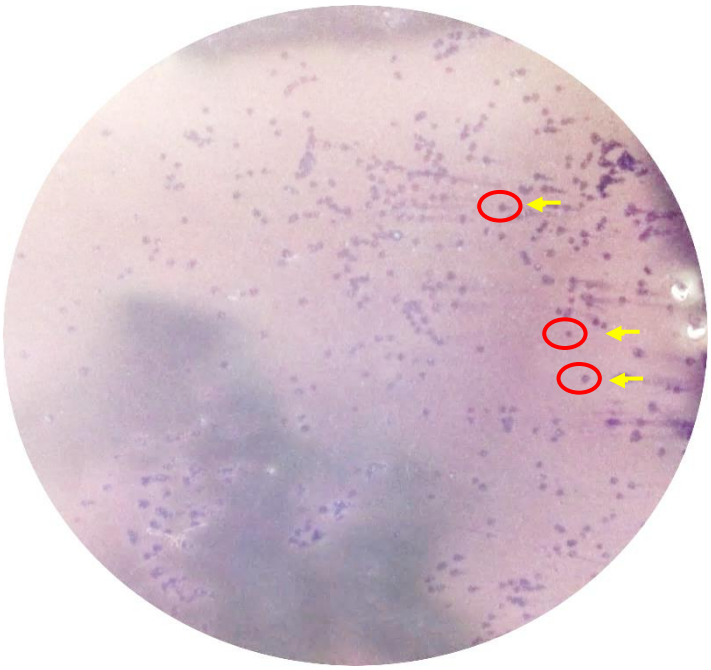


**Fig. 4.14: Colony blotting of the PPL transformed BL21 DE3 pLysS colonies. Few protein expressing colonies are shown in red circles**

#### 4.4.5.2 Western Blotting

The protein expressed was confirmed by western blotting. The protein was transferred onto PVDF membrane and incubated with primary and secondary antibody solutions. The membrane was incubated with chromogenic substrate until colored bands developed on the membrane. The western blot confirmed that the PPL gene was expressed in the *E. coli* expression system (**Fig. 4.17**).

## 4.4 Discussion

To feed the ever increasing population, it is essential to increase the food production per unit area. One of the strategies is to minimize the losses due to insect pest damage. The usual way of controlling damage is by the use of agrochemicals (insecticides and pesticides), which is not only cost intensive but also hazardous to environment and human health. Considerable progress has been made in developing transgenic plants with various genes from *Bacillus thuringiensis (Bt)* in different crops (Sharma *et al.*, 2000). Sap-sucking pests belonging to the order hemiptera generally have very low levels of proteolytic activity in their guts (Chang *et al*., 2003) hence; *Bt* toxins are unlikely to be effective against hemipteran insect pests (Rao *et al*., 1998). Therefore, there is a need to identify alternative natural entomotoxic metabolites producing genes such as lectins as insecticidal proteins through transgenic crops. The use of these novel plant genes through genetically modified plants will increase the level and range of resistance to insect pests. Plant lectins have severe effects on fecundity, growth, and development of an insect. Screenings of a wide range of lectins demonstrated that several lectins have the anti-insect potential and affect the survival and development of insect pests belonging to different orders (Macedo and Damico, 2003; Vasconcelos and Oliveira, 2004; Coelho *et al*., 2007). The anti-insect activity of plant lectins is of great potential in pest management because lectins being primary metabolic product, their genes can be good candidates to confer insect resistance to transgenic crops (Macedo and Damico, 2003). The insecticidal activity of a carbohydrate binding plant lectin *Allium sativum* leaf lectin (*ASAL)* was analyzed against various hemipteran pests

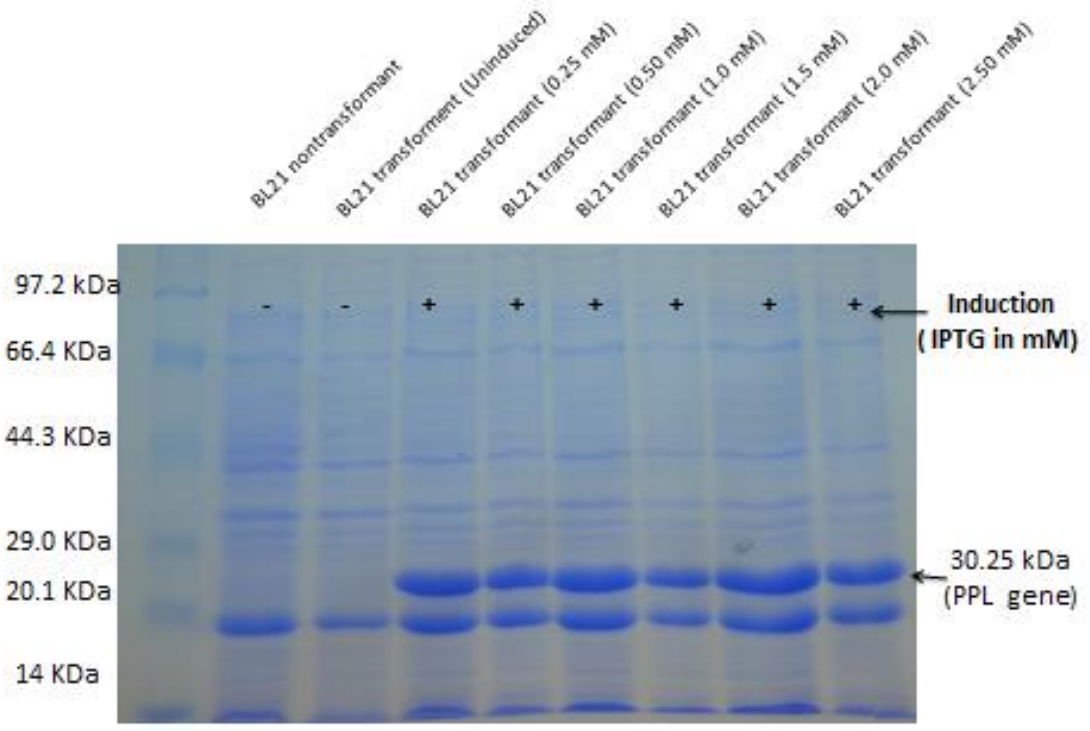



**Fig. 4.15: SDS-PAGE of the Induced protein sample of PPL transformed BL21 DE3 pLysS cells at 3 hrs of induction**

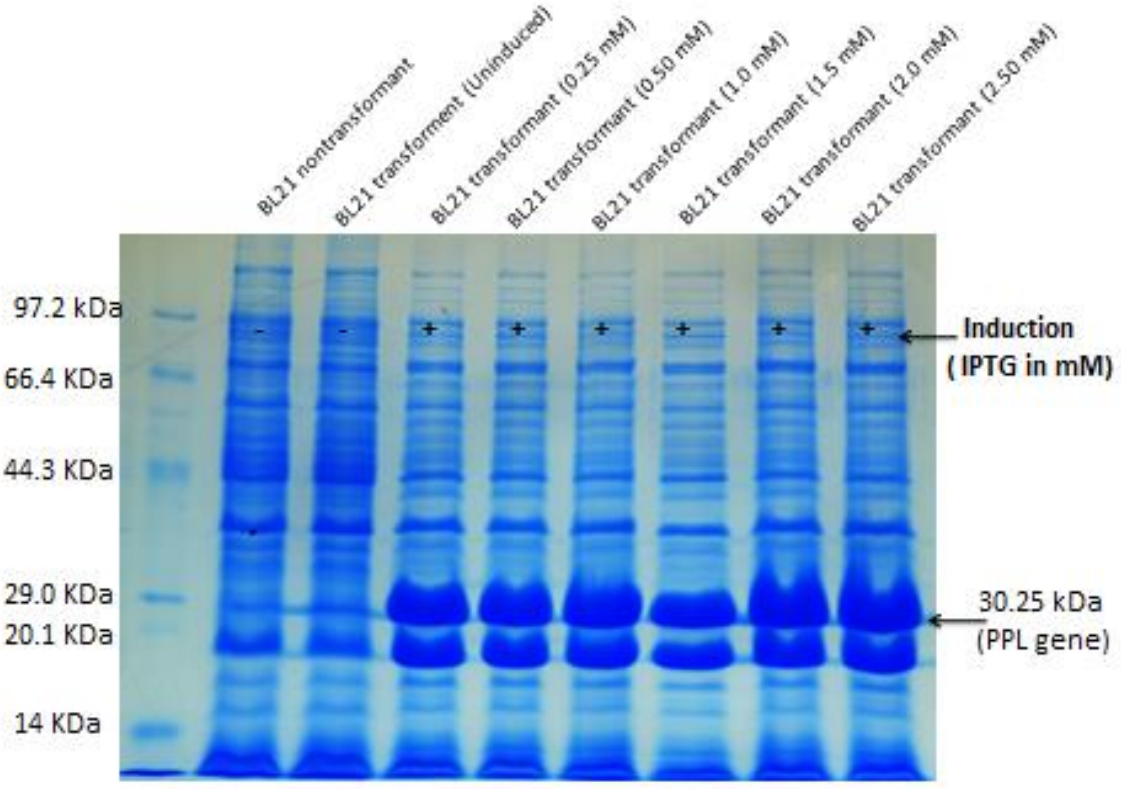



**Fig. 4.16: SDS-PAGE of the Induced protein sample of PPL transformed BL21 DE3 pLysS cells at 5 hrs of induction**

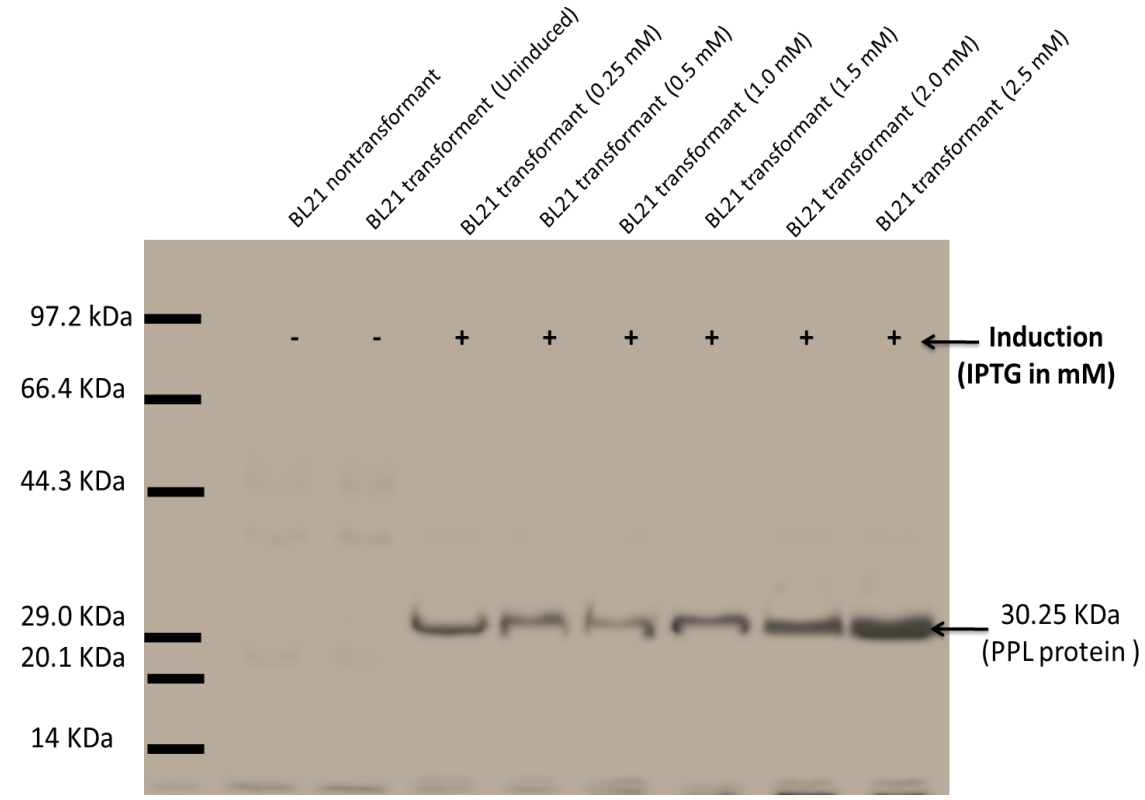



**Fig. 4.17: Western Blotting of the expressed proteins in transformed BL21 DE3 pLysS cells. Black bands represent the PPL protein having His-Tag attached to enzyme linked antibodies**

(Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004). It was shown that constitutive expression of *ASAL* in tobacco and rice exhibited significant level of resistance against *M. persicae* (Dutta *et al*. 2005), *N. lugens* and *N. virescens* (Saha *et al*., 2006) respectively. Recently transgenic rice lines resistant to sap sucking insect pest (*Nilparvata lugens*) were developed by ectopic expression of Garlic lectin (*ASAL*) in their phloem tissues (Chandrasekhar *et al*; 2014). Most widely cloned lectins belong to mannose-binding types isolated from monocots. Lectins have been cloned from seven families of angiosperms including Amaryllidaceae, Araceae, Alliaceae, Orchidaceae, Liliaceae, Iridaceae and Bromeliaceae (Van Damme *et al.*, 1991; Van Damme *et al.*, 1994; Van Damme *et al.*, 1996; Smeets *et al.*, 1997; Van Damme *et al.*, 2000; Neuteboom *et al.*, 2002; Chai *et al.*, 2003; Fei *et al.*, 2003; Kai *et al.*, 2003; Yao *et al.*, 2003; Zhao *et al.*, 2003), among which lectins from Amaryllidaceae species have been extensively studied and well documented (Van Damme *et al.*, 1992; Van Damme *et al.*, 1998; Kai *et al.*, 2003; Pang *et al.*, 2003; Zhao *et al.*, 2003). Many lectins are glycosylated and the presence or absence of the carbohydrate side chains can have significant effects on the functional and physical properties of the expressed proteins. A few reports indicated that plant lectins are non-glycosylated (Hoffman and Donaldson, 1987; Rodriguez-Arango *et al.*, 1992; Zhu *et al.*, 1996), and form insoluble inclusion bodies (Arango *et al.*, 1992; Adar *et al.*, 1997; Longstaff *et al.*, 1998). *E. coli* is also incapable of glycosylating eukaryotic proteins expressed in this host. Activity of the lectin thus produced was comparable to the native lectin. *E. coli* expressed *Xerocomus chrysenteron* lectin (XCL), like its native form could show toxicity to some insects such as dipteran *Drosophila melanogaster* and hemipteran *Acrythosiphon pisum* (Trigueros *et al.*, 2003).

Almost all the known lectin genes are intron-free and hence the molecular weight of the protein can be predicted by prokaryotic expression. In these cases, DNA or cDNA coding for lectin is cloned into expression vector with His tag and then transformed to *E. coli.* After induction with IPTG ($C_9H_{18}O_5S$), inclusion bodies from *E. coli* were solubilized and lectin was purified and characterized by SDS-PAGE.

In an effort to develop plant based lectin gene mediated resistance against various insects like lepidopterans and hemipterans, the pigeonpea lectin gene was cloned in an expression vector and its expression was analyzed. The lectin gene was cloned

using Gateway cloning system in a destination vector (pET300/NT-DEST) and transformed into *E. coli* BL21 DE3 pLysS cells for its optimum expression. The protein expression studies were conducted at two different temperatures (30 ºC and 37 ºC), but no expression was observed at 30 ºC. The pigeonpea lectin protein of ~30 KDa was expressed maximally at 37 °C, when induced using 2.5 mM IPTG for 5 hrs. The expression was also observed at lower concentrations of IPTG and time intervals, but in low quantity. The expressed protein was characterized by SDS-PAGE and western blotting and confirmed the protein of the predicted size (~30 KDa).The pET system was originally developed by Studier and colleagues and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in E. coli from the T7 promoter (Rosenberg *et al*., 1987; Studier & Moffatt, 1986; Studier *et al*., 1990). pET300/NT-DEST contain the T7 lac promoter to drive expression of the gene of interest. The T7lac promoter consists of a lac operator sequence placed downstream of the T7 promoter. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon. The lac operator serves as a binding site for the lac repressor (encoded by the *lacI* gene) and functions to further repress T7 RNA polymerase-induced basal transcription of the gene of interest in BL21 strains in absence of IPTG.

Initial attempts on cloning and expressing in *E. coli* included lectin genes from *Bauhinia purpurea* (Kusui *et al.*, 1991), *Erythrin acorallodendron* (Arango *et al.*, 1992), *Dolichos biflorus* (Chao *et al.*, 1994), lima bean (Jordan and Goldstein, 1994), *Galanthus nivalis* (Longstaff*et al.*, 1998), *Canavalia brasiliensis* (Nogueira *et al.*, 2002), *Pinellia ternate* (Lin *et al.*, 2003), *Phlebodium aureum* (Tateno *et al.*, 2003) and *Polyporus squamosus* (a mushroom) (Tateno *et al.*, 2004), *Microcystis viridis* (Li yuqin *et al.*, 2011). Many of these expressed proteins were used for insect bioassay using artifitial diet and evidenced the harmful effects of lectins on insects, which included loss in weight, mortality and feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation.

Based on the insecticidal activity of many well characterized lectins, the purified expressed protein of pigeonpea lectin will be used for insect bioassay to check its efficacy against various lepidopterans and hemipteran insect pests. Then the gene can be used to transform many agronomically important crop plants like cotton, safflower, sunflower, mustard, soybean, rice etc. to confer resistance against various economically important insect pests.

## 4.5 Conclusion

The PPL gene (~825 bp) was cloned in an expression vector (pET300/NT-DEST) and the expression was confirmed by SDS-PAGE and western blotting. The expressed protein now will be further characterized by MALDI and insect bioassay. Then the gene can be cloned in a binary vector under tissue specific promoter and used for transformation of crop plants for developing resistance against various lepidopterans and hemipterans insect pests.

# 5. DISCUSSION

Sustained agricultural production to meet the demands of growing population now depends heavily on crop management rather than bringing more area under cultivation. One way of increasing the yield would be to protect the agricultural produce from insect pest which are estimated to consume around 14 % of global agricultural output (Hilder and Boulter, 1999). Chemicals used to combat these pests are not eco-friendly and may lead to development of insecticide resistance among the pests. Therefore, there is an urgent need to develop alternative technologies, which require limited use of pesticides and yet provide adequate protection to crops within a sustainable agricultural framework. Use of novel genes through genetically modified plants to increase the level and range of resistance to insect pests is one of the potential strategies. Various genes from *Bacillus thuringiensis* (*Bt*) were transferred in different crops (Sharma *et al.*, 2000). However, there are possibilities of development of resistance to these genes and hence there is need to identify alternative genes such as lectins as insecticidal proteins through transgenic crops (Tabashnik, 1994).

Lectins are group of carbohydrate binding proteins or glycoproteins of non-immune origin, having biologically significant activities, such as ability to agglutinate cells and precipitate polysaccharides and glycoproteins (Lis and Sharon, 1986) with adverse effect against insects in terms of larval pupation, adult emergence, and larval survival and growth. Lectins have more of chronic than acute effect on insects (Shukla *et al.*, 2005). The specificity of wheat germ lectin toward chitin suggested that the peritrophic membrane, a thin porous chitin layer that covers the gut epithelium in many insects, is the target of its action. The toxicity of a lectin to a particular insect arises from type of glycoprotein on the outer membrane and sugar-specificity of the lectin (Powell *et al.*, 1993). Many plant lectins have been characterized, but the studies were concentrated more on the insecticidal activity of snowdrop lectin (GNA) (Gatehouse *et al.*, 1997; Powell *et al.*, 1998). The fungal lectins have also been purified and studied for insecticidal property. A lectin (XCL) from *Xerocomus chrysenteron* was shown to possess potent insecticidal activity to *D. melanogaster*, *Acyrthosiphon pisum* and *S.*

*littoralis*. The insecticidal activity XCL lectin on *D. melanogaster* was higher than that of GNA, one of the most toxic lectins to insects, thereby indicating their application in resistance development using transgenic approach (Trigueros *et al.*, 2003). To be expressed in plants, genes of fungal origin have advantages such as no codon bias, efficient RNA processing and enhanced protein stability.

The insecticidal activity of a carbohydrate binding plant lectin *ASAL* has been analyzed against various hemipteran pests (Bandyopadhyay *et al*., 2001; Majumder *et al*., 2004). It has also been shown that constitutive expression of ASAL in tobacco and in rice exhibited significant level of resistance against *M. persicae* (Dutta *et al*., 2005), *N. lugens* and *N. virescens* (Saha *et al*., 2006) respectively.

Almost all the known lectin genes are known to be intron-free and hence the molecular weight of the protein can be predicted by prokaryotic expression. In these cases, DNA or cDNA coding for lectin is cloned into expression vector with His tag and then transformed to *E. coli.* After induction with IPTG, inclusion bodies from *E. coli* were solubilized and lectin was purified and characterized by SDS-PAGE. The pET system was originally developed by Studier and colleagues and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in E. coli from the T7 promoter (Rosenberg *et al*., 1987; Studier & Moffatt, 1986; Studier *et al*., 1990). pET300/NT-DEST contain the T7lac promoter to drive expression of the gene of interest. The T7lac promoter consists of a lac operator sequence placed downstream of the T7 promoter. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon. The lac operator serves as a binding site for the lac repressor (encoded by the *lacI* gene) and functions to further repress T7 RNA polymerase-induced basal transcription of the gene of interest in BL21 strains in absence of IPTG.

The pigeonpea lectin gene was cloned in Destination vector (pET300/NT-DEST) using Gateway cloning system and its expression was analyzed by SDS polyacrylamide gel electrophoresis and western blotting. The lectin gene was cloned using Gateway cloning system in a destination vector (pET300/NT-DEST) and transformed into *E. coli* BL21 DE3 pLysS cells for its optimum expression. The protein expression studies were conducted at two different temperatures (30 ºC and 37 ºC), but no expression was

observed at 30 ºC. The pigeonpea lectin protein of ~30KDa was expressed maximally at 37 °C, when induced using 2.5mM IPTG for 5hrs. The protein was also expressed at lower concentrations of IPTG and time, but in low amounts.

Initial attempts on cloning and expressing in *E. coli* included lectin genes from *Bauhinia purpurea* (Kusui *et al.*, 1991), *Erythrina corallodendron* (Arango *et al.*, 1992), *Dolichos biflorus* (Chao and Etzler, 1994), lima bean (Jordan and Goldstein, 1994), *Galanthus nivalis* (Longstaff *et al.*, 1998), *Canavalia brasiliensis* (Nogueira *et al.*, 2002), *Pinellia ternate* (Lin *et al.*, 2003), *Phlebodium aureum* (Tateno *et al.*, 2003) and *Polyporus squamosus* (a mushroom) (Tateno *et al.*, 2004), *Microcystis viridis* (Li Yuqin *et al.*, 2011). Many of these expressed proteins were used for insect bioassay using artifitial diet and evidenced the harmful effects of lectins on insects, which included loss in weight, mortality and feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation.

Based on the insecticidal activity of many well characterized lectins, the purified expressed protein of pigeonpea lectin will be used for *In Vitro* insect bioassay to check its efficacy against various lepidopterans and hemipteran insect pests. Then, the gene can be cloned in a binary vector under the control of phloem specific promoters and used to transform *Brassica juncea* to confer resistance against Aphids.

# 6. Summary and Conclusion

The present study was undertaken with the aim to clone the isolated pigeon pea lectin (PPL) gene in an expression vector and characterize the expressed protein by SDS-PAGE and western blotting.

The main findings of the present study are:

1. The pigeonpea lectin gene was PCR amplified using gene specific forward and reverse primers. The stop codon was removed from the reverse primer and CACC nucleotides were added at the 5' end of the forward primer.
2. The ~825 bp amplified PCR product was ligated with pENTR-D-TOPO vector and transformed into one shot TOP10 chemically competent *E. coli* cells.
3. The transformed colonies were reselected on LA plate with kanamycin and confirmed for the presence of PPL (pigeonpea lectin) gene by colony PCR and restriction digestion of isolated plasmid DNA.
4. The plasmid DNA was recombined with Destination vector pET300/NT-DEST using LR clonase enzyme and transformed into TOP10 chemically competent host cells.
5. The transformed colonies were confirmed for the presence of gene by PCR.
6. The plasmid DNA of the positive colony was used for the transformation of BL21 DE3 pLysS competent cells for protein expression studies.
7. The colonies were confirmed for protein expression studies by the colony blot method after inducing the proteins for 4 hours at 1 mM IPTG concentration and 37 ºC.
8. The protein expressing positive colonies were streaked and used for expression of protein at different IPTG concentrations (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mM) and time intervals (3, 4, 5 hrs.) at 30 ºC and 37 ºC.
9. The maximum protein expression was obtained at 37 ºC, 2.0 mM and 2.5 mM IPTG concentrations after 5 hrs.
10. The expressed protein was confirmed by SDS- PAGE and western blotting. The size of the pigeonpea lectin was found to be approximately 30 KDa.

The expressed protein now can be further characterized by MALDI and insect bioassay. Then the gene can be cloned in a binary vector under tissue specific promoter and used for transformation of crop plants for developing resistance against various lepidopterans and hemipterans insect pests.

# *7. Abstract*

## *In vitro* Expression of Pigeonpea Lectin Gene and its Characterization


### ABSTRACT

Crop losses due to insect pests are a significant factor in limiting food production. Worldwide use of chemical insecticides and pesticides has increased the cost of pest control, resulted in insecticidal hazards to biological organisms, pollution to the environment and increasing insect resistance. Therefore, the challenge today is to achieve higher and stable crop production with safe and eco-friendly strategies. Plants accumulate a set of defense proteins including lectins, proteinase inhibitors, amylase inhibitors etc. Lectins reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran and homopteran insect pests. The harmful effects of lectins on biological parameters of insects include loss in weight, mortality, feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation. Realizing their importance, lectin gene have been isolated and characterized from pigeon pea. The isolated pigeonpea lectin (PPL) gene (~825 bp) was first cloned in pENTR-D-TOPO vector, subcloned into an expression vector (Gateway Destination vector pET300/NT-DEST) and transformed into BL21 DE3 pLysS competent cells of *E. coli* for protein expression studies. The PPL gene expression studies were carried out at different temperatures, IPTG concentrations and time intervals. The expression was maximum at 2.0 and 2.50 mM IPTG concentration at 37 ºC for 5 hrs. The size of the protein was found to be around ~30 KDa, as predicted by using the ProtParam software. The expression was confirmed by SDS-PAGE and western blotting. Thus, transferring these defense genes under the control of tissue specific promoters will be an effective tool for sustainable insect pest management programme.

# अरहर लेक्टिन जीन की *इन विट्रो* अभिव्यक्ति एवं इसका अभिलक्षणन

## सार

कीट पीड़कों के कारण फसल–क्षति, खाद्य फसलों के उत्पादन में कमी का एक महत्वपूर्ण कारक है। रासायनिक कीटनाशियों एवं पीड़कनाशियों के विश्वभर में विस्तृत उपयोग से पीड़क–नियंत्रण के खर्च में बढ़ोतरी हुई है तथा इसके परिणामस्वरूप जीवों के लिए कीटनाशी संबंधी संकटों, पर्यावरण –प्रदूषण एवं कीटों की प्रतिरोधिता में भी बढ़ोतरी हुई है। इसलिए आज चुनौति है कि सुरक्षित एवं पर्यावरण मित्र रणनीतियों के साथ उच्चतर एवं टिकाऊ फसलोत्पादन किया जाए। पौधों में रक्षात्मक प्रोटीन्स यथा लेक्टिन्स, प्रोटीनेज़ संदमकों, एमायलेज़ संदमकों आदि का संचयन होता है। लेक्टिन्स परिवर्तनीय रूप से एवं एन्ज़ायम उपयोग रहित रूप से विशिष्ट कार्बोहायड्रेट्स के साथ जुड़ते हैं और उनका समुच्चयन होने का यह गुण उन्हें लेपिडोप्टेरा एवं होमोप्टेरा गण के कीट पीड़कों के विरूद्ध उपयोगी बनाता है। कीटों के जीव विज्ञान संबंधी प्राचलों पर लेक्टिन्स के दुष्प्रभावों में, पहली एवं दूसरी संतति के भार में कमी, मर्त्यता, आहार–संदमन, विकास संबंधी कुल अवधि, वयस्क–निर्गमन एवं जननक्षमता में कमी का समावेश होता है। उनके इस महत्व को समक्षते हुए, अरहर से लेक्टिन जीन का विलगन किया गया। इस जीन (~ 825 बीपी) की pENTR–D-TOPO रोगवाहक में क्लोनिंग की गई। प्रोटीन–अभिव्यक्ति संबंधी अध्ययनों हेतु, इसे एक अभिव्यक्ति रोगवाहक (गेटवे डेस्टीनेशन वैक्टर pET300/NT-DEST) में सब–क्लोन किया गया एवं *ई. कोलाई* की BL21 DE3 pLysS कंपीटेंट कोशिकाओं में रूपांतरित किया गया। पीपीएल जीन अभिव्यक्ति–अध्ययन, विभिन्न तापमानों, आई पी टी जी सान्द्रताओं एवं समयान्तरालों पर किए गए। $37^{o}$ सें तापमान, 5 घन्टे तथा 2.0 एवं 2.50 मिमो आई पी टी जी सान्द्रता पर अभिव्यक्ति अधिकतम थी। प्रोटीन का परिमाण ~ 30 कि डा के लगभग पाया गया जैसा कि परोटपारम सॉफ्टवेयर के उपयोग से पूर्वानुमान मिला। एस डी एस – पेज एवं वैस्टर्न व्लाटिंग द्वारा इस अभिव्यक्ति की पुष्टि की गई।

अब अरहर लेक्टिन जीन की ऊतक विशिष्ट प्रोमोटर्स के नियंत्रण के अंतर्गत क्लोनिंग की जा सकती है तथा टिकाऊ कीट पीड़क प्रबंधन कार्यक्रम में एक प्रभावी उपाय के रूप में इसका उपयोग हो सकता है।

# 8. Bibliography


Adar, R., Streicher, H., Rozenblatt, S. and Sharon, N. (1997). Synthesis of soybean agglutinin in bacterial and mammalian cells. *Eur. J. Bioche.* **249**(3): 684-689.

Afzal, AJ., Wood, AJ. and Lightfoot, DA. (2008). Plant receptor-like serine threonine kinases: roles in signaling and plant defense. *Mol. Plant Microbe Int*. **21**: 507–17.

Allsop, P.G. and McGhie, T. K. (1996). Snowdrop Lectin and wheat germ lectins as antimetabolites for the control of sugarcane white grubs. *Entomol. Exp. Appln.* **80**(2): 409-414.

Anshuman, Kansal, R., Kumar, A., Kumar, P. and Koundal, KR. (2010). Isolation and characterization of lectin gene from mungbean [*Vigna radiata* (L) R.Wilczek]. *Pusa Agri. Science.* **33**: 1-7.

Arango, R., Adar, R., Rozenblatt, S. and Sharon, N. (1992).Expression of *Erythrina corallodendron* lectin in *Escherichia coli*. *Eur. J. Biochem.* **205**(2): 575-581.

Ayouba, A., Causse, H., Van Damme, E. J. M., Peumans, W. J., Bourne, Y., Cambillau, C. and Rouge, P. (1994). Interactions of plant lectins with the components of the bacterial cell wall peptidoglycan. *Biochem. Syst. Ecol.* **22**(2): 153-159.

Balzarini, J., Neyts, J., Schols, D., Hosoya, M., Van Damme, E., Peumans, W. and De Clercq, E. (1992). The mannose-specific plant lectins from *Cymbidium hybrid* and *Epipactishelleborine* and the (N-acetylglucosamine) n-specific plant lectin from *Urtica dioica* are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication in vitro. *Antiviral Res*. **18** (2): 191-207.

Bandyopadhyay, S., Roy, A. and Das, S. (2001). Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. *Plant Sci*. **161**:1025–1033.

Bandyopadhyay, S., Roy, A., Das, S. (2001). Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. *Plant Sci*. **161**:1025–1033.

Barondes, S. H., (1988). Bifunctional properties of lectins: lectins redefined. *Trends Biochem. Sci.* **13**(12): 480-482.

Bharathi, Y., Vijaya, KS., Pasalu, IC., Balachandran, SM., Reddy, VD. (2011). Pyramided rice lines harbouring *Allium sativum* (asal) and *Galanthus nivalis* (gna) lectin genes impart enhanced resistance against major sap-sucking pests. *J. Biotechnol*. **152**: 63–71.

Birnboim, HC., Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.***7**(6): 1513–23.

Boulter, D. (1993). Insect pest control by copying nature using genetically engineered crops. *Phytochemistry*. **34**: 1453-1466.

Boulter, D., Edwards, G. A., Gatehouse, A. M. R., Gatehouse, J. A. and Hilder, V. A. (1990). Additive protective effects of different plant-derived insect resistance genes in transgenic tobacco plants. *Crop Prot*. **9**(5): 351–354.

Boyko, EV., Smith, CM., Vankatappa, T., Bruno, J., Deng, Y., Starkey, S R. and Klaahsen, D. (2006). The molecular basis of plant gene expression during aphid invasion: wheat Pto- and Pti like sequences modulate aphid-wheat interaction. *J. Econ. Entomol.* **99**: 1430–1445.

Broekaert, W. F. and Peumans, W. J. (1986). Lectin release from seeds of *Datura stramonium* and interference of the *Datura stramonium* lectin with bacterial motility. In: Bog-Hansen T. C. and E. Van Driessche, eds. *Lectins, Biology, Biochemistry, Clinical Biochemistry, Berlin.* pp. 57–66.

Chai, Y., Pang, Y., Liao, Z., Zhang, L., Sun, X., Lu, Y., Wang, S. and Tang, K. (2003). Molecular cloning and characterization of a mannose-binding lectin gene from *Crinum asiaticum. J. Plant Physiol.* **160**: 913-920.

Chakraborti, D., Sarkar, A., Mondal, H. and Das, Sampa. (2009). Tissue specific expression of potent insecticidal, *Allium sativum* leaf agglutinin (ASAL) in

important pulse crop, chickpea (*Cicer arietinum* L.) to resist the phloem feeding Aphis craccivora, *Transgenic Res*. **18**: 529-544.

Chandrasekhar, K., Vijayalakshmi, M., Vani, K., Kaul, T. and Reddy, M K. (2014). Phloem-specific expression of the lectin gene from *Allium sativum* confers resistance to the sap-sucker *Nilaparvata lugens*. *Biotechnol Lett.* **36**:1059–1067.

Chang, T., Chen, L., Chen, S., Cai, H., Liu, X., Xiao, G., and Zhu, Z. (2003). Transformation of tobacco with genes encoding *Helianthus tuberosus* agglutinin (HTA) confers resistance to peach-potato aphid (*Myzus persicae*). *Transgenic Res*. **12**: 607-614.

Chao, Q. and Etzler, M. E. (1994).Incorrect targeting of plant vacuolar lectins in yeast. *J. Biol. Chem*. **269**(33): 20866-20871.

Chrispeels, M. J. and Raikhel, N. V. (1991).Lectins, lectin genes, and their role in plant defense. *Plant Cell*. **3**(1): 1-9.

Coelho, M.B., Marangoni, S. and Macedo, M.L. (2007). Insecticidal action of *Annona coriacea* lectin against the flour moth *Anagasta kuehniella* and the rice moth *Corcyra cephalonica* (Lepidoptera: Pyralidae). *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **146**: 406 -416.

Colucci, G., Moore, J. G., Feldman, M. and Chrispeels, M. J. (1999). cDNA cloning of FRIL, a lectin from *Dolichos lablab*, that preserves hematopoietic progenitors in suspension culture. *Proc. Natl. Acad. Sci*. **96**(2): 646-650.

Couty, A., Down, RE., Gatehouse, A.M.R., Kaiser, L., Pham-Delegue, MH., Poppy, GM. (2001). Effects of artificial diet containing GNA and GNA expressing potatoes on the development of the aphid parasitoid *Aphidius ervi* (Hymenoptera: Aphididae). *J. Insect Physiol.* **47:**1357–1366.

Datta, S., Kansal, R. and Koundal, KR. (2000). Construction of cowpea (*Vigna unguiculata* L.) cDNA library and characterization of an isolated lectin gene. *J. Plant Biochem.Biotechnol.* **9**: 67-71.

Dutta, I., Majumder, I., Saha, P., Ray, K. and Das, S. (2005). Constitutive and phloem specific expression of *Allium sativum* leaf agglutinin (*ASAL*) to

engineer aphid (*Lipaphis erysimi*) resistance in transgenic Indian mustard (*Brassica juncea*). *Plant Sci.* **169**: 996–1007.

Eck, J., Langer, M., Mockel, B., Baur, A., Rothe, M., Zinke, H. and Lentzen, H. (1999). Cloning of the mistletoe lectin gene and characterization of the recombinant A-chain. *Eur. J. Biochem.* **264**(3): 775-784.

Fei, J., Liao, Z., Chai, Y., Pang, Y., Yao, J., Sun, X. and Tang, K. (2003).Molecular cloning and characterization of a novel mannose-binding lectin gene from *Amorphophallus konjac*. *Mol. Biol. Rep*. **30**(3): 177-183.

Fitches, E., Audsley, N. and Gatehouse, J.A. (2002). Fusion proteins containing neuropeptides as novel insect control agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect Biochemistry and Molecular Biology*. **32**: 1653–1661.

Fitches, E., Gatehouse, A.M.R. and Gatehouse, J.A. (1997). Effects of snowdrop lectins (GNA) delivered via artificial diet and in transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. *J. Insect Physiol.* **43**: 727–739.

Fitches, E., Ilett, C., Gatehouse, A.M.R., GatehouseL.N., Greene , R., Edwards, J.P., and Gatehouse, J.A. (2001b).The effects of Phaseolus vulgaris erythro- and leucoagglutinatingisolectins (PHA-E and PHA-L) delivered via artificial diet and transgenic plants on the growth and development of tomato moth (*Lacanobia oleracea*) larvae; lectin binding to gut glycoproteins in vitro and in vivo. *J. Insect Physiol*. **47**(12): 1389-1398.

Fitches, E., Woodhouse, S. D., Edwards, J. P. and Gatehouse J. A. (2001a). *In vitro* and *in vivo* binding of snowdrop ( *Galanthus nivalis*) agglutinin; GNA) and jackbean (*Canavali aensiformis*) ConAlectins within tomato moth (*Lacanobia oleracea*) larvae: mechanisms of insecticidal action. *J. Insect Physiol*. **47**: 777–787.

Frohman, M. A., Dush, M. K. and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Nat. Acad. Sci*. **85**(23): 8998-9002.

Gatehouse, A. M. R., Powell, K. S., Peumans, W. J., Van Damme, E. J. M. and Gatehouse, J.A.(1995). Insecticidal properties of plant lectins: their potential in plant protection. In:Pusztai A. and S. Bardocz, eds. Lectins: *Biomedical Perspectives, London. Taylor and Francis* pp. 35-58.

Gatehouse, A.M.R. and Gatehouse, JA. (1998). Identifying proteins with insecticidal activity: Use of encoding genes to produce insect-resistant transgenic crops. *Pestic. Sci.* **52**:165-175.

Gatehouse, J. A., Gatehouse, A. M. R. and Fitches, E. (1997). Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. *J. Insect Physiol.* **43**(8): 727-739.

Goldstein, I. J. and Poretz, R. D. (1986).Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener I. E. N. Saron and I. J. Goldstein, eds. *The Lectins: Properties, Functions and Applications in Biology and Medicine*. pp 33–247.

Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. and Sharon, N. (1980). What should be called a lectin? *Nature.* **285**(5760): 66-66.

Habibi, JE., Backus, EA. and Czapla, TM. (1993). Plant lectins affect survival of the potato leafhopper (Homoptera: Cicadellidae). *J. Econ. Entomol.* **86**: 945-951.

Hegedus, D., Erlandson, M., Gillot, C. and Toprak, U. (2009).New insights into peritrophic matrix synthesis, architecture, and function. *Annu. Rev. Entomolol*. **54**: 285-302.

Hilder, V. A. and Boulter, D. (1999). Genetic engineering of crop plants for insect resistance–a critical review. *Crop Prot*. **18**(3): 177-191.

Hilder, V. A., Powell, K. S., Gatehouse, A. M. R., Gatehouse, J. A., Gatehouse, L. N., Shi, Y., Hamilton, W. D. O., Merryweather, A., Newell, C. A., Timans, J. C., Peumans, W. J., Damme, E. V. and Boulter, D. (1995). Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. *Transgenic Res.* **4**(1): 18-25.

Hilder, V. A., Powell, K. S., Gatehouse, A. M. R., Gatehouse, J. A., Gatehouse, L. N., Shi, Y., Hamilton, W. D. O., Merryweather, A., Newell, C. A., Timans, J. C., Peumans, W. J., Damme, E. V. and Boulter, D. (1995). Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. *Transgenic Res*. **4**(1): 18-25.

Hoffman, L. M. and Donaldson, D. D. (1987).Synthesis of mitogenic phytohemagglutinin-L in *Escherichia coli*. *Nat. Biotechnol*. **5**(2): 157-160.

Hossain, MA., Maiti, MK., Basu, A., Sen, S., Ghosh, AK. and Sen, SK. (2006). Transgenic expression of onion leaf lectin gene in Indian mustard offers protection against aphid colonization. *Crop Sci.* **46**: 2022–32.

Huang, Z., Zhang, T., Yang, J., Zhu, P., Du, G. and Cheng, K. (2005).Cloning and expression of human lectin-like oxidized low density lipoprotein receptor-1 in *Pichia pastoris*. *Biotechnol. Lett.* **27**(1): 49-52.

Jaber, K., Cuartero, Diaz.G., Haubruge, E., Francis, F. (2008). Investigation of carbohydrate binding property of a fungal lectin from *Xerocomus chrysenteron* and potential use on *Myzus persicae* aphid. *Commun. Agric. Appl. Biol. Sci*. **73**: 629–638.

Jordan, E. T. and Goldstein, I. J. (1994).The sequence of a second member of the lima bean lectin gene family and the expression and characterization of recombinant lectin in Escherichia coli. *J. Biol. Chem*. **269**(10): 7674-7681.

Kai, G., Zhao, L., Zheng, J., Zhang, L., Miao, Z., Sun, X. and Tang, K. (2004). Isolation and characterization of a new mannose-binding lectin gene from *Taxus media*. *J. Biosci*. **29**(4): 101-000.

Kai, G., Zheng, J., Zhang, L., Pang, Y., Liao, Z., Li, Z., Zhao, L., Sun, X. and Tang, K. (2003). Molecular cloning of a new lectin gene from *Z. grandiflora*. *DNA Seq*. **14**(4): 335-338.

Kanrar, S., Venkateshwari, J., Kirti, PB.and Chopra, VL. (2002). Transgenic Indian mustard (*Brassica juncea*) with resistance to mustard aphid *(Lipaphis erysimi* Kalt.). *Plant Cell Rep.* **20**: 976-981.

Karimi, J., Francis, F., Haubruge, E. (2010). Development of entomotoxic molecules as control agents: illustration of some protein potential uses and limits of lectins. *Biotech. Agro. Society Environ.* **14**(1): 225-241.

Klingler, J., Creasy, R., Gao, LL., Nair, RM., Calix, AS., Jacob, HS., Edwards, OR. and Singh, KB. (2005). Aphid resistance in *Medicago truncatula* involves antixenosis and phloemspecific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. *Plant Physiol.* **137**: 1445–1455.

Kusui, K., Yamamoto, K., Konami, Y. and Osawa, T. (1991). cDNA cloning and expression of *Bauhinia purpurea* lectin. *J. Biochem.* **109**(6): 899-903.

Lannoo, N., Vervecken, W., Proost, P., Rouge, P. and Van Damme, E. J. (2007). Expression of the nucleocytoplasmic tobacco lectin in the yeast *Pichia pastoris*. *Protein Expr.Purif.* **53**(2): 275-282.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**(5259): 680-5.

Leal-Bertioli, S. C. M., Pascoal, A. V., Guimaraes, P. M., Sa, M. F. G., Guimaraes, R. L., Monte, D. C. and Bertioli, D. J. (2003). Transgenic tobacco plants expressing Tarin 1inhibit the growth of *Pseudomonas syringae* pv. tomato and the development of *Spodoptera frugiperda*. *Ann. Appl. Biol*. **143**(3): 349-357.

Leonidas, D. D., Swamy, B. M., Hatzopoulos, G. N., Gonchigar, S. J., Chachadi, V. B., Inamdar, S. R., Zographos, S. E. and Oikonomakos, N. G.(2007). Structural basis for the carbohydrate recognition of the *Sclerotium rolfsii* lectin. *J. Mol. Biol*. **368**(4): 1145-1161.

Lin, J., Yao, J., Zhou, X., Sun, X. and Tang, K. (2003).Expression and purification of a novel mannose-binding lectin from *Pinellia ternata*. *Mol. Biotechnol*. **25**(3): 215-221.

Lis, H. and Sharon, N. (1986).Lectins as molecules and as tools. *Annu. Rev. Biochem.* **55**(1): 35-67.

Liu, J., Xu, X., Liu, J., Balzarini, J., Luo, Y., Kong, Y., Li, J., Chen, F., Van Damme, E. and Bao, J. (2007).A novel tetrameric lectin from Lycoris aurea with four mannose binding sites per monomer. *Acta Biochim. Pol*. **54**(1): 159-166.

Li, Yuqin., Liao, Xiaoxia., Chen, Gu., Yap, Yeeleng., Zhang, Xuewu. (2011). Cloning, Expression and Purification of *Microcystis viridis* Lectin in *Escherichia coli*. *Mol. Biotechnol*. **47**(2): 105-110.

Longstaff, M., Powell, K. S., Gatehouse, J. A., Raemaekers, R., Newell, C. A. and Hamilton, W. D. O. (1998).Production and purification of active snowdrop lectin in *Escherichia coli*. *Eur. J. Biochem*. **252**(1): 59-65.

Luo, Y., Xu, X., Liu, J., Li, J., Sun, Y., Liu, Z., Liu, J., Van Damme, E., Balzarini, J. and Bao, J. (2007). A novel mannose-binding tuber lectin from *Typhonium divaricatum* (L.)Decne (family Araceae) with antiviral activity against HSV-II and anti-proliferative effect on human cancer cell lines. *J. Biochem. Mol. Biol*. **40**(3): 358-367.

Macedo, M.L.R. and Damico, D.C.S. (2003). Purification and characterization of an o-Acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effects on the larval development of *Callosobruchus maculates* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera : Pyralidae). *J. Agric. Food. Chem.* **51**: 2980-2986.

Maddock, S. E., Hufman, G., Isenhour, D. J., Roth, B. A., Raikhel, N. V., Howard, J. A. and Czapla, T. H. (1991). Expression in maize plants of wheat germ agglutinin, a novel source of insect resistance. Paper presented In: *3rd International Congress of Plant Molecular Biology*, Tucson, Arizona, USA.

Majumder, P., Banerjee, S., Das, S. (2004). Identification of receptors responsible for binding of the mannose specific lectin to the gut epithelial membrane of the target insects. *Glycoconjugate J.* **20**:525–530.

Majumder, P., Saha, P., Dutta, I., Ray, T., Roy, S. C. and Das, S. (2006). Transgenic rice expressing *Allium sativum* leaf lectin with enhanced resistance against sap sucking insect pests. *Planta.* **223**: 1329–1343.

Maqbool, S. B., Riazuddin, S., Loc, N. T., Gatehouse, A. M. R., Gatehouse, J. A. and Christou, P. (2001). Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Mol. Breed.* **7**(1): 85-93.

Murdock, L. L., Huesing, J. E., Nielsen, S. S., Pratt, R. C. and Shade, R. E. (1990).Biological effects of plant lectins on the cowpea weevil. *Phytochemistry*. **29**(1): 85-89.

Nagadhara, D., Ramesh, S., Pasalu, IC., Rao, YK., Krishnaiah, NV., Sarma, NP., Brown, D P.,Gatehouse, JA., Reddy, VD. and Rao, KV. (2003). Transgenic indica rice resistant to sapsucking insects. *Plant Biotechnol. J.* **1**: 231–240.

Nagadhara, D., Ramesh, S., Pasalu, IC., Rao, YK., Sarma, NP., Reddy, VD. and Rao, KV. (2004). Transgenic rice plants expressing the snowdrop lectin gene (*gna*) exhibit high-level resistance to the whitebacked planthopper (*Sogatella furcifera*). *Theor. Appl. Genet.* **109**: 1399–1405.

Nagahora, H., Ishikawa, K., Niwa, Y., Muraki, M. and Jigami, Y. (1992). Expression andsecretion of wheat germ agglutinin by *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **210**(3): 989-997.

Nathan, S. and Halina Lis. (2004). History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology*. **14**: 11.

Neuteboom, L. W., Kunimitsu, W. Y., Webb, D. and Christopher, D. A. (2002). Characterization and tissue-regulated expression of genes involved in pineapple (*Ananas comosus* L.) root development. *Plant Sci.* **163**(5): 1021-1035.

Nogueira, N. A., Grangeiro, M. B., Da Cunha, R. M., Ramos, M. V., Alves, M. A., Teixeira, E. H., Barral-Netto, M., Calvete, J. J., Cavada, B. S. and Grangeiro, T. B. (2002). Expression and purification of the recombinant ConBr (*Canavalia brasiliensis* lectin) produced in *Escherichia coli* cells. *Protein Pept.Lett.* **9**(1): 59-66.

Pang, Y. Z., Shen, G. A., Liao, Z. H., Yao, J. H., Fei, J., Sun, X. F., Tan, F. and Tang, K. X. (2003). Molecular cloning and characterization of a novel lectin gene from *Zephyranthes candida*. *DNA Seq*. **14**(3): 163-167.

Peumans, W. J. and Van Damme, E J M. (1995). Lectins as plant defense proteins. *Plant Physiol.* **109**(2): 347-352.

Powell, K. S., Spence, J., Bharathi, M., Gatehouse, J. A. and Gatehouse, A. M. R. (1998). Immunohistochemical and developmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). *J. Insect Physiol.* **44**(7-8): 529-539.

Powell, KS., Gatehouse, AMR., Hilder, VA. and Gatehouse, JA. (1993). Antimetabolic effects of plants lectins and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*. *Entomol. Exp. Appl*. **66**: 119-126.

Pusztai, A., Ewen, S. W. B., Grant, G., Brown, D. S., Stewart, J. C., Peumans, W. J., Van Damme, E. J. M. and Bardocz, S. (1993). Antinutritive effects of wheat-germ agglutinin and other N-acetylglucosamine-specific lectins. *Br. J. Nutr.* **70**: 313-321.

Pusztai, A., Ewen, S. W., Grant, G., Peumans, W. J., Van Damme, E. J., Rubio, L. and Bardocz, S. (1990). Relationship between survival and binding of plant lectins during small intestinal passage and their effectiveness as growth factors. *Digestion*. **46**(2): 308-316.

Pusztai,A. and Bardocz, S. (1996). Biological effects of plant lectins on the gastro-intestinal tract: metabolic consequences and application. *Trends in Glycoscience and Glycotechnology*. **8**: 149- 165.

Qureshi, IA., Dash, P., Srivastava, PS. and Koundal, KR. (2006).Purification and characterization of an *N*-acetyl-D-galactosamine-specific lectin from seeds of chickpea (*Cicerarietinum* L.). *Phytochem. Analysis.* **17**: 350-356.

Raemaekers, R. J. M., De Muro, L., Gatehouse, J. A. and Fordham-Skelton, A. P. (1999). Functional phytohemagglutinin (PHA) and *Galanthus nivalis* agglutinin (GNA) expressed in *Pichia pastoris*. Correct N-terminal

processing and secretion of heterologous proteins expressed using the PHA-E signal peptide. *Eur. J. Biochem.* **265**(1): 394-403.

Rahbe, Y., Sauvion, N., Febvay, G., Peumans, W. and Gatehouse, A. (1995). Toxicity of lectins and processing of ingested proteins in the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* **76**(2): 143-155.

Rao, K. V., Rathore, K. S., Hodges, T. K., Fu, X., Stoger, E., Sudhakar, D., Williams, S., Christou, P., Bharathi, M. and Bown, D. P. (1998). Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper. *Plant J.* **15**(4): 469-477.

Rodriguez-Arango, E., Arango, R., Adar, R., Galili, G. and Sharon, N. (1992). Cloning, sequence analysis and expression in *Escherichia coli* of the cDNA encoding a precursor of peanut agglutinin. *FEBS Lett*. **307**(2): 185-189.

Rosenberg, Alan, H. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene.* **56**(1): 125-135.

Rossi, M., Goggin, FL., Milligan, SB., Kaloshian, I., Ullman, DE.and Williamson, VM. (1998). The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci.* **95**: 9750–4.

Roy, A., Banerjee, S., Majumder, P and Das, S. (2002).Efficiency of mannose binding plant lectins in controlling a homopteran insect, the red cotton bug. *J Ag Food Chem.* **50**: 6775-6779.

Sadeghi, A., Broeders, S., Greve, HD., Hernalsteens, J., Peumans, WJ., Damme, EJV. and Smagghe, G. (2007). Expression of garlic leaf lectin under the control of the phloem specific promoter *Asus*1 from *Arabidopsis thaliana* protects tobacco plants against the tobacco aphids (*Myzus nicotianae*). *Pest. Manag. Sci.* **63**: 1215–23.

Saha, P., Majumder, P., Dutta, I., Ray, T., Roy, SC. and Das, S. (2006). Transgenic rice expressing *Allium sativum* leaf lectin with enhanced resistance against sap sucking insect pests. *Planta.* **223**: 1329–43.

Sauvion, N., Rahbe, Y., Peumans, W. J., Damme, E. J. M., Gatehouse, J. A. and Gatehouse, A. M. R. (1996). Effects of GNA and other mannose binding

lectins on development and fecundity of the peach-potato aphid *Myzus persicae*.*Entomol. Exp. Appl*. **79**(3): 285-293.

Schlumbaum, A., Mauch, F., Voegeli, U. and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature.* **324**(6095): 365-367.

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

Sharma, H. C., Sharma, K. K. and Crouch, J. H. (2004). Genetic transformation of crops for insect resistance: potential and limitations. *Crit. Rev. Plant Sci*. **23**: 47-72.

Sharma, H. C., Sharma, K. K., Seetharama, N. and Ortiz, R. (2000).Prospects for using transgenic resistance to insects in crop improvement. *Electron. J. Biotechnol*. **3**: 21-22.

Shukla, S., Arora, R. and Sharma, H. C. (2005). Biological activity of soybean trypsin inhibitor and plant lectins against cotton bollworm/legume pod borer, *Helicoverpa armigera*. *Plant Biotechnol*. **22**(1): 1-6.

Singh, D., Arya, R A., Chandra, N., Niwas, R. and Salisbury, P. (2010). Genetic diversity studies in relation to seed yield and its component traits in Indian mustard (*Brassica juncea* L. Czern & Coss.). *Journal of Oilseed Brassica*. **1**: 19-22.

Smeets, K., Van Damme, E. J. M., Verhaert, P., Barre, A., Rougé, P., Van Leuven, F. and Peumans, W. J. (1997). Isolation, characterization and molecular cloning of the mannose-binding lectins from leaves and roots of garlic (*Allium sativum* L.). *Plant Mol. Biol.* **33**(2): 223-234.

Studier, F. W., & Moffatt, B. A. (1986).Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of molecular biology*. **189**(1): 113-130.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol*. **185**: 60–89.

Swamy, B. M., Bhat, A. G., Hegde, G. V., Naik, R. S., Kulkarni, S. and Inamdar, S. R. (2004). Immunolocalization and functional role of *Sclerotium rolfsii* lectin in development of fungus by interaction with its endogenous receptor. *Glycobiology*. **14**(11): 951-957.

Tabashnik, B. E. (1994). Evolution of resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol.* **39**(1): 47-79.

Tague, B. W. and Chrispeels, M. J. (1987). The plant vacuolar protein, phytohemagglutinin, is transported to the vacuole of transgenic yeast. *J. Cell Biol*. **105**(5): 1971-1979.

Tateno, H., Winter, H. C. and Goldstein, I. J. (2004). Cloning, expression in *Escherichia coli* and characterization of the recombinant Neu5Acalpha2, 6Galbeta1, 4GlcNAc-specific high-affinity lectin and its mutants from the mushroom *Polyporus squamosus*. *Biochem. J*. **382(**Pt2): 667-675.

Tateno, H., Winter, H. C., Petryniak, J. and Goldstein, I. J. (2003). Purification, characterization, molecular cloning, and expression of novel members of jacalin related lectins from rhizomes of the true fern *Phlebodium aureum* (L) J. Smith (Polypodiaceae). *J. Biol. Chem.* **278**(13): 10891-10899.

Trigueros, V., Lougarre, A., Ali-Ahmed, D., Rahbe, Y., Guillot, J., Chavant, L., Fournier, D. and Paquereau, L.(2003). *Xerocomus chrysenteron* lectin: identification of a new pesticidal protein. *Biochim.Biophys. Acta*. **1621**(3): 292-298.

Trung, N. P., Fitches, E. and Gatehouse, J. A. (2006). A fusion protein containing a lepidopteran-specific toxin from the South Indian red scorpion (Mesobuthus tamulus) and snowdrop lectin shows oral toxicity to target insects. *BMC Biotechnology*. **6**: 18.

Vajhala, CSK., Sadumpati, VK., Nunna, HR., Puligundla, SK., Vudem, DR. (2013). Development of transgenic cotton lines expressing Allium sativum agglutinin (ASAL) for enhanced resistance against major sap-sucking pests. *PLoS ONE*. **8**: e72542.

Van Damme, E. J. M., Astoul, C. H., Barre, A., Rouge, P. and Peumans, W. J. (2000). Cloning and characterization of a monocot mannose-binding lectin from *Crocus vernus* (family-Iridaceae). *FEBS J*. **267**(16): 5067-5077.

Van Damme, E. J. M., Brike, F., Winter, H. C., Leuven, F., Goldstein, I. J. and Peumans, W. J. (1996). Molecular cloning of two different mannose-binding lectins from tulip bulbs. *Eur. J. Biochem*. **236**(2): 419-427.

Van Damme, E. J. M., Goldstein, I. J., Vercammen, G., Vuylsteke, J. and Peumans, W. J. (1992). Lectins of members of the Amaryllidaceae are encoded by multigene families which show extensive homology. *Plant. Physiol.* **86**(2): 245-252.

Van Damme, E. J. M., Kaku, H., Perini, F., Goldstein, I. J., Peeters, B., Yagi, F., Decock, B. and Peumans, W. J. (1991). Biosynthesis, primary structure and molecular cloning of snowdrop (*Galanthus nivalis* L.) lectin. *Eur. J. Bioche.* **202**(1): 23-30.

Van Damme, E. J. M., Peumans, W. J., Barre, A. and Rouge, P. (1998). Plant lectins: A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* **17**(6): 575-692.

Van Damme, E. J. M., Smeets, K., Torrekens, S., Leuven, F. and Peumans, W. J. (1994). Characterization and molecular cloning of mannose-binding lectins from the Orchidaceae species *Listera ovata*, *Epipactis helleborine* and *Cymbidium hybrid*. *Eur. J. Biochem*. **221**(2): 769-777.

Vasconcelos, I.M. and Oliveira, J.T. (2004). Antinutritional properties of plant lectins. *Toxicon*. **44**: 385-403.

Vijayan, M. and Chandra, N. (1999). Lectins. *Curr.Opin. Struct. Biol*. **9**(6): 707-714.

Von Schaewen, A. and Chrispeels, M. J. (1993). Identification of vacuolar sorting information in phytohemagglutinin, an unprocessed vacuolar protein. *J. Exp. Bot*. **44**: 339-342.

Yao, J., Pang, Y., Qi, H., Wan, B., Zhao, X., Kong, W., Sun, X. and Tang, K. (2003). Transgenic tobacco expressing *Pinellia ternata* agglutinin confers enhanced resistance to aphids. *Transgenic Res.* **12**(6): 715-722.

Yarasi, B., Sadumpati, V., Immanni, CP., Reddy. VD., Rao, KV. (2008). Transgenic rice expressing Allium sativum leaf agglutinin (ASAL) exhibits high-level resistance against major sap-sucking pests. *BMC Plant Biol.* **8**:102.

Zhao, X., Yao, J., Sun, X. and Tang, K. (2003). Molecular cloning and characterization of a novel lectin gene from *Lycoris radiata*. *DNA Seq.* **14**(3): 223-226.

Zhu, K., Huesing, J. E., Shade, R. E., Bressan, R. A., Hasegawa, P. M. and Murdock, L. L. (1996). An insecticidal N-acetylglucosamine-specific lectin gene from *Griffonia simplicifolia* (Leguminosae). *Plant Physiol*. **110**(1): 195-202.

# APPENDIX

## Solutions for Gel Electrophoresis

**1. TAE Buffer (50X)**

| | |
|---|---|
| Tris base (0.04M) | 242.0 g |
| Glacial acetic acid | 57.1 g |
| 0.5M EDTA (pH 8.0) | 100 ml |

Autoclaved at 121$^{o}$C at 15 psi for 20mins

**2. TAE Buffer (1X) (1L)**

| | |
|---|---|
| TAE buffer (50X) | 20 ml |
| DD water | 980 ml |

**3. 6X Gel Loading Dye**

0.25% (W/V) Bromophenol Blue, 0.25% (W/V) Xylene Cyanol and 15% (Ficoll type 400) dissolved in water and stored at room temperature.

## Antibiotics

**Ampicillin (100mg/ml stock solution)**

1.0 g ampicillin was dissolved in 10 ml of distilled water and filter sterilized with 0.22 µM filter membrane.

**Kanamycin (50mg/ml stock solution)**

0.5 g kanamycin was dissolved in 10 ml of distilled water and filter sterilized with 0.22 µM filter membrane.

# Solutions for SDS-PAGE

**1. 30% Acrylamide**

- Acrylamide - 30 g
- Bis-acrylamide - 0.8 g

Dissolve in SDW and filter sterilize with whattman filter paper and maintain volume to 100 ml.

**2. 1.5 M Tris-HCl (pH 8.8)**

Dissolve 18.18 g of Tris basein 80 ml waterand maintain volume and pH with HCl and then autoclave.

**3. 0.5 M Tris-HCl (pH 6.8)**

Dissolve 6 g of Tris base in 80 ml water and maintain PH and volume with HCl and then autoclave.

**4. 5X Tris Glycine Running Buffer**

Mix 15 g Tris Base, 72 g Glycine and 5 g SDS and make volume to 1000 ml. Adjust pH 8.3 using concentrated HCl.

**5. Loading Buffer:** For 8 ml

| | |
|---|---|
| Deionised water | 3.8 ml |
| 0.5 M TrisHCl (Ph 6.8) | 1 ml |
| Glycerol | 0.8 ml |
| 10% SDS(W/V) | 1.6 ml |
| B- mercaptoethanol | 0.4 ml |
| 1% Bromophenol blue | 0.4 ml |
| Total | 8 ml |

**6. Staining Solution:** For 500 ml

| | |
|---|---|
| Methanol | 250 ml |
| Acetic acid | 50 ml |
| Coomassie brilliant blue | 0.5 g |
| SDW | 200 ml |
| Total | 500 ml |

**7. Destaining Solution:** For 500 ml

| | |
|---|---|
| Methanol | 200 ml |
| Glacial acetic acid | 50 ml |
| SDW | 250 ml |
| Total | 500 ml |

## Solutions for Colony Blotting

**1. Denaturing solution**

- 0.5 M NaOH
- 1.5 M NaCl

**2. Neutralization solution**

- 1.5 M NaCl
- 0.5 M Tris-HCl (pH 7.4 at RT )

**3. 20X SSC (for 500 ml):**

- 87.65 g NaCl
- 50.25 g Trisodium citrate.$2H_2O$

**4. TBS Buffer**

- 10 Mm Tris-Cl (pH7.5)
- 150 Mm NaCl

## Solutions for Competent Cell Preparation and Transformation

**TSS Buffer**

It consists of LB broth with 10% (W/V) PEG, 5% (V/V) DMSO and 20-50 mM $Mg^{2+}$ (MgSO4 or MgCl2), at a final pH of 6.5.

**5X KCM**

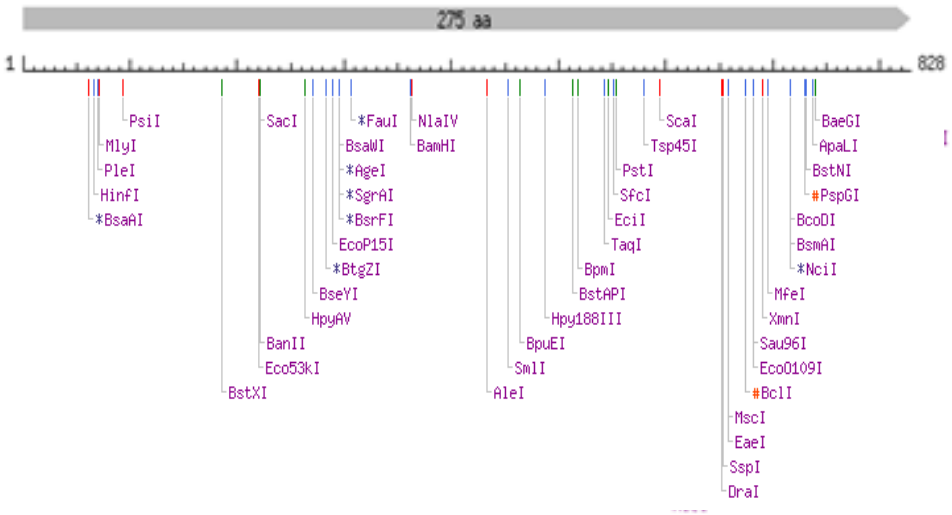
Mix 6.0 ml of 0.5 M KCl, 4.5 ml of 0.15 M $CaCl_2$ and 7.5 ml of 0.25 M $MgCl_2$ and the volume is maintained to 30 ml using SDW, filter sterilize and store at 4ºC.

# In Silico Analysis of PPL Gene (GenBank JN561784.1)

## 1. PPL gene cDNA sequence: from NCBI

GATGGGACATGTGTGTATATGTGTTTCATGTAACGCTCTATAAAGACACGTAGAATGAGTCATCACCACTATATAAAC
AAGTACAGCATGTGCATGCATGCAATTATAACCAATA**ATG**GCTTCTCTTCAAACCCAAATGATCTCATTCTATCTGAT
ATTTCTATCCATTCTCTTAACAACAATCTTTTTCTTCAAGGTGAACTCAACAGAAACCACTTCCTTTTCCATCACCAAGT
TTAGCCCAGACCAAAAAAACCTAATCTTCCAAGGAGATGCTACACCACAAAAGGGAAGTTGACACTGACCAAGGCAG
TAAAGAGCACTGTTGGCAGAGCTCTCTATTCCACACCTATCCGTATATGGGATAGAGATACAGGTAACGTTGCTAATT
TTGTAACTTCCTTCACTCTTGTCATAGATGCGCCCAGCAGTTACAACGTTGCCGATGGGTTTACGTTCTTCATCGCACC
GGTGGATACTAAGCCGCAGACTGGCGGGGGATACCTCGGAGTTTTCAATAGCAAAGAATATGATAAAACTAGTCAAA
CTGTTGCTGTGGAGTTTGACACTTTCTATAACGCTGCTTGGGATCCAAGCAATAAAGAAAGACATATTGGGATTGATG
TTAACAGTATCAAATCCGTAAATACTAAGTCGTGGAATTTGCAGAATGGTGAACGGGCTAATGTTGTGATAGCTTTTA
ATGCTGCCACTAATGTGTTAACTGTTACTTTAACTTATCCTAATTCACTTGAGGAAGAGAATGTAACTAGTTATACTCT
TAATGAAGTTGTGCCTTTGAAAGATGTTGTTCCTGAGTGGGTGAGGATTGGTTTCTCAGCTACCACTGGAGCAGAATTT
GCAGCACATGTAGTTCATTCATGGTCTTTTCATTCTGAGTTGGGCGGAACTTCGAGTTCTAAGCAAGCTGCAGATGCA**T**
**AG**TTTTTTTGCTTTTCATCATCATGCATGTCAAGTCATGTGTGACAGATCCAGTTTATATGCAGTACTTTTGTAATGTTG
TTATGTATGTTACTTGATGCGTTTATTAATCATGTGTGTGATTAATTGTTTGAGTTATGTGTGTTAAATTAGATAGTTAC
TCTCTTTTTAAATATTATTTGGCCAAATGCAAAAACTGTTAAGCATTAGTGATCACTAGTATAGGGCCTAATAGAACCC
ATTCTTGGCAATTGGGACATGCATACCTATAAATAAACTGCGCTCCGGGGCAGAGACACCTTGTTTTGTACCCAGGGG
TTCATGTGCACCTCTCAACATTTTGGATTTACACATACGTTATTTTATGTTTTGAAAAAAAAAAAAAAAA

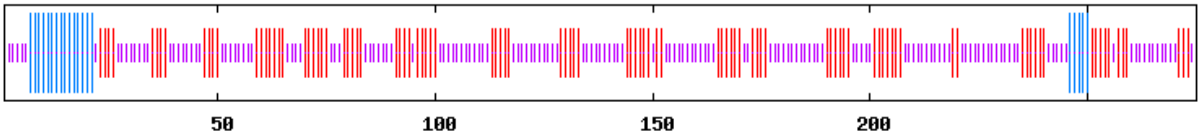
## 2. Restriction Sites on the PPL Gene: NEB cutter Results



## 3. PPL Protein Length and Structural Components: GOR4 Results

```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
MASLQTQMISFYLIFLSILLTTIFFFKVNSTETTSFSITKFSPDQKNLIFQGDGYTTKGKLTLTKAVKST
ccccccchhhhhhhhhhhhhhhceeeecccccccceeeecccccccceeeecccccccceeeeeeecccc
VGRALYSTPIRIWDRDTGNVANFVTSFTLVIDAPSSYNVADGFTFFIAPVDTKPQTGGGYLGVFNSKEYD
eeeeeeccceeeeecccccceeeeceeeeecccccccccccceeeeecccccccccccceeeeecccccc
KTSQTVAVEFDTFYNAAWDPSNKERHIGIDVNSIKSVNTKSWNLQNGERANVVIAFNAATNVLTVTLTYP
cccceeeeeecceccccccccccceeeeeecceeeecccccccccccceeeeeecccccceeeeeeecc
NSLEEENVTSYTLNEVVPLKDVVPEWVRIGFSATTGAEFAAHVVHSWSFHSELGGTSSSKQAADA
ccccccccceecccccccccccccceeeeeecccccchhhhheeeeeceeecccccccccceeec

Sequence length:   275
GOR4:


   Alpha helix      (Hh):    20 is   7.27%
   Extended strand  (Ee):    99 is 36.00%
   Random coil      (Cc):   156 is 56.73%
```

## 4. Graphical Representation of the PPL Protein Structural Components



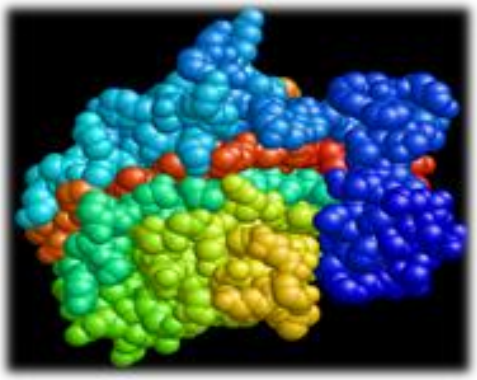

## 5. Physico-Chemical Properties: ProtParam Results

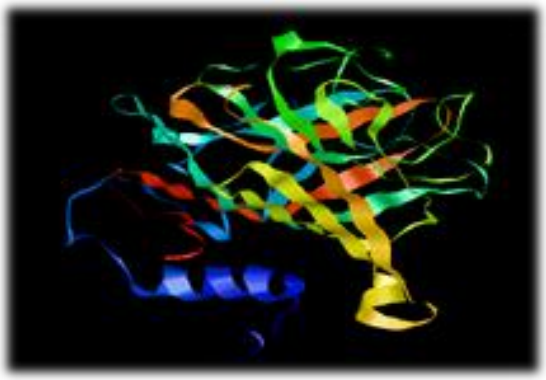
**User-provided sequence:**

```
          10         20         30         40         50         60
MASLQTQMIS FYLIFLSILL TTIFFFKVNS TETTSFSITK FSPDQKNLIF QGDGYTTKGK

          70         80         90        100        110        120
LTLTKAVKST VGRALYSTPI RIWDRDTGNV ANFVTSFTLV IDAPSSYNVA DGFTFFIAPV

         130        140        150        160        170        180
DTKPQTGGGY LGVFNSKEYD KTSQTVAVEF DTFYNAAWDP SNKERHIGID VNSIKSVNTK

         190        200        210        220        230        240
SWNLQNGERA NVVIAFNAAT NVLTVTLTYP NSLEEENVTS YTLNEVVPLK DVVPEWVRIG

         250        260        270
FSATTGAEFA AHVVHSWSFH SELGGTSSSK QAADA
```

- **Number of amino acids:** 275
- **Molecular weight:** 30234.9
- **Theoretical pI:** 5.59

## 6. Predicted PPL Protein Structure and its Binding Sites: I-TASSER Results





**Predicted Binding Site**

| Residue | Amino acid | contact | av distance | JS divergence |
|---|---|---|---|---|
| 110 | ALA | 16 | 0.00 | 0.47 |
| 111 | ASP | 17 | 0.00 | 0.51 |
| 128 | GLY | 22 | 0.36 | 0.39 |
| 129 | GLY | 19 | 0.07 | 0.30 |
| 153 | PHE | 26 | 0.08 | 0.31 |
| 155 | ASN | 17 | 0.04 | 0.30 |
| 246 | GLY | 22 | 0.08 | 0.55 |
| 247 | ALA | 25 | 0.08 | 0.00 |
| 248 | GLU | 23 | 0.12 | 0.00 |

**Heterogens present in Predicted Binding Site**

| Heterogen | Count | source structures |
|---|---|---|
| GAL | 2 | 1dzq_A,1dbn_A |
| NAG | 3 | 2arb_B,2auy_A,1qoo_A |
| FRU | 1 | 1n3p_A |
| MAN | 23 | 2anx_A,2arb_B,2phu_B,2pht_B,2phf_B, 2auy_A,1q8p_A,1q8o_A,2phr_A,1q8v_A, 1q8s_A,1q8q_A |
| GLC | 3 | 2bqp_B,1n3q_B,1n3p_A |