# CLONING AND EXPRESSION OF cDNA ENCODING RIBOSOME INACTIVATING PROTEINS

**Thesis submitted to the**
*University of Agricultural Sciences, Dharwad*
*In partial fulfillment of the requirements for the*
*Degree of*

**MASTER OF SCIENCE (AGRICULTURE)**

**In**

**PLANT BIOTECHNOLOGY**

**By**

**SALAR ABBAS**

**DEPARTMENT OF BIOTECHNOLOGY**
**COLLEGE OF AGRICULTURE, DHARWAD**
**UNVERSITY OF AGRICULTURAL SCIENCES,**
**DHARWAD – 580 005**

**JULY, 2007**

**ADVISORY COMMITTEE**

**DHARWAD**
**JUNE, 2007**

**(P.U. KRISHNARAJ)**
MAJOR ADVISOR

**Approved by:**

**Chairman: _____**
**(P.U. KRISHNARAJ)**

**Members: 1. _____**
**(M.S. KURUVINASHETTI)**

**2. _____**
**(O. SRIDEVI)**

**3. _____**
**(SUMANGALA BHAT)**

# CONTENTS

# LIST OF TABLES



# LIST OF FIGURES

# LIST OF PLATES

# LIST OF APPENDICES

# I. INTRODUCTION

Nature is a finite resource that provides people with food, feed, fibre and all our requirements. Continuously growing population and the insatiable desire for new sources of raw materials for daily life and industry has led to high stress on nature. Modern cultivation practices have resulted in changing insect pest disease scenario. Of late viral diseases are causing increasing damage to crops. In many research breeding programmes around the world, increased virus resistance in crop plants is one of the often stated objective.

Conventional breeding programmes have partly succeeded in developing resistance or tolerance to plant pathogenic viruses. Many higher plants have developed a variety of defense systems to combat pathogen attack that is essential for their survival. Certain plant species are known to synthesize proteins with antimicrobial properties (Broekaert *et al.,* 1997). Some of these proteins belong to a family of ribosome inactivating proteins (RIPs), which directly inhibit the protein synthesis on ribosomes. RIPs are capable of removing a single specific adenine residue in a conserved GAGA tetraloop of the major ribosomal RNA (Barbieri *et al*., 1993). In addition to plant antiviral activity; RIPs have anti-human immunodeficiency virus (HIV) (Zarling *et al*., 1990), anti-tumor (Lee-haung *et al*., 1995), and anti-fungal (Wang *et al*., 1998) activity.

Virus resistance, previously observed in transgenic plants expressing coat protein genes or the read-through component of the virus replicase gene, have been specific for the virus from which the genes are derived or to closely related viruses (Beachy *et al*., 1990; Golemboski *et al*., 1990). However, the expression of pokeweed antiviral protein (PAP) in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses (Jennifer *et al*., 1993)

In the light of the above background, this research programme had the following objectives;

1. Cloning a gene encoding an antiviral peptide/RIP from *Bougainvillea spectabilis*
2. Cloning a full-length cDNA encoding antiviral peptide/RIP from *Boerhaavia diffusa.*
3. Analysis of expression of these genes in *Escherichia coli.*

# II. REVIEW OF LITERATURE

Proteins with selective toxicity have been investigated for varied uses including increased plant defense against pathogens (Lodge et *al*., 1993; Logemann *et al*., 1992; Madin *et al*., 2000), cancer therapy (Frankel *et al.,* 1996; Kreitman, 1999; Olsnes and Pihl, 1982; Paston and Fitzgerald, 1991; Spooner and Lord, 1990; Thorpe *et al.,* 1982), and even as biological weapons (Christopher *et al*., 1997; Weiner, 1996). One class of proteins with selective toxicity, called ribosome-inactivating proteins (RIPs), is found in fungi, bacteria, and the plant kingdom. RIPs are N-glucosidases that inhibit translation through their activity against ribosomal RNA.

Due to the selective toxicity of RIPs, a primary focus of research has been to use them as the toxic agent in immunotherapies (Olsnes and Pihl, 1982). As a result, much of the RIP literature involves isolation and characterization of RIPs from new plant species and their use as anticancer agents (Barbieri *et al*., 1993; Gasperi-Campani *et al*., 1985; Stirpe *et al*., 1992). Many RIPs have high activity against non-plant ribosomes whereas plant ribosomes are resistant to depurination (Bass *et al*., 1992; Hartley and Beevers, 1982; Krawetz and Boston, 2000), These studies have led researchers to propose a role for RIPs in plant defense. Antiviral activity has also been found in a number of type 1 and 2 RIPs (Barbeiri *et al*., 1993; Hartley *et al*., 1996; Jackman *et al*., 1999).

## 2.1 MECHANISM OF RIBOSOME-INACTIVATING ACTIVITY

The N-glycosidation of RIPs involves the removal of a single, specific adenine corresponding to residue A4324 in rat 28S ribosomal RNA (Endo *et al*., 1987; Endo and Tsurugi, 1987). This adenine residue lies within a 14-nucleotide region known as the $\alpha$- sarcin loop having a GAGA sequence, with the first adenine being the RIP substrate, at the core of a putative loop with a short base-paired stem (Correll *et al*., 1998; Gutell *et al*., 1993; Orita *et al*., 1993). This adenine residue is required for elongation factor (EF)-1 and EF-2 dependent GTPase activities. Irreversible depurination of the target adenine residue by a RIP blocks the binding of EF-2, thereby blocking translation (Nilsson *et al*., 1986). The $\alpha$-sarcin loop is evolutionarily conserved in large rRNAs from bacteria to humans (Fig. 1) and the RIP-targeted adenine residue is absolutely conserved (Mehta and Boston, 1998). Thus, all large rRNAs could be susceptible to depurination by RIPs. The activity of RIPs depends on both the source of the RIP and the target ribosome (Hartley *et al*., 1996). Native RIPs do not affect ribosomes, while they may inhibit protein synthesis by heterologous plant ribosomes (Maria *et al*., 2005). RIPs such as PAP are very active against plant, animal, and bacterial ribosomes, whereas RIPs from cereals generally have low activity against plant ribosomes. For example, $10^3$ -fold more ricin is required to depurinate wheat ribosomes than to depurinate mammalian ribosomes (Cawley *et al*., 1977). Differences in efficiency are also observed between depurination of ribosomes and free rRNA. Ricin is needed $10^4$ fold more to depurinate oligonucleotides than to kill mammalian cells (Endo *et al*., 1991). There have been reports of interaction between ricin and the ribosomal proteins L9 and P0, which are located in a region of the ribosome called the acidic stalk (Vater *et al*., 1995). The acidic stalk is known to be involved in interactions with elongation factors (Rich and Steitz, 1987; Uchiumi *et al*., 1987). Vater and colleagues claim that there is enough sequence divergence in the L9 and P0 proteins to account for lack of prokaryotic recognition by ricin (Vater *et al*., 1995). Further, an additional component of the acid stalk, P3, appears to be unique to higher plants and may contribute to structural differences that affect RIP interactions with plant ribosomes (Bailey-Serres, 1998; Bailey-Serres *et al*., 1997).

Thermodynamic studies of the α-sarcin loop suggest that the loop is not very stable (Szewczak *et al*., 1991). Thus, the conformation of the α-sarcin loop may change during translation (Szewczak and Moore, 1995). Studies have shown that ribosomes have different sensitivities to RIPs, depending on their conformation (Holmberg and Nygard, 1996; Girbes *et al*., 1996; Alegre *et al*., 1996). Differences in RIP tertiary structure could also account for specificity toward certain ribosomes. X-ray crystallography with RIPs having differing ribosome specificity have shown that overall tertiary structure is similar (Wang *et al.,* 2000; Savino *et al*., 2000; Poyet *et al*., 1998; Katzin *et al*., 1991; Zhuo *et al*., 1994). Amino-terminal, carboxy-terminal, and central domains of two RIPs with differing ribosome-specificity were swapped, The mutants produced by swapping domains of pokeweed antiviral protein (PAP),

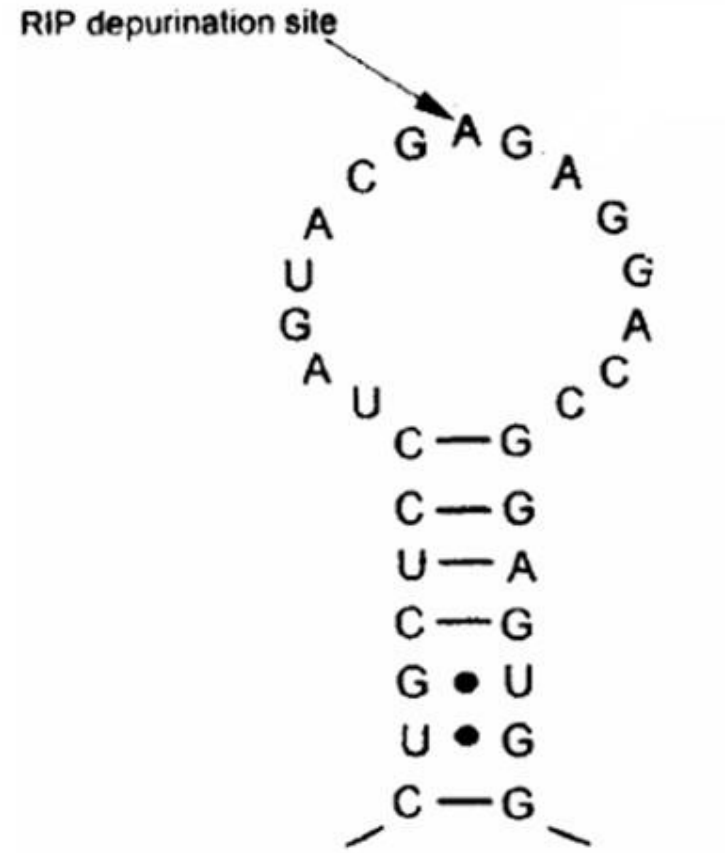


**Fig 1. The open conformation showing α-sarcin loop and depurination site of N-glycosidase ribosome-inactivating proteins (RIP)**

which normally has activity against both prokaryotic and eukaryotic ribosomes, and ricin, which normally has activity only against eukaryotic ribosomes, only showed activity against eukaryotic ribosomes *in vitro* (Chaddock, *et al* , 1996). When interpreted in terms of tertiary structure, the swapped domains included surface loop structures between ricin and PAP. Differences in structure between ricin and Mirabillis antiviral protein (MAP30) also occur mainly at loops of the two proteins (Wang *et al*., 2000). Much is still unknown about how the interaction of RIPs and their target ribosomes affect specificity. These interactions are quite complex with small variations in both the structure of the RIP and the structure of ribosomal proteins conferring specificity. However, the specificity of RIPs towards certain ribosomes appears to involve specific positive or negative interactions between the surface of the RIP and the surface of the ribosome and not large structural differences between the RIPs.

## 2.2 STRUCTURE OF RIPs

The three-dimensional (3D) structure for several type 1 and 2 RIPs has been determined by X-ray diffraction (Rutenber *et al*., 1991; Monzingo *et al*., 1993; Husain *et al*., 1994; Wang *et al*., 1999; Savino *et al*., 2000). Comparison of the structures of these RIPs has shown certain features that seem to be consistent between these proteins. For example, a prominent cleft that contains the active site has been observed in all RIPs crystallized to date (Wang *et al*., 1999). Ricin was the first RIP whose three-dimensional structure was solved by X-ray crystallography (Montfort *et al.,* 1987) and refined to 2.5 $A^0$ resolution (Rutenber *et al.,* 1991). The family of RIPs shares only about eight invariant amino acids, and most of this cluster in the active site. These include Tyr 80, Tyr 123, Glu 177, Arg 180, and Trp 211. Among these, aromatic Tyr80, Tyr123, and Trp211 are thought to contribute to substrate binding (Montfort *et al*., 1987 Rutenber *et al*., 1991; Watanabe *et al*., 1992) by intercalating their aromatic rings between the bases of the ribosomal RNA. The ricin A-chain (RA) has been characterized for correlations between structure and function. The mutational analysis identified 32 of the 267 amino acids present in RA that were required for the ribosome-inactivating activity (Munishkin *et al.,* 1995). These 32 amino acids are congregated in or near either the putative active site cleft or the hydrophobic core behind it. The largest concentration

of these amino acids is in Helix E and the adjacent unstructured region (Morris *et al* 1994). Helix E (residues 161-180) forms one side of the active site cleft and is the longest helix in RA. Helix E has a distinct bend (residues 177-180) near its carboxyl-terminal end that disrupts the local hydrogen-bonding pattern and is thought to produce the active site cavity (Munishkin and Wool, 1995).

Saporin, a type 1 RIP, belongs to a multigene family that encodes several isoforms. The saporin seed isoform 6 has significantly higher N-glycosidase and cytotoxic activities as compared to the seed isoform 5. Although the two have identical active sites, the amino acid changes outside the active sites of these isoforms have been implicated in the catalytic activity (Paroma *et al*., 2006).

## 2.3 CLASSIFICATION OF RIPS

RIPs are defined by their N-glycosidase ribosome-inactivating activity and not by sequence homology. Three classes of RIPs have been identified based on their physical properties (Mundy *et al*., 1994; Nielsen and Boston, 2001). a) Type 1 RIPs; these are monomeric enzymes with an Mw of about 30,000 and a basic isoelectric point that can contain a signal peptide and/or C-terminal extensions. The majority of RIPs characterized to date are type 1 RIPs (Barbieri *et al*., 1993). b) Type 2 RIPs; these are heterodimeric proteins with ribosome-inactivating activity and lectin properties on separate polypeptides. A disulfide bond links the subunits. c) Type 3 RIPs; these are produced as inactive single polypeptide precursors that must be proteolytically processed to produce the active protein (Mundy *et al*., 1994). Type 3 RIPs are less prevalent than type 1 or type 2 RIPs. To date, type 3 RIPs have been characterized only in cereals (Bass *et al*., 1992; Reinbothe *et al*., 1994a; Reinbothe *et al*., 1994b; Walsh *et al*., 1991). One of the best-characterized type 3 RIPs is a kernel specific protein from maize, designated proRIP1 (Walsh *et al*., 1991; Bass *et al*., 1992). ProRIP1 is an acidic protein that is cleaved during kernel germination to release 26 internal amino acid residue as well as residues from both the amino- and carboxy-termini (Bass *et al*., 1992; Walsh *et al*., 1991). This proteolysis converts the proRIP1 from a single peptide of 32 KDa to two tightly associated subunits with basic isoelectric points and of 16.5 KDa and 8.5 KDa (Walsh *et al*., 1991). The two subunits interact to form the enzymatic site for the complex. The proteolytically activated form, RIP1, has a ribosome-inactivating activity *in vitro* 1000-fold higher than that of proRIP1 (Walsh *et al*., 1991).

## 2.4 ENTRY OF RIPs INTO CELL

RIPs are localized extracellularily as well as in compartments that vary among different host cells. Most RIPs appear to have N-terminal signal sequences that target them for cotranslational entry into the endomembrane system (Hartley and Lord, 1993). The endomembranes then provide a barrier between the RIP and the orthologous ribosomes. RIPs, however, can be transported bidirectionally – both forward and reverse through the endomembrane system. As a result, additional protective steps are necessary to prevent subsequent retrograde transport of newly synthesized RIPs back to the ribosome-containing cytosol. One strategy is, to synthesize them as inactive pro-proteins. *Phytolacca insularis* antiviral protein (PIP2), saporin, sechiumin, trichoanguin, and tricosanthin are all type 1 RIPs with short carboxy terminal extensions that are encoded by the corresponding genes but not found on proteins isolated from plants (Benatti *et al,* 1991; Carzaniga *et al*., 1994; Chow *et al*., 1999; Song *et al*., 2000; Wu *et al*., 1998). The C-terminal extensions are similar in sequence to those found on vacuolar proteins and may act as vacuolar targeting signals (Neuhaus and Rogers, 1998; Vitale and Raikhel, 1999). Recombinant tricosanthin and sechiumin that contain the C-terminal amino acid peptides have fivefold less enzymatic activity than the corresponding native proteins (Wu *et al*., 1998; Zhu *et al.,* 1992). Thus, in addition to targeting, extensions may prevent these RIPs from becoming active prior to reaching the storage organelles. The importance of sequential processing and localization of RIPs away from ribosomes was recently demonstrated by studies to express various ricin constructs in transgenic tobacco protoplasts and determine their cellular fates (Frigerio *et al*., 1998). Ricin, a type 2 RIP, undergoes a complex maturation process. It is produced in castor bean as a precursor polypeptide (preproricin) that contains a signal peptide as well as the A- and B-chains joined by a short linker peptide (Butterworth and Lord, 1983; Lamb *et al*., 1985). Proricin is generated by cleavage of the N-terminal signal peptide from preproricin and transported from its site of synthesis at the endoplasmic reticulam (ER) to vacuoles via the

Golgi complex (Lord, 1985). Only after proricin has reached the vacuole is the linker peptide cleaved to produce the mature active protein. When the ricin A-chain was expressed alone in transgenic tobacco protoplasts, it could be detected in ER, but its continued expression was toxic (Frigerio *et al*., 1998). This toxicity indicates that the active A-chain can most likely be transported from ER into the cytosol where it would interact with ribosomes. When the native preproricin construct was assayed, processing, glycosylation, and targeting to the vacuole occurred normally and no toxicity was seen (Sehnke and Ferl, 1999). Thus, it appears that to prevent toxicity to the host cell, ricin, and most likely a number of other type 2 RIPs, must be produced as preproteins and targeted to the vacuole prior to activation by cleavage of the peptide linker between the A- and B-chains. Besides those RIPs localized in vacuoles, there are others such as PAP that are secreted to a final destination in the cell wall matrix (Carzaniga *et al*., 1994; Ready *et al*., 1986). Like RIPs localized in vacuoles, these proteins would be physically separated from orthologous ribosomes. Furthermore, the host cell would not be a barrier for activity against target cells.

## 2.5 ANTIVIRAL ACTIVITY OF RIPs

It has been known since the 1920s that crude extracts of pokeweed leaves inhibit plant virus infection when the extracts and virus were mixed and rubbed onto the surface of a test plant (Duggar *et al.,* 1925). Later, the active protein component was isolated and named as pokeweed antiviral protein. Subsequently, purified RIPs from many sources have been shown to potently inhibit the infection of test plants by diverse plant viruses. All RIPs with proven antiviral activity toward plant viruses are type 1 RIPs except the type 2 RIP from *Eranthis thyemalis* (Barbieri *et al.,* 1993; Hartley *et al*., 1996; Kumar *et al.,* 1993). Three possible explanations were put forward for the antiviral activity. First, RIPs may act directly on the viral nucleic acids through their rRNA N-glycosylase activity (Wang *et al.,* 1999). Second, RIPs may act directly on the host, selectively killing the infected cells, thus preventing the virus from replicating and spreading to neighboring cells and finally, RIPs may act indirectly through inactivation of the plant's defense system (Zoubenko *et al*., 1997 and Peumans *et al*., 2001).

Several lines of evidence however, suggest that antiviral activity can be separated from depurination at the $\alpha$-sarcin loop (Aron *et al.,* 1980 and Zhang *et al.,* 2000). Experiments with transgenic plants expressing PAP and PAP mutants were able to dissect to some extent the mechanism of the *in planta* antiviral activity of RIPs (Nilgun *et al.,* 1997; Wang *et al.,* 2000). The most definitive demonstration was made by Nilgun *et al.* (1997) who assayed a deletion mutant of a PAP gene in transgenic tobacco. Even though the mutant protein was unable to depurinate the $\alpha$-sarcin loop, it still conferred viral resistance against *Potato virus X,* by depurinating tobacco ribosomes.

There has been a report of novel activity of PAP to selectively depurinate RNAs that had a 5' terminal m7GpppG cap (Hudak *et al.,* 2000) which could explain the antiviral activity of PAP against the Sindbis-like viruses, *Tobacco mosaic virus* (TMV), *Brome mosaic virus* (BMV), and *Cucumber mosaic virus (*CMV), all of which have 5' terminal caps (Goldbach *et al*., 1986; Strauss *et al.,* 1988)

A PAP mutant with two amino acid substitutions, L20R and Y49H, showing antiviral activity and toxicity caused a constitutive expression of the acidic form of PR-1 (class II pathogenesis related protein PR-1). This acidic PR-1 is normally induced in tobacco by salicylic acid (SA). However, upon expression of PAP the synthesis of acidic PR-1, acidic PR-2 and isoform of basic PR-3 occurred independently of SA accumulation (Zoubenko, *et al,* 2000). Reciprocal grafting experiments concluded that PAP generates, through its enzymatic activity, a soluble signal that can be translocated to the upper and lower parts of the grafted plants and induces resistance to virus infection by a mechanism that is not linked to accumulation of salicylic acid or induction of PR 1 (Smirnov *et al.,* 1997).

## 2.6 ANTIFUNGAL ACTIVITY OF RIPs

RIPs have intrinsic antifungal activity due to their ability to inactive fungal ribosomes *in vitro* and, presumably, *in situ*. A 30-kDa ribosome-inactivating protein was purified from barley *(Hordeum vulgare* L.) seeds which synergistically inhibited the growth of fungi measured in a microtiter well assay. Northern hybridization analysis showed accumulation of this protein in starchy endosperm during late seed development (Leah *et al,* 1991). Two novel

type I RIPs have been isolated from the storage root of the Andean root crop species *Mirabilis expansa.* These proteins, named ME1 and ME2, were active against root-rot fungi (Vivanco, *et al.,* 1999). An abundant maize kernel ribosome-inactivating protein 1 (RIP1) decreased hypal proliferation of *Aspergillus nidulans* and *Aspergillus flavus,* thus protecting seed from fungal invasion (Nielsen *et al.,* 2001). Both type 1 and type 2 RIPs show broad activity against a number of plant and human pathogenic fungi (Oda *et al.,* 1997; Rezzonico *et al.,* 1998). Recent studies with a type 2 RIP showed that the cell-binding B chain (lectin) binds to fungal cells, forming a channel allowing the *N*- glycosidase A-chain entry into cells, resulting in RNA damage (Xia *et al.,* 2000; Zhang *et al.,* 1999). Precisely how type I RIPs, which do not have a cell-binding chain inhibit fungi, i.e., how are they internalized, is not known.

## 2.7 INSECTICIDAL ACTIVITY OF RIPs

Insect bioassays with ricin and saporin showed extreme toxicity to larvae of two *Coleopteran* species but had no detrimental effect on *Lepidoptera* (Gatehouse *et al.,* 1990). However Cinnamomin, a type 2 RIP from seeds of the camphor tree *Cinnamomum camphora*, was toxic to larvae of mosquito *Culex pipines pallens* and cotton bollworm *Helicoverpa armigera* (Zhou *et al.,* 2000). In both studies, the capacity of insect gut homogenates to hydrolyze the RIP was associated with a decrease in effectiveness of the RIP against insects. Thus, the apparent resistance seen in these studies may simply indicate that the RIP was degraded before it could be internalized into target cells.

Another demonstration of insect susceptibility came from two type 2 RIPs of *Eranthis hyemalis* that were highly toxic (90%–100% mortality at 1.0 mg/ml) to the southern corn rootworm *Diabrotica undecimpunctata howardii* in feeding trials (Kumar *et al.,* 1993). Toxicity to insects has also been shown for maize proRIP and active RIP, which deterred feeding of adult beetles of *Carpophilus freemani*, *C. lugubris*, *Stelidota germinata*, and *Sitophilus zeamais* in assays in which the insects were given a choice of diets. In contrast, only active RIP was toxic to caterpillars in no-choice assays (Dowd *et al.,* 1998). Cabbage loopers (*Trichoplusia ni*), which do not feed on maize, were most severely affected, whereas Indian meal moths (*Ploidia interpunctella*), which commonly feed on stored maize grain, showed no significant difference from controls. Fall armyworms (*Spodoptera frugiperda*, preferential leaf feeders), corn earworms (*Helicoverpa zea*), and corn borers (*Ostrinia nubilalis*; preferential immature seed feeders) had intermediate levels of susceptibility. Thus, differences in susceptibility of caterpillar species to active RIP varied in apparent accordance to host adaptation (or lack thereof), but factors contributing to the differential susceptibility or resistance of these insects have not been identified. Cinnamomin and ricin exhibited a different toxicity to domestic silkworm (*Bombyx mori*) larvae by oral feeding bioassay. The $LC_{50}$ of ricin to the silkworm larvae at third instar was much lower than that of cinnamomin. The purified A-chains of both cinnamomin and ricin showed a slightly different RNA N-glycosidase activity to the domestic silkworm pupal ribosome. It was proposed that the difference of their toxicity to domestic silkworm larvae was not related to their A-chains but to the properties of their B-chains (Guo-qing Wei *et al.,* 2004).

## 2.8 CLONING AND EXPREESION OF RIPs

Ricin was the first of plant RIPs to be cloned (Roberts *et al.,* 1985); both cDNA and genomic clones of preprocin were isolated. For expression in heterologous system, DNA encoding individual A or B chains rather than full preproricin precursor was utilized (O'Hare *et al.,* 1987), cDNA of Mirabilis antiviral protein (MAP) was cloned by constructing a cDNA library using λZAP cloning vector and probing with a synthethic MAP gene, the cDNA consisted of 1066 nucleotides and encoded 278 amino acids. The MAP cDNA was expressed in *E.coli* strain JM109 (DE3) based on T7 expression system and the product encoded by the cDNA was confirmed to be MAP precursor by western blot analysis followed by immunological analysis (Jiro *et al.,* 1991). Pokeweed (*Phytolacco americana)* produces three forms of antiviral proteins (PAPs) *viz*., PAP, PAP-S and PAP II, a complete cDNA encoding Pokeweed antiviral protein II (PAP II) was cloned and expressed in *E.coli*. The PAP II cDNA is composed of 1187 nucleotides and encoding a mature protein of 285 amino acids, the predicted amino acid sequence was having 33% similarity to PAP and PAP-S. The PAP II which was expressed in *E.coli*, inhibited protein synthesis in a rabbit reticulocyte translation system (Jean *et al.,* 1994).

The cDNA of Sechiumin has been cloned by RACE approach and expressed using a pET expression system in *E.coli.* The Sechiumin cDNA is composed of 951 nucleotides, encoding a protein with 285 amino acids that has nearly 60% similarity to several type-I RIPs purified from the Cucurbitaceae family (Tsann-hueiwu *et al.,* 1998). cDNA clones were isolated from a leaf cDNA library of *Chenopodium album* using a DNA probe derived from the N-terminal amino acid sequences giving two full length cDNA clones CAP30 and CAP30B both containing a putative signal peptide of 25 amino acids and a conserved domain commonly found in RIPs, transformed *E.coli* cells expressing pre or mature CAP had greatly reduced growth rates (Joug-sug *et al.,* 2003). A small cDNA fragment containing a Ribosome-inactivating site was isolated from a leaf cDNA population of *Clesoia cristata* by polymerase chain reaction (PCR) using a degenerate primer designed from the partially conserved peptide of Ribosome-inactivating/ antiviral proteins, the 150 bp cDNA fragment was sequenced and expressed in *E.coli* giving a 57 Kda fused protein and was confirmed by western blot analysis. The recombinant protein was purified and showed antiviral activity towards tobacco mosaic virus on host plant leaves, *Nicotiana glutinosa* (Gholizadeh *et al.,* 2005).

## 2.9 TRANSGENICS

Genes encoding RIPs have been analysed for their expression in plants. For the evaluation of the maize RIP b-32 gene ( *pZmcrip3a*), as an anti-fungal agent in transgenic rice, two constructs pARP7 and pBY605RR were made based on plasmid pBY505 (B. Wang and R. Wu, unpublished), which contains the *bar* gene expression cassette. R2 plants were tested for response to fungal pathogens of rice, *Rhizoctonia solani* and *Magnaporthe grisea*. Disease severity caused by infection with the fungal pathogens was not significantly reduced in the transgenic plants expressing the b-32 gene as compared to control plants, suggesting that processing of b-32 protein may be required to have antifungal activity *in planta*. (Ju-Kon *et al.,* 1999). The antiviral activity of the type-2 ribosome-inactivating protein (RIP) IRAb from Iris was analyzed by expressing IRAb in tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants and challenging the transgenic plants with tobacco mosaic virus (TMV). Transgenic tobacco lines expressing IRAb showed enhanced resistance against TMV infection but the level of protection was markedly lower than in plants expressing IRIP, the type- 1 RIP from Iris that closely resembles the A-chain of IRAb. Expression of the type-1 and type-2 RIPs from Iris confers tobacco plants local protection against two unrelated viruses. The antiviral activity of both RIPs was not accompanied by an induction of pathogenesis-related proteins, so observed antiviral activity of both Iris RIPs relies on their RNA N-glycohydrolase (Vandenbussche *et al*., 2004)

Transgenic tobacco and potato plants that expressed either pokeweed antiviral protein (PAP) PAP or a double mutant derivative of PAP showed resistance to infection by different viruses. Resistance was effective against both mechanical and aphid transmission. Analysis of resistance in transgenic plants suggests that PAP confers viral resistance by inhibiting an early event in infection. Expression of PAP in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene (Lodge *et al.,* 1993).

## 2.10 RIPS IN MEDICINE

Due to the selective toxicity of RIPs, the primary focus of research has been to use RIPs as the toxic agent in immunotherapies (Olsnes and Pihl, 1982). The target specificity of monoclonal antibodies induced the development of immunotoxins, which deliver toxins, such as RIPs, to specific cells. PAP linked to specific antibodies has been shown to prevent the growth of leukemia cells (Ramakrishnan *et al.,* 1984).

Another relatively successful immunotoxin is TP3-PAP. This antibody (TP3) is reactive against an antigen on human and canine osteosarcoma. This tumor-associated antigen is expressed at very high levels on the surface membrane of human osteosarcoma cells (Bruland *et al.,* 1988). Furthermore, RIP gelanin was also shown to be effective on malarial parasites when linked to human transferin (Surolia *et al*., 1996). Curcin had a powerful inhibitory action upon protein synthesis in reticulocyte lysate with an $IC_{50}$ (95 % confidence limits) value of 0.19 (0.11-0.27) nmol/L. The $IC_{50}$ (95 % confidence limits) of curcin on SGC-7901 (gastric cancer cell line), Sp2/0 (mouse myeloma cell line), and human

hepatoma was 0.23 (0.15-0.32) mg/L, 0.66 (0.35-0.97) mg/L, 3.16 (2.74-3.58) mg/L, respectively. Curcin was not found to be toxic to HeLa cells and normal cells (human embryo lung diploid cell line) (Lin *et al*., 2003). These studies indicate that RIPs could be very effective drugs when linked to proper targeting antibodies, and could thus be used as antitumor drugs.

# III. MATERIALS AND METHODS

The present study was undertaken to clone and express, RIP genes from *Bougainvillea spectabilis and Boerhaavia diffusa* belonging to family Nyctaginaceae. Matured leaves from both the plants were used in this study.

## 3.1 RNA ISOLATION FROM LEAVES

The total RNA was extracted from leaves of *Bougainvillea spectabilis* and *Boerhaavia diffusa* using Eppendorf Perfect RNA$^{TM}$, Eukaryotic Mini RNA Isolation kit. Diethylpyrocarbonate (DEPC) 0.1%, beta merceptoethanol (14.3M), Absolute ethanol (96-100%) was from Himedia. All the solutions and glassware were treated with 0.1% DEPC water and used after sterilization.

RNA isolation and RT-PCR reaction was done as per the manufacturer's instructions. One gram of the samples was ground in liquid nitrogen and approximately 100 mg ground material was taken in a tube containing 350µl lysis solution and homogenized. It was transferred to a 1.5 ml microcentifuge tube and centrifuged at 13000rpm for 1 minute. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube, to which 350 µl 70 % ethanol was added and mixed by gentle inversions. Later, 200 µl of Perfect Binding Matrix Solution was added and mixed gently. The lysate/ Binding Matrix mixture was pipetted out into a Perfect RNA Binding Matrix Spin Column and centrifuged at 13000rpm for 30 seconds. Two successive washes with 700µl wash solution 1 and 500 µl wash solution 2 were given by centrifugation for 30 seconds at 13000 rpm, before eluting RNA from the column with RNase free water. About 50 µl of RNA preparation was collected and stored in -20°C.

## 3.2 REVERSE TRANSCRIPTION

cMaster RTplus PCR System and cMaster RT Kit (Eppendorf) were used for cDNA synthesis. 4µl of total RNA was taken in a sterile 0.5-ml microcentrifuge tube, 3µl RNase-free water, 1µl Oligo (dT) primer (10mM) and 2µl dNTPs (10mM) each were added to it. The contents in the tube were spun briefly and incubated at 65°C in a thermal cycler for 5 min. it was later cooled on ice for 2 minutes and 5µl RNase-free water, 4µl RTplusPCR Buffer, 1µl cMaster RT Enzyme were added, gently vortexed, briefly centrifuged and incubated at 42°C for 1.5 hours in a thermal cycler. The reaction was stopped by heating the sample at 85°C for 5 minutes. The cDNA obtained was stored at -20°C.

### 3.2.1 Amplification of full length bouganin cDNA (*bgn*)

Primers Specific for the full length *bgn*

The cDNA specific primers for full-length *Bougainvillea* antiviral peptide of *Bougainvillea spectabilis* were designed and synthesized based on the sequences available in the database using the GENETOOL software. The primer sequences were;

Primers for Bouganin gene with *Xba*I and *Bam*HI sites

| | |
|---|---|
| BSXF | 5' GCTCTAGACATGGGTTGGTGGGCTATCA 3' |
| BSBAMR | 5' CGGATCCAGGCGATGAAAGGTGATTAGGC 3' |

### 3.2.2 Standardization of primer concentration

Primer concentrations *viz.,* 5 pM, 10 pM, 20 pM, 30 pM, 40 pM, 50 pM were used to optimize amplification. Based on the results, 10 pM of *bgn* primers, which gave single intense amplicon, were used for large scale amplification.

### 3.2.3 Reaction mix

The master mix required for all samples were prepared afresh to avoid handling errors. The master mix (20 µl) (Appendix I) was distributed to the tubes and 5µl of template DNA added to make the total reaction volume to 25 µl.

### 3.2.4 PCR amplification

PCR amplification was done with the following PCR conditions;

A) Condition for PCR amplification of Bouganin

| Stage | Step | Temperature (℃) | Duration (min) | No. of cycles |
|---|---|---|---|---|
| I | Initial denaturation | 94 | 5 | 1 |
| II | Denaturation | 94 | 1 | 39 |
| | Annealing | 51 | 1 | |
| | Extension | 72 | 1 | |
| III | Final extension | 72 | 20 | 1 |
| | Hold | 4 | - | - |

## 3.3 SEPARATION OF AMPLIFIED PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

About 10 µl of amplified products from each tube along with 2 µl of loading dye (Appendix IIA) were separated on 1 percent agarose gel (Appendix IIB) using 1X TAE buffer prepared from 50X TAE (Appendix IIC) along with *HindIII / EcoRI* double digest as DNA molecular weight marker. The image of the gel was documented using gel documentation system (UVitec, Cambridge, England).

## 3.4 CLONING OF BOUGAINVILLEA ANTIVIRAL PEPTIDE GENES

PCR based cloning was attempted starting from elution of PCR fragment from agarose gel to the confirmation of clones.

### 3.4.1 Gel elution of PCR fragment

The intense band of ~938bp PCR fragment obtained using the specific primers were excised from low melting agarose gel with a sharp sterile scalpel blade under low intensity (70%) UV transilluminator. The agarose gel piece containg the fragment was collected in a sterile pre-weighed micro-centrifuge tube. The DNA from the excised gel was eluted using Qiagen gel extraction kit following the method described in the user's manual. The purified PCR product was quantified by ethidium bromide spotting method as described by Sambrook and Russel (2001).

### 3.4.2 Cloning of pcr product

Purified PCR products of 938bp (50ng/µl) were ligated to pTZ57R/T vector (2886bp) as described in InsT/A clone™ PCR product cloning kit (#k1214) from MBI Fermentas, USA. For ligation, optimal molar ratio of ends of 1:3 of vector: insert was calculated as per the table given in (Appendix III). The components of ligation mixture was mixed into a 0.5 ml microcentrifuge tube and incubated at 16℃ overnight in a thermal cycler. A control ligation reaction was performed using control PCR fragment provided in the kit by adding components described in (Appendix IIIC). *E.coli* DH5α was transformed with this recombinant.

## 3.5 TRANSFORMATION

### 3.5.1 Preparation of competent cells

The competent cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russell (2001) with minor modification as described below.

An isolated colony from *E. coli* DH5 α plate was inoculated in 5 ml Luria broth and incubated at 37°C overnight at 200 rpm. The next day, the culture was diluted to 1:100 using Luria broth *i.e.*, 0.5 ml of culture was added to 50 ml of Luria broth and incubated for 2 to 3 hours till it attained 0.3-0.4 at $OD_{600}$. The culture was chilled in ice for 30 min and 25 ml of culture was dispensed into two 50 ml centrifuge tubes. The cells were pelleted at 6000 rpm for 5 min. The supernatant was discarded and pellet was resuspended in 12.5 ml of ice-cold 0.1 M $CaCl_2$. The centrifuge tubes were again kept in ice for 45 min and later centrifuged at 4000 rpm for 10 min. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$ and to this 88 µl of dimethyl sulfoxide (DMSO), a cryoprotectant was added.

### 3.5.2 Transformation of *E. coli* DH5 α

About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligated mixture was added into the tube and mixed gently. The mixture was chilled in ice for 45 min. Later, heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2 min. Immediately, it was transferred onto ice and chilled for 5 minutes. To this 800 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 minutes to allow bacteria to recover and express the antibiotic marker encoded by plasmid. The culture was centrifuged at 13,000 rpm for 1 min and about 700 µl of supernatant was discarded and the pellet was resuspended in remaining supernatant and spread on the plates having Luria agar (Appendix IID) with $Amp_{100}$, X-gal, IPTG and incubated overnight at 37°C.

The recombinant clones were identified by blue/white colony assay. After overnight incubation, only white colonies, having recombinant vectors were picked up and streaked on plates having Luria agar with $Amp_{100}$, X-gal, IPTG and incubated at 37°C overnight for further multiplication.

### 3.5.3 Confirmation of recombinant clones

The confirmation for the presence of desired DNA fragment in cloning vector was done by specific PCR and by restriction analysis as follows. For PCR confirmation of clones the template DNA from plasmids was isolated following the alkaline lysis protocol of Brimbion and Dolly (1979).

White colonies were inoculated to 10 ml Luria broth with ampicillin (100µg/ml) overnight at 37°C over shaker at 175 rpm. Overnight grown culture was centrifuged at 5000 rpm for 2 min at 4°C in 2.0 ml microcentrifuge tubes. The supernatant was removed and pellet was washed with STET (Appendix IV) (0.25 volume of original culture). It was centrifuged at 5000 rpm for 2 min. The pellet was resuspended in 200 µl of ice-cold alkaline lysis solution I (Appendix IV). by vigorous vortexing. Later, 400 µl of freshly prepared alkaline lysis solution II (Appendix IV) was added to each tube and the contents were mixed by inverting the tubes 4 to 5 times and kept in ice for about 5 min. To this suspension, 300 µl of alkaline lysis solution III (Appendix IV) was added and again mixed thoroughly by gently inverting the tubes 4-5 times. The tubes were stored on ice for 5 minutes and centrifuged at 13,000 for 8 min. The supernatant was transferred to fresh tubes and equal volume of phenol: chloroform isoamyl alcohol (25:24:1) was added to precipitate proteins and was mixed well. It was centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to a fresh tube and two volumes of isopropanol was added. The contents were mixed and allowed to stand for 2 minutes at room temperature. The solution was later centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and pellet was washed with 70 per cent ethanol and spun for 1 min at 13,000 rpm to recover the plasmid. The supernatant was discarded, pellet was dried completely and dispensed into 25 µl of $T_{10}E_1$ (pH=8.0) containing 3 µl of RNase A (10 mg/ml). The solution was kept at 50°C for 15 min and then stored at -20°C. The plasmid DNA was visualized on 0.8 per cent agarose gel as described, using *HindIII/EcoRI* double digest, as DNA marker

The confirmation of the presence of cloned fragment in pSKK was done by PCR amplification of clones with primers. The total DNA and cloning vector were used as positive and negative controls in the PCR.

The confirmation was also done through comparative restriction analysis of selected clones and the control vector to detect the presence of the desired insert with *BamHI* and *XbaI* for *bgn* gene.

### 3.5.4 Sequencing of clones

The full length *bgn* amplicon cloned in pTZ57R/T was sequenced using M13 primers, at Bangalore Genei Private Ltd., Bangalore. The sequences were subjected to analysis using BLAST algorithm available at http://www.ncbi.nim.nih.gov.

## 3.6 SUB CLONING OF BOUGANIN cDNA INTO A PROKARYOTIC EXPRESSION VECTOR

### 3.6.1 Vector and clone isolation

The *bgn* gene was subcloned into pET28a (+), prokaryotic expression vector. The alkaline lysis protocol of Brimbiom and Doly (1979 and 1983) with certain modifications was used for isolation of the plasmids as described earlier (3.5.3).

### 3.6.2 Elution of linearized vector pET28a (+)

Single digestion of pET28a (+) was done with restriction enzyme–*Bam*HI. The linearized vector was eluted. The eluted sample was extracted using gel extraction kit (Eppendorf, Germany) and the purified as per the protocol given in the user's manual.

### 3.6.3 Elution of inserts and ligation

The inserts were obtained by digesting pSKK3680 clone with *Bam*HI. The inserts were eluted as described earlier. The purified vector DNA and inserts were quantified using spectrophotometer. The ligation reaction was carried out with an optimal molar ratio of 1:3 vector: insert in a 0.5 ml micro centrifuge tube and incubated at 16℃ for 16 hours. A control ligation reaction was performed using vector with cohesive ends, adding components as described in (Appendix -VI).

### 3.6.4 Competent cell preparation

Competent cells of *E. coli* BL21 (pLysS) were prepared following the protocol of Sambrook and Russel (2001) with minor modifications as described earlier (3.5.1).

### 3.6.5 Transformation of *E. coli* BL21

Transformation of competent *E. coli* BL21 (pLysS) cells was transformed as described earlier (3.5.2).

### 3.6.6 Confirmation of clones

The confirmation of the clones was done by PCR amplification using *bgn* specific primers on recombinant plasmids. PCR amplification on total DNA and the control pET28a (+) vector were used as positive and negative controls respectively.

Further confirmation was done by the comparative restriction analysis of the recombinant plasmid of selected clones and control vector with *Bam*H1.

## 3.7 EXPRESSION OF *bgn* cDNA IN *E. coli*

The expression analysis was done using procedure outlined in Sambrook and Russell (2001).

### 3.7.1 Protein extraction

About 5 ml of Luria broth with kanamycin (50 µg/ml) was inoculated with 50 µl of overnight culture and incubated at 37℃ with shaking. One ml culture was taken and was

induced by adding 1mM IPTG. It was again incubated for 3 to 4 hours at 37°C over a shaker. After induction, the protein was extracted and analyzed by SDS-PAGE.

For extraction of proteins, the cell culture was centrifuged at 13,000 rpm for 1 min at room temperature. The pellet was resuspended in 100 µl of $T_{10}E_1$ and 100 µl of 2x SDS gel loading buffer added to it. The mixture was heated at 95°C for 10 minutes and centrifuged at 5000 rpm for 5 minutes at room temperature. About 60 µl of each such suspension was loaded on 10 per cent polyacrylamide gel. The protein extract from cells containing vector alone was used as control. The gels were stained by the procedure described in Sambrook and Russell (2001) and visualized under transilluminator (Appendix V).

## 3.8 CLONING FULL LENGTH cDNA OF *Boerhaavia diffusa* RIBOSOME INACTIVATING PROTEIN.

The total RNA was extracted from leaves of *Boerhaavia diffusa* using Eppendorf Perfect RNA™, Eukaryotic Mini RNA Isolation kit. Accordingly, one gram samples was ground in liquid nitrogen and then about 100 mg ground material was taken in a tube containing 350µl lysis solution and homogenized, and rest of the procedure followed was as described in section 3.1.

## 3.9 DEGENERATE PRIMER FOR RAPID AMPLICATION OF cDNA ENDS BY PCR (RACE-PCR)

*In silico* analysis of 12 RIPs was done before designing degenerate primer to identify the gene-encoding novel *RIP* gene from *Boerhaavia diffusa*. The protein and nucleotide sequences of different RIPs were downloaded from the NCBI database (http://www.ncbi.nim.nih.gov). The protein sequences were aligned using Bioedit software (www.mbio-ncsu.edu/bioedit/bioedit.html).

As per the requirements of the SMART RACE PCR kit (Clontech, TakaraBio Japan), 30bp long primer with an annealing temperature of 72°C was designed manually is given below.

Sequence of Degenerate primer for 3' RACE-PCR

| | | |
|---|---|---|
| 3'RACE-PCR | BDDEG | 5 'ATYCAATGRYKKCNKARGCAGCATT 3' |

### 3.9.1 Standardization of primer concentration

Primer concentration viz., 5.0 pM, 10 pM, 15 pM, 20 pM and 25 pM, were used to optimize amplification. Based on the results, 10 pM of primer was used further.

## 3.10 3'RACE-PCR

### 3.10.1 First strand cDNA synthesis for 3' RACE- ready cDNA

SMART RACE (Rapid Amplification of cDNA Ends) cDNA Amplification kit (Clontech, TakaraBio Japan) was used for cDNA synthesis. 4 µl of total RNA was taken in a sterile 0.5-ml microcentrifuge tube and 1 µl 3'-CDS primer (10µM) was added to it. The contents in the tube were spun briefly and incubated at 70°C in a thermal cycler for 2 min and cooled on ice for 2 minutes. Later it was briefly centrifuged and 2µl of 5X First-Strand Buffer, 1µl of dNTP Mix (10 mM each), 1 µl DTT (20mM) and 1 µl of PowerScript Reverse Transcriptase was added. The tubes were then gently vortexed, briefly centrifuged and incubated at 42°C for 1.5 hr in an incubator. The cDNA obtained was further diluted to 40µl using Tricine-EDTA buffer and incubated at 72°C for 7 minutes. Diluted sample was stored at -20°C.

3.10.1.1 Preparation of PCR master mix

The master mix was prepared by adding the following components in a sterile 0.5 ml tube.

| Reagent for 3' RACE-PCR | Volume |
|---|---|
| Sterile water | 69.0µl |
| 10X Advantage 2 PCR buffer | 10.0µl |
| dNTP mix (10mM) | 2.0µl |
| 3'RACE Ready cDNA | 5.0µl |
| 50X Advantage 2 polymerase mix | 2.0µl |
| Total volume | 88.0µl |

The components were mixed by brief centrifugation and 44µl of master mix was distributed to two 0.5 ml PCR tubes. 1 µl of BDDEG primer and 5 µl of universal primer mix (UPM) were added to both the tubes and thermal cycling was commenced immediately.

PCR amplification: Following Touch down PCR programme was used for amplification.

| Steps | Stage | Temp. ($^0$C) | Duration | No. of cycles in each stage |
|---|---|---|---|---|
| 1 | I | 94$^0$C | 30 sec | 5 |
| 2 | | 72$^0$C | 3.0 min | |
| 3 | II | 94$^0$C | 30 sec | 5 |
| 4 | | 70$^0$C | 30 sec | |
| 5 | | 72$^0$C | 3.0 min | |
| 6 | III | 94$^0$C | 30 sec | 35 |
| 7 | | 68$^0$C | 30 sec | |
| 8 | | 72$^0$C | 3.0 min | |
| 9 | IV | 72$^0$C | 30 min | 1 |

The products obtained after the PCR reaction were kept at 4$^0$C till they were used for further analysis.

3.10.1.2 Separation of amplified 3'RACE products, elution and quantification

The PCR products in 5 µl from each tube along with 2 µl of loading dye (Appendix IIA) were separated on 2% per cent agarose gel along with 100 bp DNA ladders as markers. The gels were observed under a mid range UV trans-illuminator and documented using a gel documentation system (UVtech, Cambridge, England). Remaining 95µl RACE-PCR product was electrophoresed and the major amplification fragment corresponding to 450bp region was excised from the 0.8 per cent low melting agarose gel and eluted and quantified as described earlier

## 3.10.2 Construction of recombinant vector

Eluted and purified PCR fragments of size 450bp were ligated to pTZ57R/T vector (pTZ57R/T is a 2,868bp cloning vector) as described in InsT/A clone$^{TM}$ PCR product cloning

kit (MBI Fermants) (Fig. 2). Protocols used for ligation, transformation of competent *E.coli* cells and confirmation of the recombinant vectors was as described earlier in section 3.4 and 3.5.

3.10.2.1 Sequencing of clones

The insert in p3' bd1 clone with 450bp amplicon cloned in pTZ57R/T was sequenced using M13 primers by employing primer walking technique at Bangalore Genei Private Ltd., Bangalore. After removal of vector sequences, the sequence homology search was done using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

## 3.10.3 5' RAPID amplification of cDNA ends (5'-RACE-PCR)

3.10.3.1 Construction of Gene Specific Antisense Primer

After deducing the 3' sequence information and confirming it to be the 3' region of *RIP* genes, by using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. A gene specific antisense primer was designed which is as follows

Sequence information of specific primer for 5'RACE-PCR

| | | |
|---|---|---|
| 5'RACE-PCR | SR2 | 5 'GCCGTGGTGGAGGTCTTGGATTGAT 3' |

3.10.3.2 First strand cDNA synthesis for 5'-RACE- ready cDNA

Similar to 3'-RACE ready cDNA synthesis, SMART RACE kit (Clontech, TakaraBio Japan) was used for 5'-RACE-ready cDNA synthesis. 3 µl of Poly A+ RNA was taken in a sterile 0.5-ml microcentrifuge tube, 1 µl 5'-CDS primer (10 µM) and 1 µl SMART II A oligo primer was added to it. The contents of the tube were treated as in 3.9.1

3.10.3.3 PCR amplification: Preparation of PCR master mix

As in case of 3.4.2 for 5'-RACE in place of 3'-RACE ready cDNA, 5'-RACE ready cDNA was added. The components were mixed by brief centrifugation and 44µl of master mix was distributed to two 0.5 ml PCR tubes. 1 µl of SR2 primer and 5 µl of universal primer mix (UPM) (Clontech, TakaraBio Japan) were added to both the tubes and the thermal cycling was commenced immediately.

PCR amplification: Following Touch down PCR programme was used for amplification in thermal cycler.

| Steps | Stage | Temp ($^0$C) | Duration | No. of cycles in each stage |
|---|---|---|---|---|
| 1 | I | 94$^0$C | 30sec | 5 |
| 2 | | 72$^0$C | 3.0 min | |
| 3 | II | 94$^0$C | 30sec | 5 |
| 4 | | 70$^0$C | 30sec | |
| 5 | | 72$^0$C | 2.0 min | |
| 6 | III | 94$^0$C | 30sec | 35 |
| 7 | | 68$^0$C | 30sec | |
| 8 | | 72$^0$C | 2.0 min | |
| 9 | IV | 72$^0$C | 30 min | 1 |

The product obtained after the PCR were kept at $4^0$C till they are used for further analysis. Separation of amplified products was done on 1.2% gel electrophoresis as described earlier.

3.10.3.4 Cloning of the 5'RACE-PCR product

The procedure for gel elution of expected 650bp 5' RACE-PCR product, quantification of the elute, cloning of PCR product into pTZ57R/T cloning vector, ligation reaction and transformation were same as that followed for 3' RACE-PCR.

Blue-white assay was employed for selection of recombinant clones and recombinant clones were confirmed by plasmid isolation, restriction analysis and by PCR using nested universal (NUP) and gene specific primer SR2. Few random clones which passed all the confirmation tests for a recombinant were picked and sent for sequencing.

3.10.3.5 Sequence analysis

Computer aided similarity searches of nucleotide sequences available in the public database were carried out using the BLAST algorithm (Altschul *et al.,* 1990, 1997). Multiple sequence alignments of nucleic acid sequences were carried out with CLUSTAL W software.

## 3.11 GENERATION OF FULL-LENGTH cDNA SEQUENCE

By comparing and aligning the sequences of 3' and 5' RACE products, the full-length cDNA sequence of *bdavp* gene homologue was deduced using the VECTOR NTI 10.0 SOFTWARE and then complete CDS was obtained through RT PCR reaction using new set of primers UPM and BDFR, Sequence of the primer is listed here.

| | |
|---|---|
| BDFR | 5'-TCAGTAACATAAGATGCACCATATAAC -3' |

PCR was carried out using the SMART RACE (rapid amplification of cDNA ends) cDNA Amplification kit (Clontech, TakaraBio Japan) in a total volume of 20µl following PCR conditions were used to amplify full-length CDS

| Steps | Stage | Temp ($^0$C) | Duration | No. of cycles in each stage |
|---|---|---|---|---|
| 1 | I | $94^0$C | 30 sec | 5 |
| 2 | | $72^0$C | 3.0 min | |
| 3 | II | $94^0$C | 30 sec | 5 |
| 4 | | $70^0$C | 30 sec | |
| 5 | | $72^0$C | 2.0 min | |
| 6 | III | $94^0$C | 30 sec | 35 |
| 7 | | $68^0$C | 30 sec | |
| 8 | | $72^0$C | 2.0 min | |
| 9 | IV | $72^0$C | 30 min | 1 |

The procedure for gel elution of PCR product, ligation with pTZ57R/T vector, transformation of DH5$\alpha$ and sequencing were described earlier.

### 3.11.1 In silico analysis of sequence

Complete sequence of the cDNA was subjected to homology search in the BLAST algoritm and restriction analysis was done in BTI software GENETOOL. Using the GENSCAN software *in silico* translation of cDNA was performed and general features of the protein (molecular weight and amino acid composition) were assessed using Prot. Param tool. Further, for the purpose of alignment, predicted protein sequence of Boerhaavin and known RIP proteins of other crops were aligned using CLUSTAL W (Thompson *et al*., 1994) with default option.

## 3.12 SUB CLONING OF Bdavp cDNA INTO A PROKARYOTIC EXPRESSION VECTOR

### 3.12.1 Vector and Clone isolation

The *bdavp* gene was subcloned into pET28a (+), prokaryotic expression vector. The alkaline lysis protocol of Brinbiom and Doly (1979 and 1983) with certain modifications was used for isolation of the plasmids as described earlier (3.5.3).

### 3.12.2 Elution of linearized vector pET28a (+)

Double digestion of pET28a (+) was done with restriction enzyme–*Sac*I and *Hind*III. The linearized vector was eluted. The eluted sample was extracted using gel extraction kit (Eppendorf, Germany) and purified as per the protocol given in the user's manual.

### 3.12.3 Elution of inserts and ligation

The inserts were obtained by digesting pbdavp clone with *Sac*I and *Hind*III. The inserts were eluted as described earlier. The purified vector DNA and inserts were quantified using spectrophotometer. The ligation reaction was carried out with an optimal molar ratio of 1:3 vector: insert in a 0.5 ml micro centrifuge tube and incubated at 16℃ for 16 hours. A control ligation reaction was performed using vector with cohesive ends, adding components as described in (Appendix VI).

### 3.12.4 Competent cell preparation

Competent cells of *E. coli* BL21 (pLysS) were prepared following the protocol of Sambrook and Russel (2001) with minor modifications as described earlier (3.5.1).

### 3.12.5 Transformation of *E. coli* BL21

Transformation of competent *E. coli* BL21 (pLysS) cells were transformed as described earlier (3.5.2).

### 3.12.6 Confirmation of clones

The confirmation of the clones was done by PCR amplification using T7 promoter primer and BDFR primers from recombinant plasmids. Further confirmation was done by the comparative restriction analysis of recombinant Plasmid of selected clones and control vector with *Sac*I and *Hind*III.

## 3.13 EXPRESSION OF Bdavp cDNA IN *E. Coli*

The expression analysis of *bdavp* was done using the procedure outlined in Sambrook and Russel (2001) with minor modifications as described earlier (3.7.1).

# IV. EXPERIMENTAL RESULTS

The present study was conducted to clone and express full length cDNA encoding Ribosome inactivating protein from *Bougainvillea spectabilis and Boerhaavia diffusa.* The results of the experiments conducted towards achieving the objectives are presented herein.

## 4.1 RNA ISOLATION

cDNA of *Bougainvillea spectabilis* synthesised from total RNA was used for further amplification.

## 4.2 AMPLIFICATION AND CLONING OF FULL LENGTH BOUGANIN GENE (*BGN*) FROM *Bougainvillea spectabilis*

Different concentrations of primers were used in the PCR amplification to find out optimum primer concentration. The primer concentrations used were 5, 10, 20, 30, 40 and 50 pM/µl. Though amplification of desired length was observed in all the cases, at 10pM/µl a unique band was observed without leaving primer dimers, which was used routinely for further PCR amplification. Keeping all the other components such as dNTPs, primers, Taq DNA polymerase and template DNA constant, the reaction volume of PCR mixture was adjusted with sterile water to 20 or 25 µl. A sharp unique band was observed with 20µl reaction volume and it was used routinely.

### 4.2.1 Cloning of *bgn* cDNA

#### 4.2.1.1 Isolation of the Amplicons

The large-scale amplification of full length gene encoding Bouganin (*bgn*)(938bp) from *Bougainvillea spectabilis* was achieved using Bouganin (*bgn*) specific primers. The products of PCR were separated in a low melting 1% agarose gel along with 100 bp DNA marker as a standard DNA marker. A very sharp amplicon of expected size (0.9kb) was obtained (Plate 1) and it was eluted from the preparative gel.

### 4.2.2 Ligation and confirmation of recombinants

The eluted fragments were ligated to pTZ57R/T cloning vector. The recombinant product pTZ57R/T : : 938bp *bgn* gene were transferred to *E.coli* DH5α.

The transformed white colonies were picked up streaked on LA+Amp+X-gal+IPTG for selection of clones. The recombinant cells were selected based on LA+Amp+X-gal+IPTG. In case of clones of *bsavp* gene, out of 20 transformants, 11 were white. All showed the presence of 938 bp insert, clones were confirmed through PCR and restriction analysis (Plate 2 and 3) and clones were named pSKK3680 (Fig.2).

### 4.2.3 Sequence Analysis

Using M13 primers one of *bgn* clones was sequenced. The complete sequence of nucleotide and amino acids of bgn are presented in (Fig. 4). The available sequence information from cloned fragments was analysed further using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. The nucleotide sequence of *bgn* showed 97 per cent homology to published *Bougainvillea spectabilis (RIP) gene* (DQ98945.1) (Table.1), The translated BLAST results also showed 97 per cent amino acid homology with it (Fig. 5). The conserved domains of the sequence analyzed using NCBI CDD search database (Fig. 6) showed conserved domain of the Ribosome inactivating proteins.

The sequences were subjected to further analysis in BTI software of GENETOOL for finding restriction sites and open reading frame (Fig. 7, 8). Bouganin sequence did not have any internal restriction sites for *Bam*H1*, Xba*I*, Not*I and *Hind*III.

## 4.3 SUB CLONING IN PROKARYOTIC EXPRESSION VECTOR

Following restriction, the gene in pSKK3680 (Bouganin) was cloned into *BamHI* sites of the expression vector pET28a (+) and introduced into *E. coli* BL 21 (plysS) cells. The transformants with *bsavp* were picked and streaked on Luria agar plates containing 50 µl/ml

**Plate 1: Large scale amplification of Bouganin (*bgn*) gene**

M : 100 bp DNA ladder

Lane 1-2 : Large scale amplification of Bouganin (*bgn*) gene

**Plate 2: PCR Confirmation of pSKK3680 clones**

M : λ Hind III/EcoRI double digest DNA marker

Lane 1-2 : pSKK clones

3 : Negative Control

4 : 1 kb DNA ladder

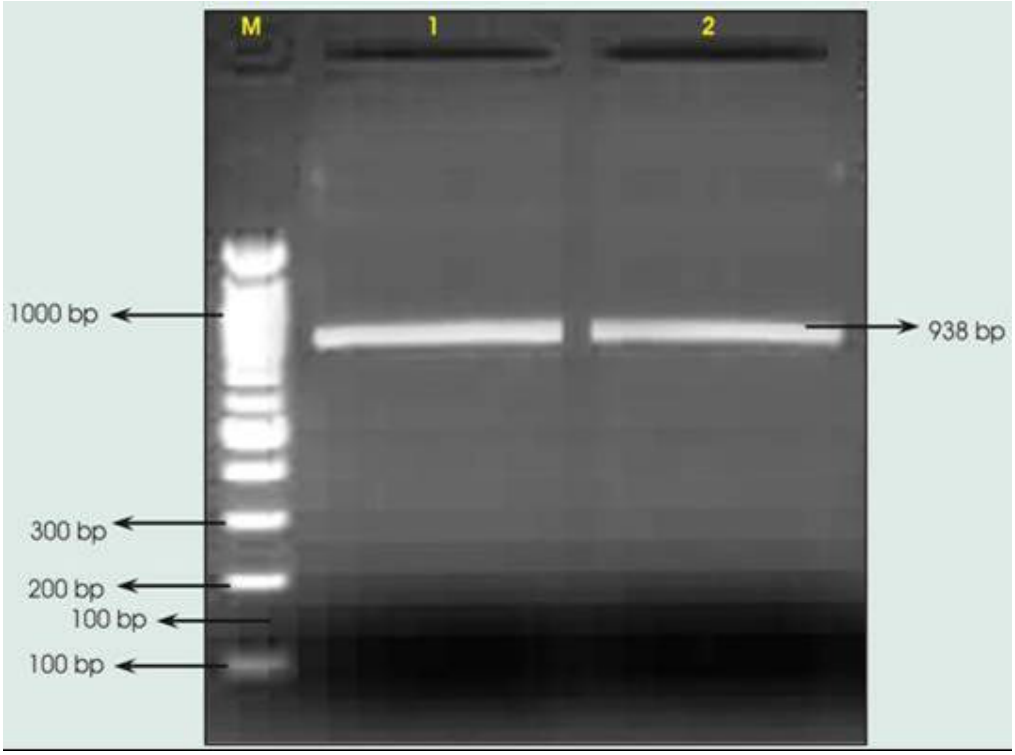


**Plate 1: Large scale amplification of Bouganin (*bgn*) gene**

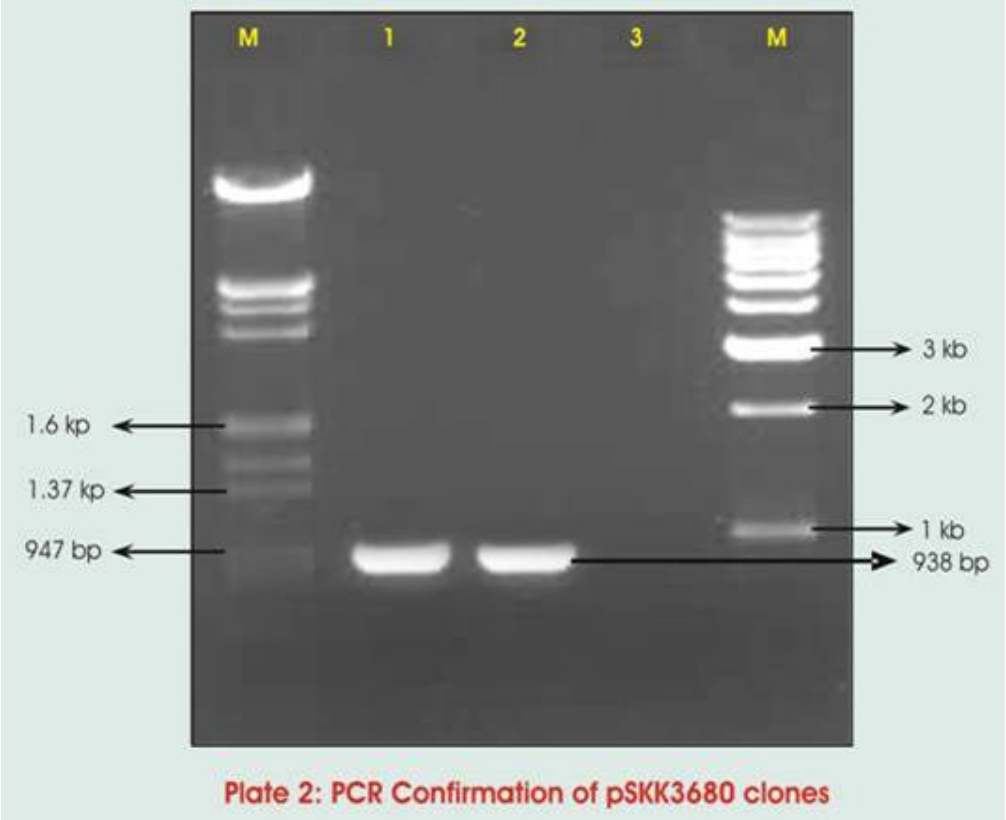


**Plate 2: PCR Confirmation of pSKK3680 clones**

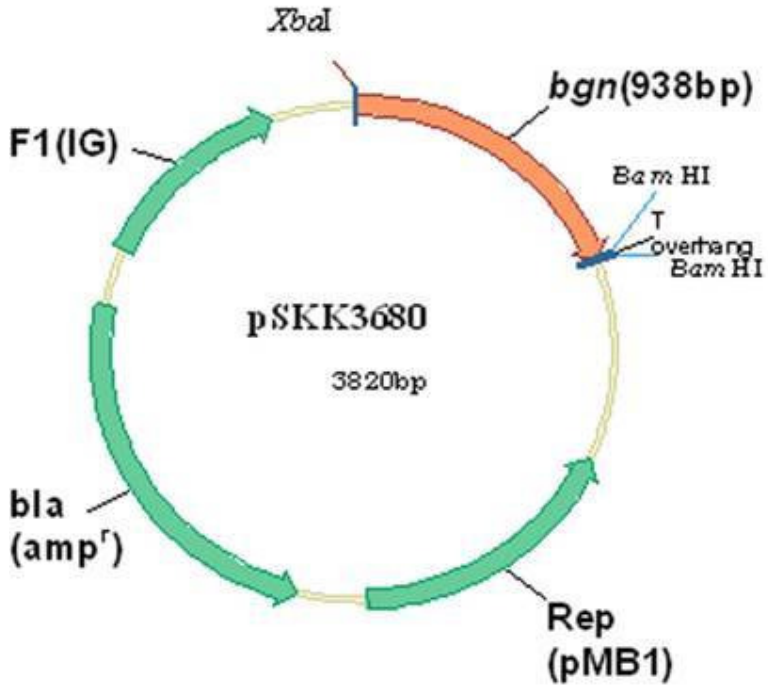


**Fig 2. Restriction map of Construct of pSKK3680 containing full length *bgn gene* in pTZ57R/T**

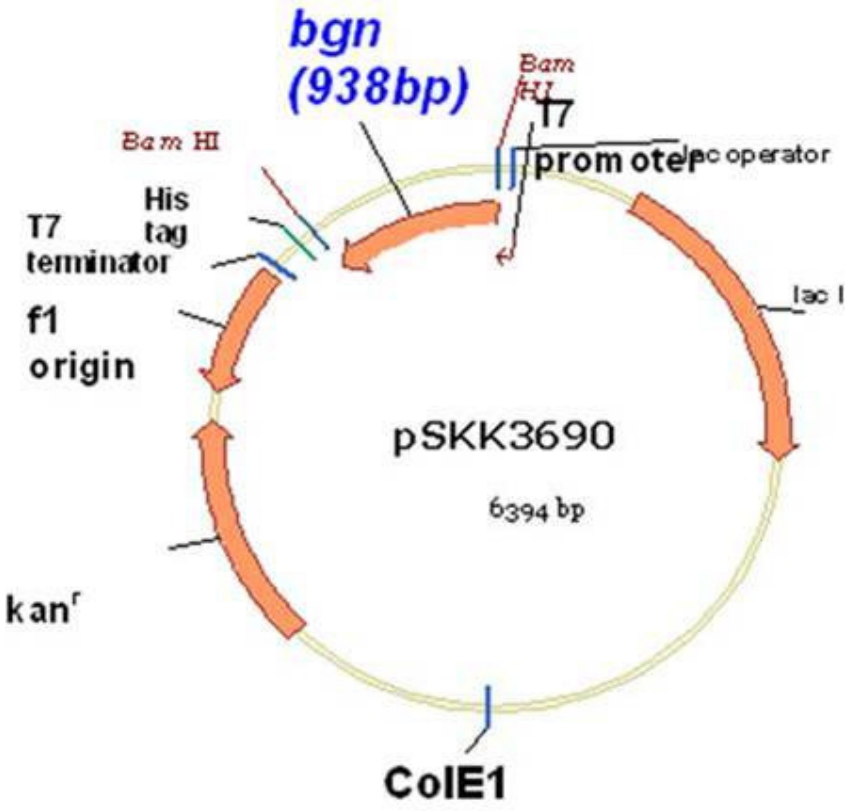


**Fig 3. Restriction map of construct pSKK3690 containing full length *bgn* gene in pET28 (a+)**

kanamycin. The pSKK3690 (pET28a+ : : Bouganin) (Fig. 3) clones were PCR checked using specific primers. The recombinants gave a 938 bp amplicons. These clones were further confirmed by digestion with *Bam H*I for the release of 938 bp insert following electrophoresis (Plate 4).

## 4.4 EXPRESSION STUDIES

To check the expression of the cloned *bsavp* gene in pET28a (+) in E. *coli* BL 21, with pET28a (+) and BL21 with recombinant construct was subjected to SDS-PAGE. The gel showed differential banding pattern in all Bouganin recombinants except pSKK3690E when compared with controls. The protein band corresponding to approximately 27 kDa (Plate 5) indicated the *bgn* gene expression in *E.coli.*

## 4.5 BIOASSAY FOR ANTIFUNGAL ACTIVITY

Cell free extract from culture of *E.coli* BL-21 harbouring recombinants of pET28a+ *bgn* gene upon induction with IPTG was used to assess the toxicity of the recombinant protein against *Sclerotium rolfsi*. The results showed that the transformed *E.coli* produced a biologically active fungicidal protein, which was toxic to the fungal pathogens. After three days, there was reduction in the growth of *Sclerotium rolfsi* exposed to Bouganin (3.2 cm dia) compared to control (9 cm dia) (Plate 6).

In another set of experiments an attempt was made to clone full length cDNA of Ribosome inactivating protein from *Boerhaavia diffusa* by performing 5' & 3' RACE PCR.

## 4.6 RNA ISOLATION

Total RNA from leaves of *Boerhaavia diffusa* was used for further experiments.

### 4.6.1 Primer synthesis and Tm standardization

A new set of primers were designed and custom synthesized as per the recommendations made in the RACE PCR Kit. Annealing temperatures of the primers were

**COMPLETE NUCLEOTIDE SEQUENCE**

ATGGGTTGGTGGGCTATCATAGTTGAGATAGTGCTTGCAAAGCCATCAATAATAACCACCAAA
GAAACAGCCTCACTTGGTTACAAAACCGTGTCTTTTAACCTTGGAGAGGCCGAAGAGTACTCC
ACTGTTATACAAGAATTGCGCAATGCATTGGCTAATGGTGCACCAGTATGTTATTTTCCAGTGA
CAGCAAAAACCATAGCCAATGATAAGAGATTTGTTCTAGTTGATCTCACTACGACCTCGATGAA
AACTATTACGCTTGCTATAGATGTGACGAATGTGTACGTGGTGGGTTATCGCGACCTATACAA
TAACAAAGATCGAGCCGTTTTCCTTGCCGAGGTTCCTACTGTTGCAATCGATGATCTTTTCCCA
GGGGTGACAAATCGTGAAATGTTAACATTTTCTGGACACTATCAAAAACTTCAAGAGGCTGCC
AAAGTGAATAGAGAGAATCTCGAACTGGGGGTTAACAAATTGGGATTTGCCATTGAATCTATCT
ATGGTAGTAAAACGCTAAATGGTAAAGACATAGCCAGGTTCTTTCTTATTGCAATCCAAATGAT
GTCAGAGGCAGCACGGTTTAAGTATATTGAGAATGAGGTGTCTAATAACGGATTATATGGATC
ATTCACACCCAACCCCAAAGTATTGAACTTGGAGAACAATTGGGGTGACATCTCTGATGCCAT
TCACAAATCATCCCCTAAATGTACCACTATTAAGCCAGCACTTCAGTTGAAAACCCCCTCAAAT
GACCCATGGGTTGTGCATGAAGTAAGTGAAATCCGTCCTGATTTGGGTATCCTCAAGTTTAAA
AGCTCCAAATTAACTCAGTTTATTACAATAATAAGGTCTATTGTGGTTGAAGAGTTAGATGGTG
GTGAACTTGAAATACTAGAGCCAAACATTGCCTAATCAGAATTCCGA

**COMPLETE AMINO ACID SEQUENCE**

MGWWAIIVEIVLAKPSIITTKETASLGYKTVSFNLGEAEEYSTVIQELRNALANGAPVCYFPVTAKTIA
NDKRFVLVDLTTTSMKTITLAIDVTNVYVVGYRDLYNNKDRAVFLAEVPTVAIDDLFPGVTNREMLT
FSGHYQKLQEAAKVNRENLELGVNKLGFAIESIYGSKTLNGKDIARFFLIAIQMMSEAARFKYIENEV
SNNGLYGSFTPNPKVLNLENNWGDISDAIHKSSPKCTTIKPALQLKTPSNDPWVVHEVSEIRPDLGI
LKFKSSKLTQFITIIRSIVVEELDGGELEILEPNIA

**Fig 4. Complete nucleotide and amino acid sequence of *bgn* gene in pSKK3680**

**Plate 3: Restriction confirmation of pSKK3680 clones**

M : 1kb ladder

**Lane 1-2: pSKK clones**

M : 100 bp DNA ladder

**Plate 4: Restriction confirmation of pSKK3690 clones**

**M: 1 kb DNA ladder**

Lane 1-2: pSKK Clones

M: 100 bp DNA ladder

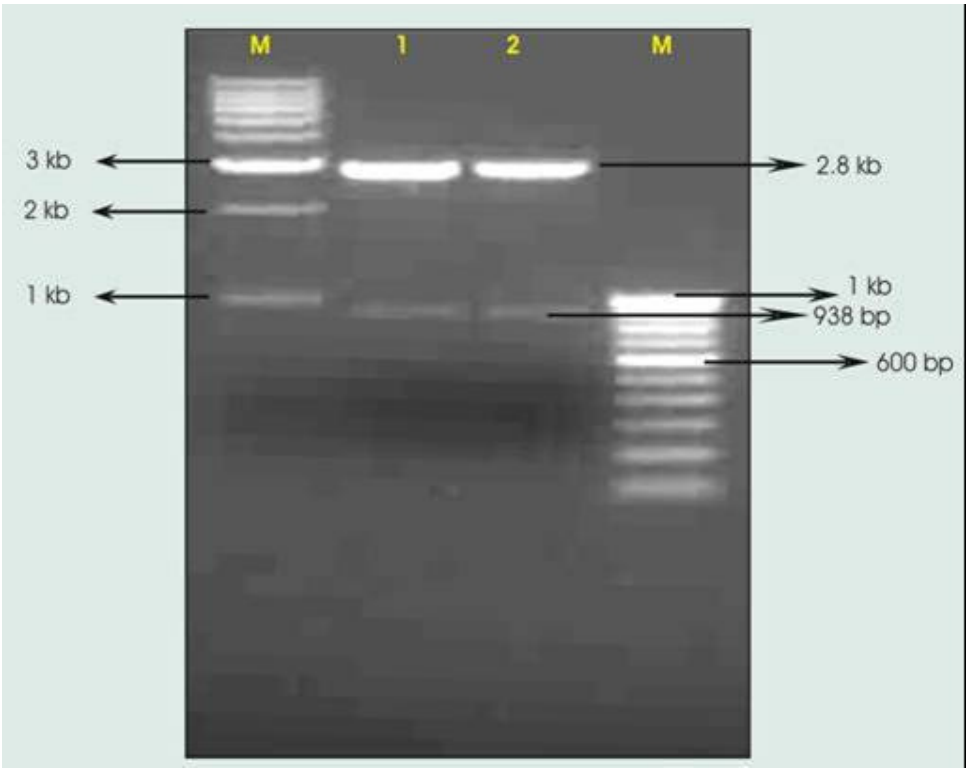


**Plate 3: Restriction confirmation of pSKK3680 clones**

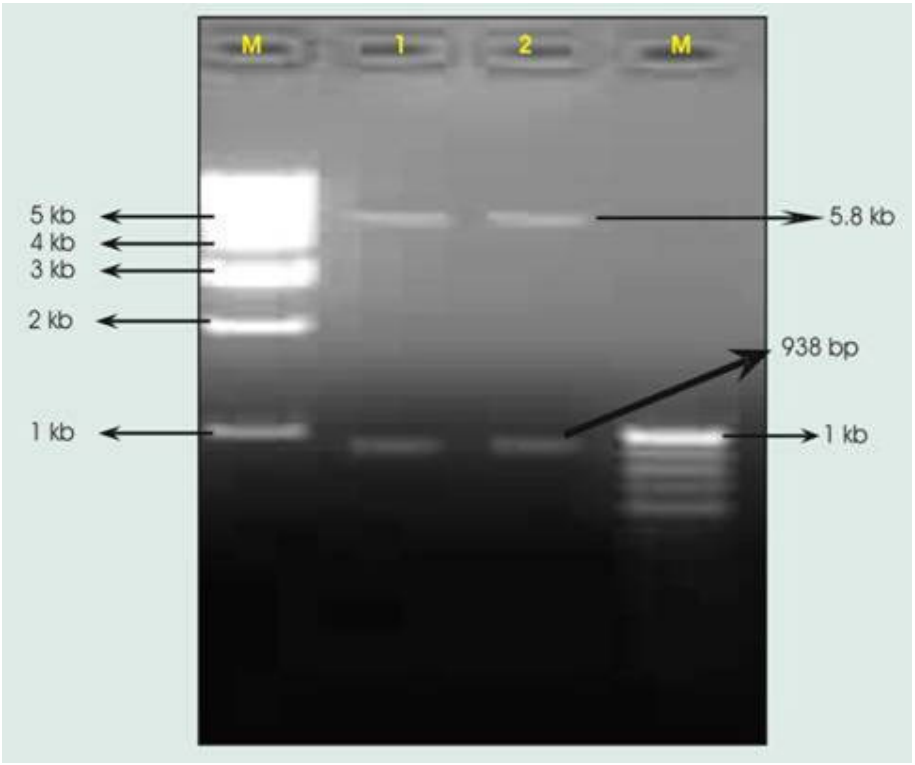


**Plate 4: Restriction confirmation of pSKK3690 clones**

>gi|114325706|gb|ABI64066.1| ribosome inactivating protein [Bougainvillea spectabilis]
Length=305

Score = 577 bits (1486), Expect = 3e-163, Method: Composition-based stats.
Identities = 297/307 (96%), Positives = 300/307 (97%), Gaps = 2/307 (0%)

```
Query  1    MGWWAIIVEIVLAKPSIITTKETASLGYKTVSFNLGEAEEYSTVIQELRNALANGAPVCY  60
Sbjct  1    ............................................................N  60

Query  61   FPVTAKTIANDKRFVLVDLTTTSMKTITLAIDVTNVYVVGYRDLYNNKDRAVFLAEVPTV  120
Sbjct  61   ................................D.......................S.  120

Query  121  AIDDLFPGVTNREMLTFSGHYQKLQEAAKVNRENLELGVNKLGFAIESIYGSKTLNGKDI  180
Sbjct  121  ..H.....................................................-.A.....  179

Query  181  ARFFLIAIQMMSEAARFKYIENEVSNNGLYGSFTPNPKVLNLENNWGDISDAIHKSSPKC  240
Sbjct  180  ........................-.......................................  238

Query  241  TTIKPALQLKTPSNDPWVVHEVSEIRPDLGILKFKSSKLTQFITIIRSIVVEELDGGELE  300
Sbjct  239  ...N....S.....................................................Q  298

Query  301  ILEPNIA  307
Sbjct  299  .......  305
```

**Fig 5. Blast result of pSKK3680 Bgn protein with NCBI database**

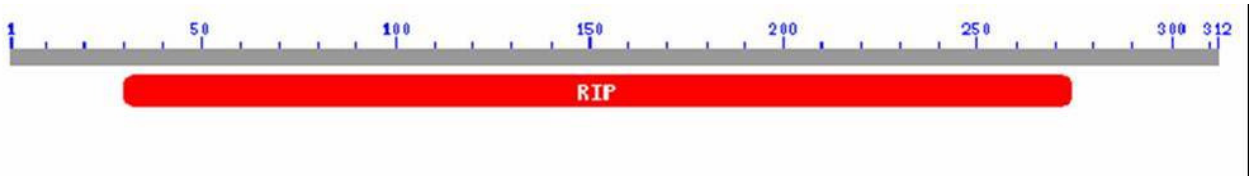


**Fig 6. Conserved domain of *bgn* gene**

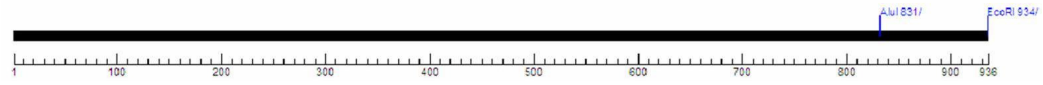


**Fig 7. Restriction map of *bgn* gene in pSKK3680**

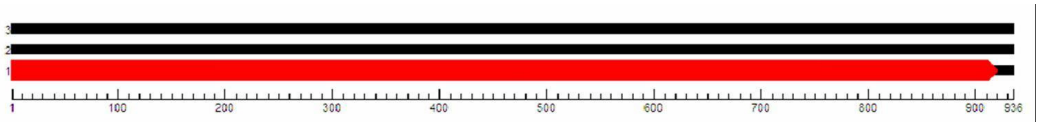


**Fig 8. Open reading frame of *bgn* gene in pSKK3680**

**Table 1: Nucleotide-Nucleotide blast (BLASTn) result of cloned antiviral protein gene from *Bougainvillea spectabilis* (pSKK3680)**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| DQ989495.1 | *Bougainvillea spectabilis* ribosome inactivating protein (RIP) gene, complete cds | 1613 | 1613 | 100% | 0.0 | 97% |
| AF445416.1 | *Bougainvillea spectabilis* bouganin mRNA, complete cds | 942 | 942 | 99% | 0.0 | 85% |
| AY437531.1 | *Bougainvillea spectabilis* antiviral protein (Ap1) mRNA, complete cds | 931 | 931 | 99% | 0.0 | 85% |

optimized by gradient PCR. Primers showed amplification between annealing temperatures of 68-72$^0$ C, facilitating the use of touchdown PCR programme.

### 4.6.2 RACE PCR

For 3' RACE, cDNA synthesis was performed with the 3' RACE system. Essentialy an aliquot of isolated Total RNA (100 ng) was reverse transcribed with 3'CDS primer. The 3'RACE PCR was carried out in a 50 µl volume, as per manufacturer's instructions. The desired amplicons of 450bp, along with a few minor bands were seen when 5µl of 3' RACE product was examined on 1% agarose gel. The remaining 95µl of RACE product from two reactions was loaded on 1% agarose gel and the expected fragment corresponding to 450 bp was excised from the gel and eluted (Plate 7)

Similarly, the 5' RACE system was used for 5' cDNA cloning. RT & PCR amplification was conducted as described for the Kit. After gel electrophoresis, the target DNA bands corresponding to 650 bp (Plate 8), was purified using gel extaction Kit.

### 4.6.3 Ligation and Confirmation of Recombinants

The eluted fragments were ligated into pTZ57R/T cloning vector and *E.coli* DH5α was transformed with the recombinant plasmids. White colonies were picked up, streaked on LA+Amp+X-gal+IPTG for selection of clones. In case of clones of 5' RACE, out of 10 putative transforments, 8 were white and of them six showed the presence of 650 bp insert. This was confirmed by PCR amplification and restriction analysis with *Bam*HI and *Sac*I. While 12 clones were obtained from 3'RACE-PCR product, eight showed presence of the insert (Plate: 10, 9, 11). The clones were named as p5BD1, p5BD2 and p5BD3 in case of 5'RACE and p3BD1, p3BD8 and p3BD11 in case of 3' RACE (Fig 9, 10)

### 4.6.4 Sequencing and generation of full-length cDNA

Partial sequencing of three clones p3BD1, p3BD8 and p3BD11 of 3' RACE and three clones of 5'RACE-PCR viz, p5BD1, p5BD2, and p5BD3 were done using M13 forward and reverse primers. Sequencing of more than one clone was necessary for 5' RACE in order to elucidate maximum possible sequence at 5'end. When the sequences from the clones were subjected to analysis using the BLAST algorithm available at http://www.ncbi.nim.nih.gov, they showed homology with few reported RIP genes sequences. Among the sequenced clones, a maximum of 650bp sequence, excluding the universal primer sequences was obtained from clone p5bd1 of 5'RACE. While, clone p3BD1 of 3' RACE contained 450bp long cDNA fragment.

Further, by comparing and aligning sequences of 3' and 5' RACE products, which had an overlapping region of 100bp, a composite 1167bp sequence of *Boevrhaavia diffusa*

**Plate 5: SDS-PAGE for expression of *bgn* gene**

M. Protein marker

1. Induced BL21 (pET28a (+))
2. BL21
3. Uninduced BL21(pET28a (+))
4. Uninduced pSKK3690
5. Induced pSKK3690 A
6. Induced pSKK3690 B
7. Induced pSKK3690 C
8. Induced pSKK3690 D
9. Induced pSKK3690 E
10. Induced pSKK3690F
11. Induced pSKK3690G
12. Induced pSKK3690H
13. Induced BL21 (pET28a (+))
14. BL21
15. Uninduced BL21(pET28a (+))
16. Uninduced pSKK3690
17. Induced BL21 (pET28a (+))

**Plate 6: Plate bioassay for antifungal activity of *bgn* gene in pET28**

A. Positive control

B. Negative control

C. pSKK3690

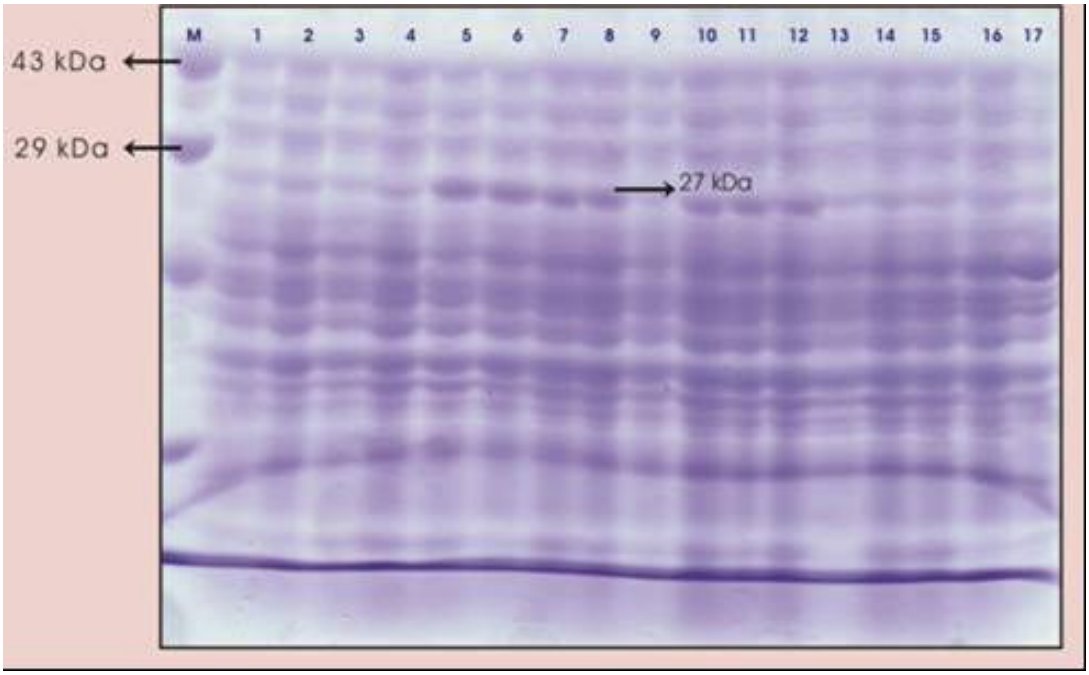


**Plate 5: SDS-PAGE for expression of *bgn* gene**

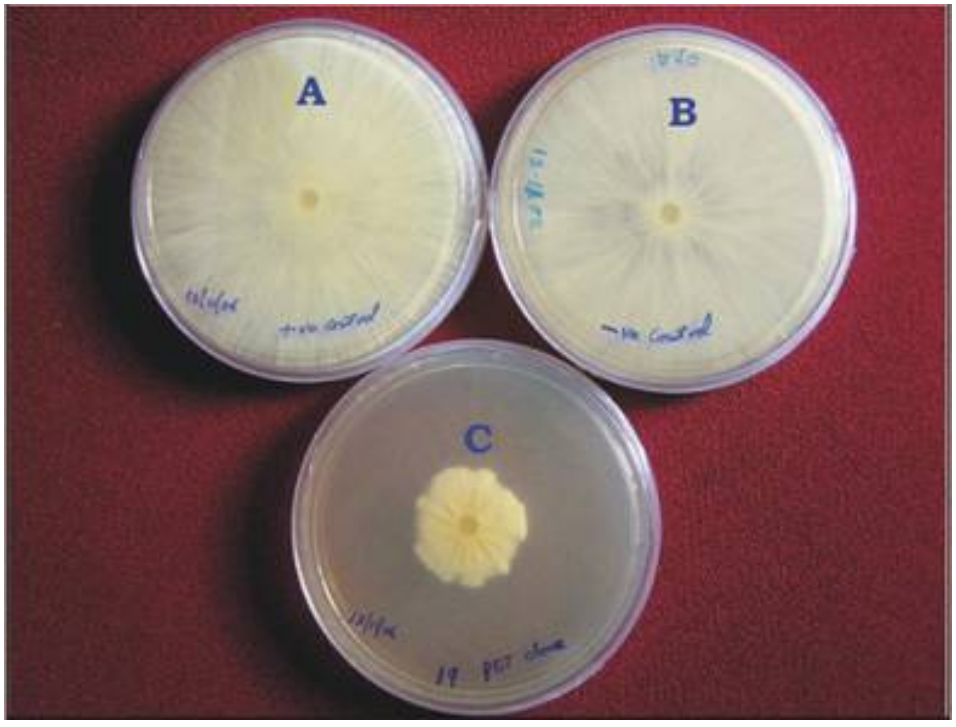


**Plate 6: Plate bioassay for antifungal activity of *bgn* gene in pET28**

**Plate 7: *bdavp* 3'RACE amplicons**

M: 100bp DNA ladder

Lane 1-2: 450 bp *bdavp* 3'RACE amplicons

**Plate 8: bdavp 5'RACE amplicons**

M: 100 bp DNA ladder

Lane 1-2: 650 bp amplification

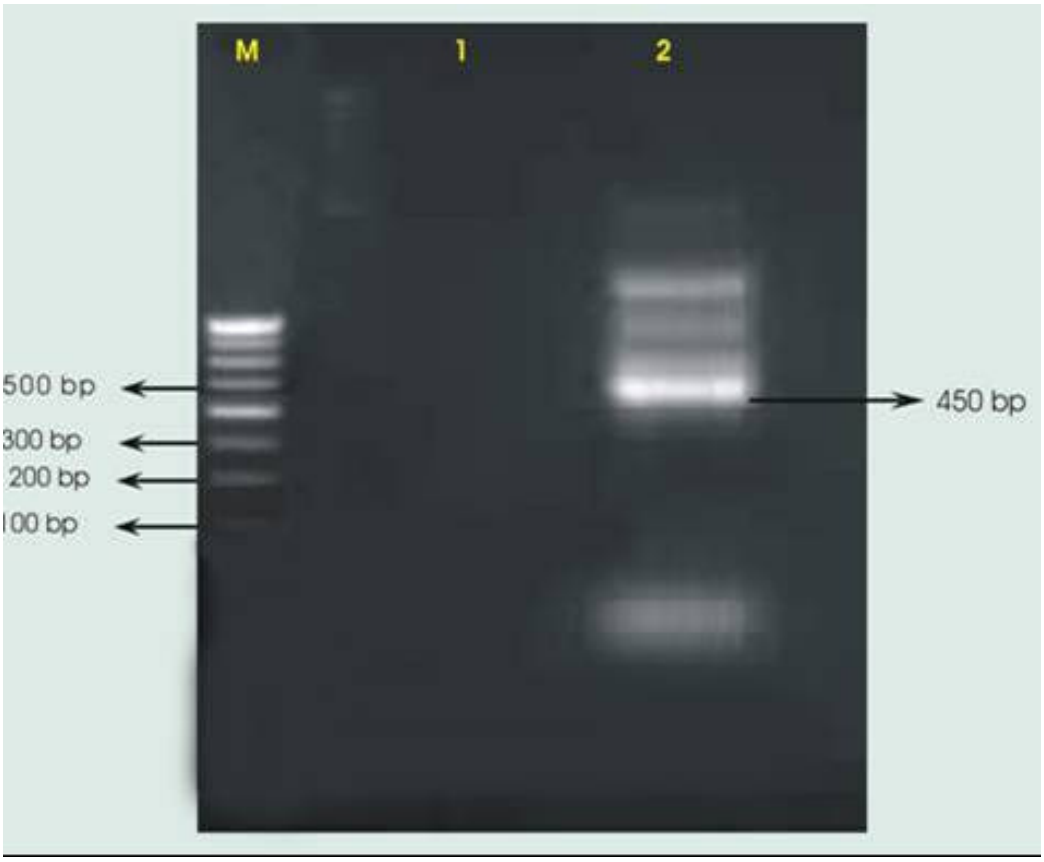


**Plate 7: *bdavp* 3'RACE amplicons**

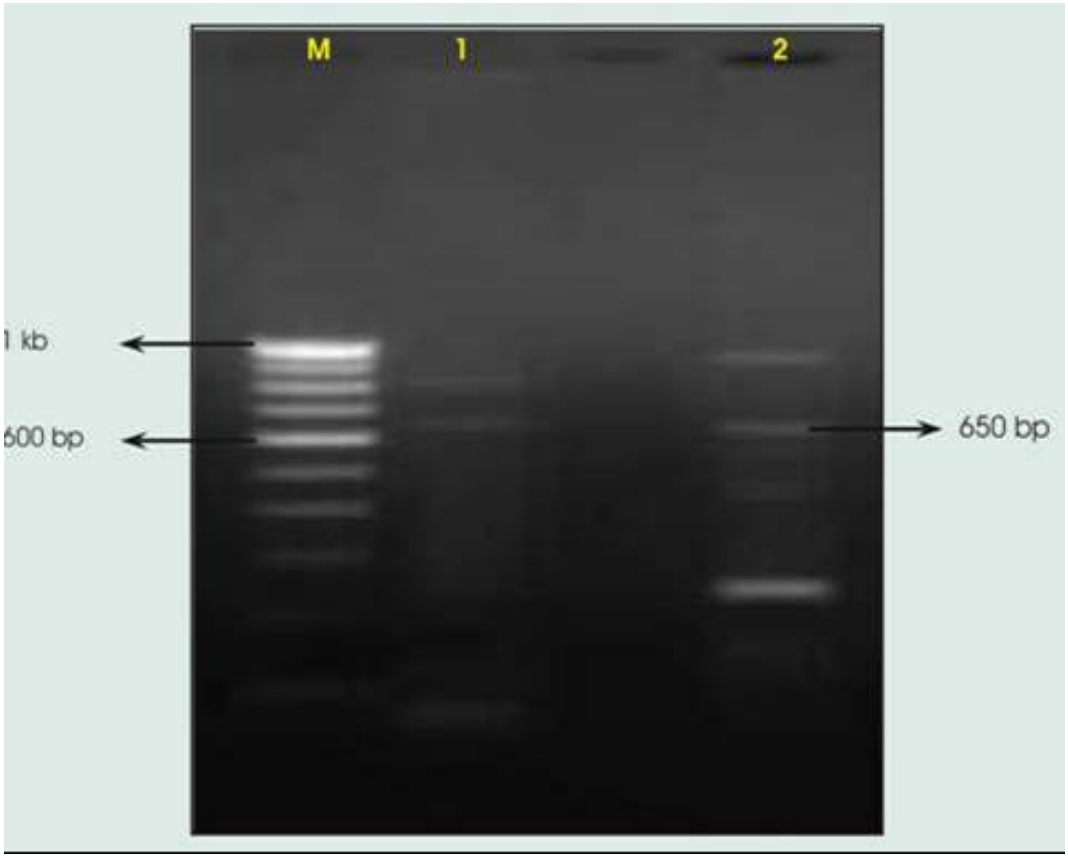


**Plate 8: bdavp 5'RACE amplicons**

**Plate 9: PCR confirmation of 3'RACE clones**

M: 100 bp DNA ladder

Lane 1-8: 3' Bdavp clones

**Plate 10: PCR confirmation of 5'RACE clones**

M: 100 bp DNA ladder

Lane 1-3: 5' Bdavp clones

**Plate 11: Restriction confirmation of 5'RACE and 3'RACE clones**

M:100 bp DNA ladder

Lane 1-4: 5' Bdavp clones

Lane 5-8: 3' Bdavp clones

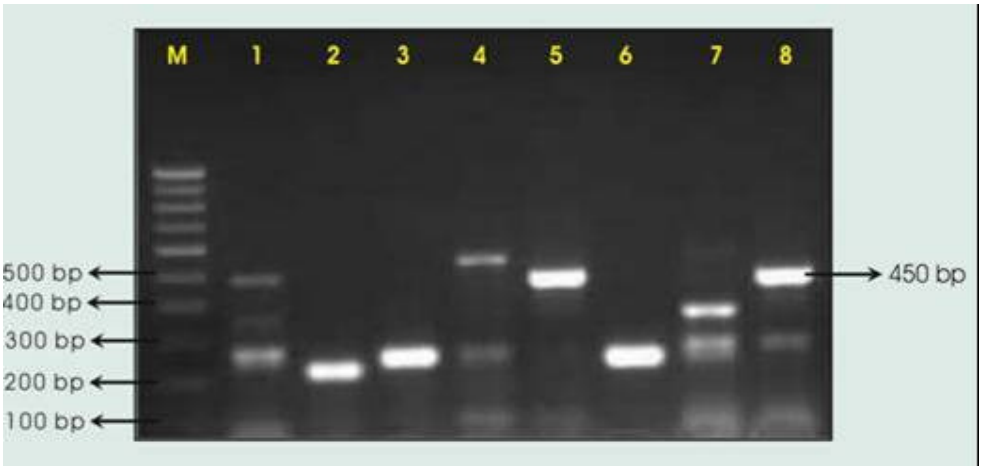


**Plate 9: PCR confirmation of 3'RACE clones**

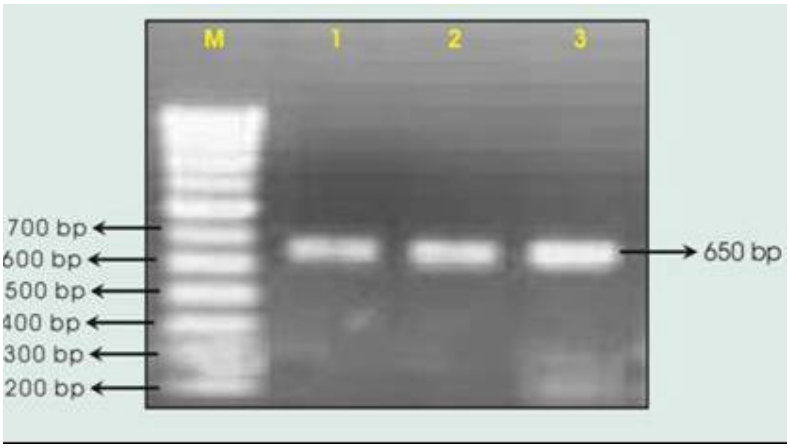


**Plate 10: PCR confirmation of 5'RACE clones**

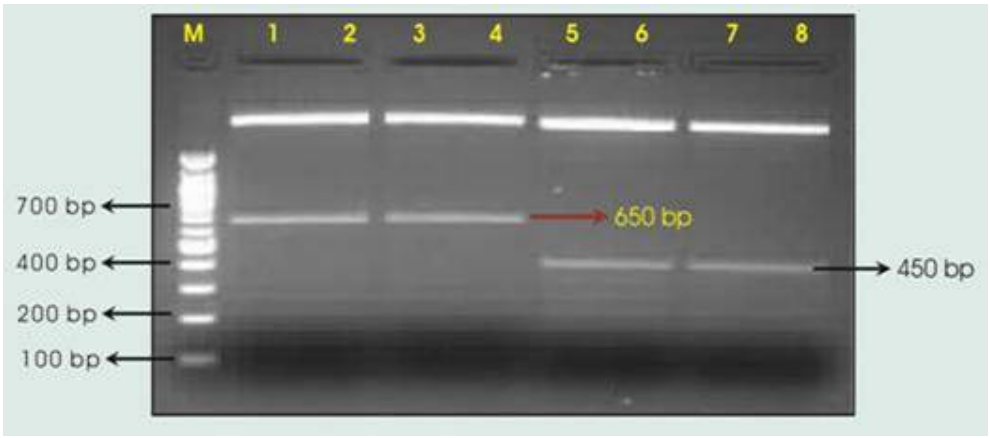


**Plate 11: Restriction confirmation of 5'RACE and 3'RACE clones**

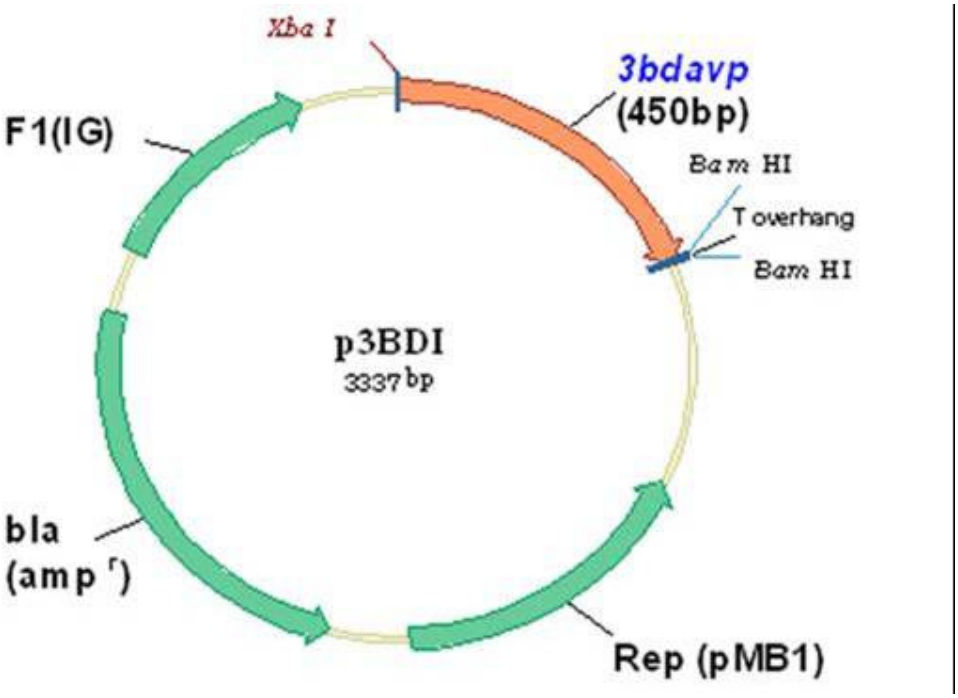


**Fig 9. Construct map of p3BD1 containing *3'bdavp* gene in pTZ57R/T**

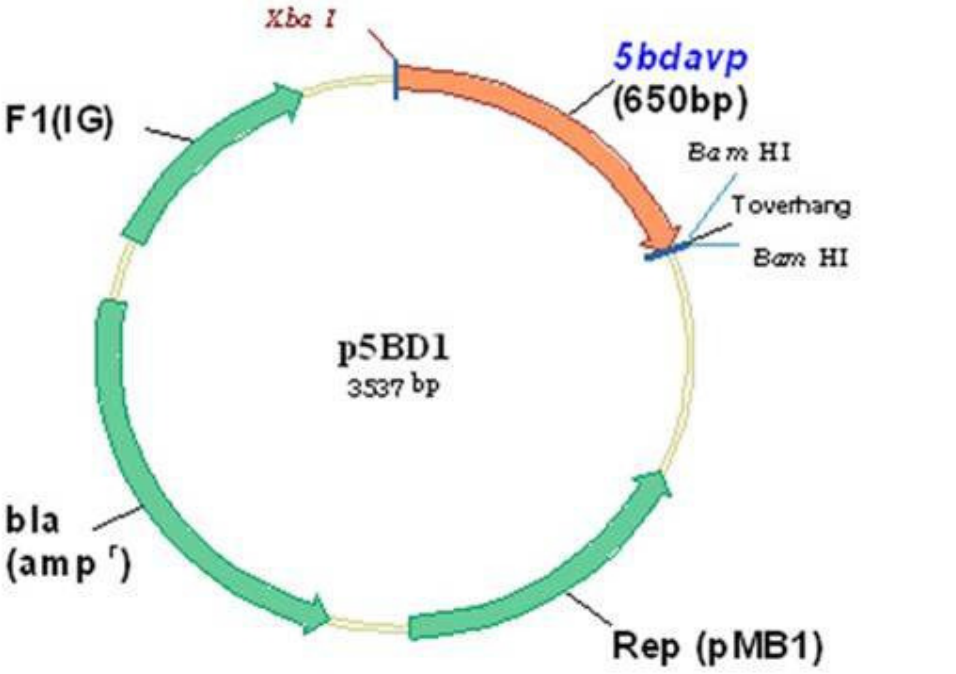


**Fig 10. Construct map of p5BD1 containing *5'bdavp* gene in pTZ57R/T**

ribosome-inactivating protein gene (*bdavp*) was deduced using VECTOR NTI suite 10.0 software. UPM as forward and a new primer BDHR as reverse primer were used to amplify and clone full- length cDNA by carrying out RACE –PCR. pBDAVP clones containing full length cDNA were confirmed through PCR and restriction analysis (Plate 12,13). Further these clones were verified by sequencing. Sequencing information of full- length cDNA and construct map are given respectively (Fig.12, 11).

### 4.6.5 Structure of *bdavp* gene

The full-length (1146bp) cDNA was annotated using GENSCAN software. The full-length cDNA contained 1023 bp open reading frame (ORF) with 5' untanslated region (UTR) 94bp upstream from start codon and 30bp 3' UTR down stream of the stop codon. The 5' UTR had moderate G+C content (41.9 per cent), while, 3' UTR region had low G+C content (36.7 per cent). The start codon (ATG) and stop codon (TAG) were positioned at 94 and 1116 respectively.

Homology search for the complete cDNA sequence and in silico analysis was carried out using BLASTn search. The search results indicated, extensive low similarity to the Ribosome inactivating protein gene homologues reported from other plant species, the cloned gene was designated as *bdavp*. Low similarity of *bdavp* was noticed with *Mirabilis jalapa* antiviral protein (D10227) and gleonin mRNA (L12243) (Fig.13) Further the sequences were subjected to further analysis in BTI software of GENETOOL for finding restriction sites. *bdavp* sequence did not have any internal restriction sites for*, Xba1, Not1* and *HindIII* (Fig. 14)

### 4.6.6 Characterization of predicted *Bohervia Ribosome*-inactivating protein

The encoded amino acid sequence of *bdavp* was deduced using the GENETOOL software. *bdavp* gene contains a 1023bp ORF with a possible initiation codon at position 94 of the deduced nucleotide sequence (Fig.15). Translation starting from the ATG codon yields a protein of 340 amino acids, with a calculated molecular weight of 37.8 kDa.

Sequence comparison of the deduced Boerhaavin (Bdavp) polypeptide by performing BLASTp search in GenBank base data revealed that, Boerhaavin (Bdavp) had moderate homology to over hundered reported Ribosome-inactivating proteins (Fig. 16).

### 4.6.7 Multiple alignment and motif analysis

Multiple alignment of Bdavp polypeptide sequence with known Ribosome-inactivating proteins was done using Clustal W software. Alignment of Bdavp showed 48 per cent identity with the *Mirabilis expansa* antiviral protein (AAN65450). While it had 40 and 30 per cent identity with *Mirabilis jalapa* (BAA01079) and *Bougainvillea spectabilis* antiviral protein (AAR9737), respectively. Bdavp shared 24 per cent identity with PAP (AAL15442), while Gleonin (AAA16312) and Momardin (AAV68558) showed 21 per cent similarity (Table 2). Bdavp contained 21 conserved amino acid residues, including the amino acid residues at the proposed active site (Glu $^{220}$ and Arg $^{223}$ ) (Fig.19).

The analysis of the deduced amino acid sequence from *bdavp* cDNA using SignalP 3.0 software, showed that the N-terminal of *bdavp* cDNA encodes a signal peptide of 26 amio acid residues composing mostly hydrophobic amino acids, a feature common to the signal peptides of both eukaryotes and prokaryotes.

Bdavp protein was subjected to motif analysis using Quasi MotiFinder software. The protein harbored region of similarity to the Shiga/ricin ribosomal inactivating toxins active site signature VxMVxEAARFTxISxxI (Fig. 17). ScanProsite results also indicated the presences of Proline-rich region profile sequence PrrpppppprappppP (29-43) in addition to Shiga/ricin ribosomal inactivating toxins active site signature VqMVsEAARFTyISdkI (215-231) (Fig. 18).

## 4.7 SUB CLONING IN PROKARYOTIC EXPRESSION VECTOR

Following restriction, the gene in pBDAVP (Bdavp) was cloned into *Sac*I and *Hind*III sites of the expression vector pET28a (+) and introduced into *E. coli* BL 21 (plysS) cells. The transformants with *bdavp* were picked and streaked on Luria agar plates containing 50 µl/ml kanamycin. The pSKK12 (pET28a+ : : *bdavp*) (Fig. 20) clones were PCR checked using T7 promoter and BDFR primers. The recombinants gave a ~ 1200 bp amplicons (Plate 14).

**Plate 12: PCR confirmation of full length *bdavp* gene**

M: 100 bp DNA ladder

Lane 1-8: Bdavp clones

**Plate 13: .Restriction confirmation of full length *bdavp* clones**

M : 1 kb ladder

Lane 1-6 : Bdavp clones

**Plate 14: PCR confirmation of pSKK12 clones**

M : 1 kb ladder

1 -2 : pSKK12 clones

3 : Positive control p*bdavp* clone

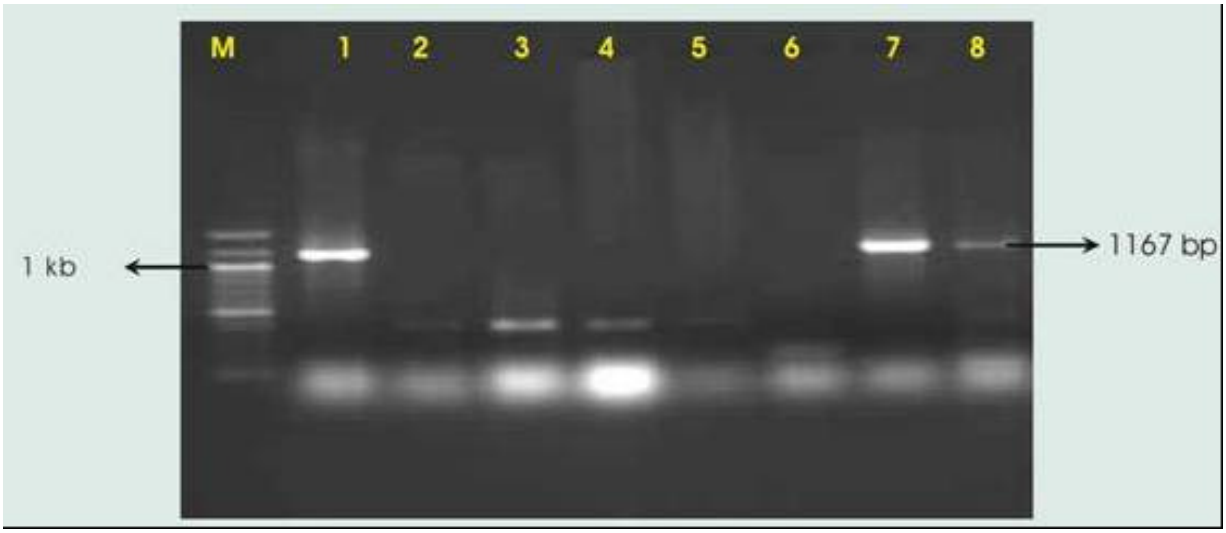


**Plate 12: PCR confirmation of full length *bdavp* gene**

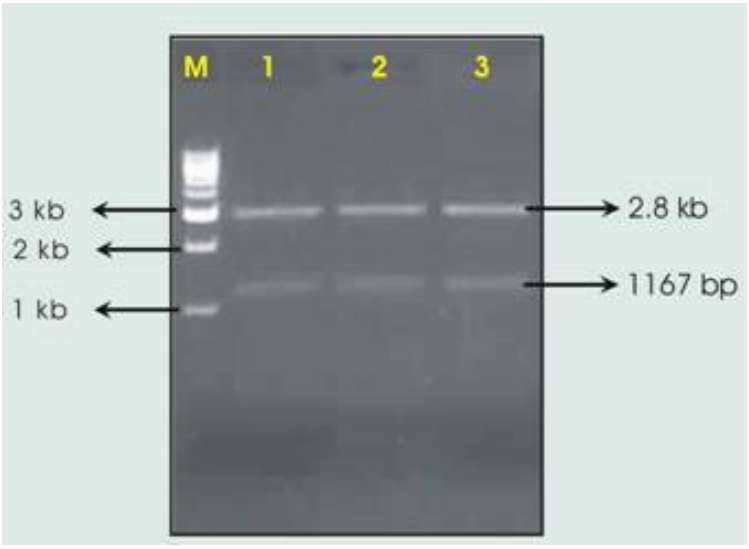


**Plate 13: .Restriction confirmation of full length *bdavp* clones**

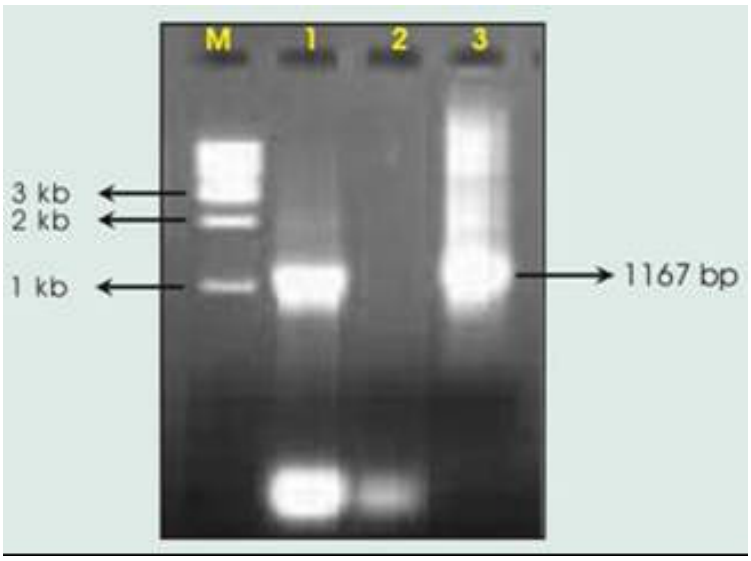


**Plate 14: PCR confirmation of pSKK12 clones**

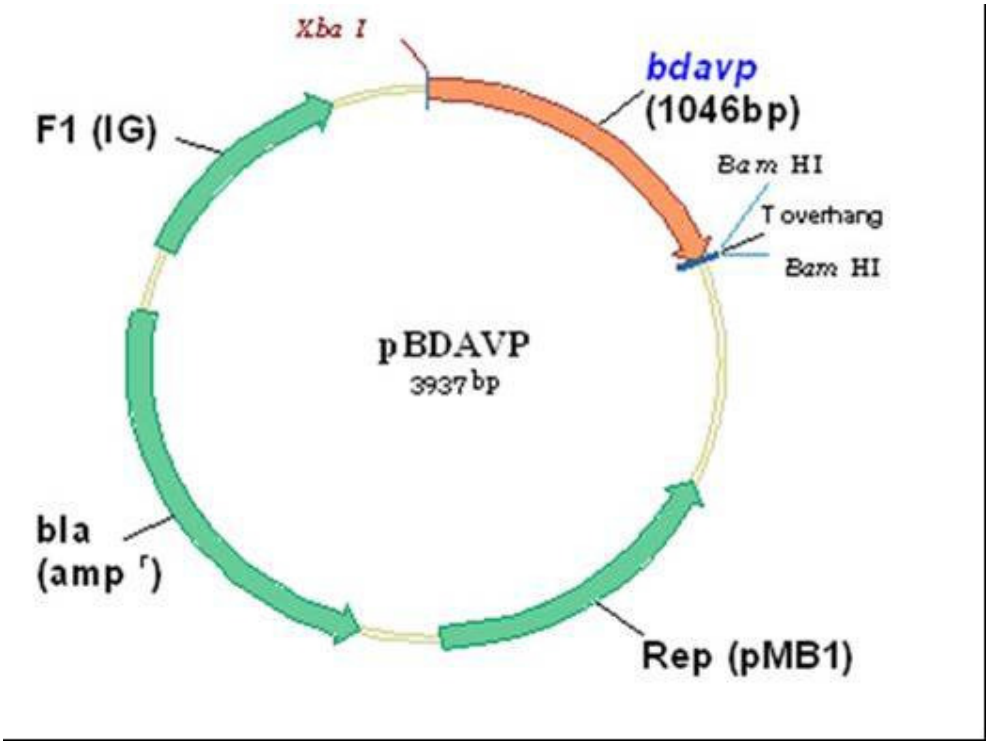


**Fig 11. Construct map of pBDAVP containing *full bdavp* gene in pTZ57R/T**

These clones were further confirmed by digestion with *Sac*I and *Hind*III for the release of ~1200 bp insert following electrophoresis .

## 4.8 EXPRESSION STUDIES

To check the expression of the cloned *bdavp* gene in pET28a (+) in E. *coli* BL21, with pET28a (+) and BL21 with recombinant construct was subjected to SDS-PAGE. The gel showed differential banding pattern in all Bdavp recombinants when compared with controls. The protein band corresponding to approximately 27 kDa (Plate 15) indicated that *bdavp* gene expressed in *E.coli.*

## COMPLETE NUCLEOTIDE SEQUENCE

GCGGGGAAATCTTCAATCCTTAAAATTGCAAATAGATTTTTAGAGCTGCCACCATTTGTTGACC
GAAAACAAGACTTGACCCTGCTGATAGGCATGGGAACTATGAAATCATATTTTCTCTTGCTGAC
AATATGTATCCATTGGATGGCCATTGCCAATCCTGTATCGGCTCAGGCACCTCGTCGGCCTCC
ACCACCGCCAAGGGCACCACCACCACCACCATCGATCCAAAAGACATCAACTTTAGACCTGAA
TCAACCTACCACATATCGCACATTTATCACAGATGTACGAAATAAATTAGCAGAAAAAGACAGT
AAAGGAAATTATATATTGAAATGTACCATGCAAAAAATAAGTAAAGTGGTCCCACCAGAGGGAT
TTATGTATGTGGAACTCATAGCCTCCTCATCAAAGACAATTACGATAGCATTGGATCTACCGAA
TGCCTATGTGATTGGGCTATCGCGCAAAGTTAAAAATAAAAGATCGTGCTTTCTTCTTCAAAGA
GTATTATGCAAAACCAAATGTTATATCAAATGTGTTCCTGATGCAACCGGTAAAGAAAACCGAC
TTGATTTGGGATTTACCGGAAGATACCAAGATCTTGAGAAGTATGGTGATAGAAGAAGCACTC
CATTAGGAATCTCTAAGCTACAAAGCAGCATAACGGACATCTATGGGAAAAATATAAAGGATAT
TCAGAAGGGGCAGCGTAAGTTTCTTCTCAGTGCAGTGCAGATGGTGTCTGAGGCAGCTCGAT
TCACATATATTTCTGATAAGATTCCGCAAGATGCACACGACACTTTGACAGTCGATGCCAAAGT
GCTTGCTTTAGAAAACGGATGGGATCCATTGTCAACAGCAGTTTATAAATCTAAGACCTCCACC
ACGGCCACTAAGTGTGCATTGACAGGGCCCGTATCATTAACTTTAGATCAAGGCAAACAATGG
ACCTTCAACACAGTGGAGGATATAAAGCAAGTCATAGGCATTCTCAAGGCCGAGGGCACCAC
TTTGTTTACTGGCAATGATGTTGAATTTGGTAAAACTGCTAATAATATAGGACCATATGGTAATT
TAATAACTTGCAATAAGGAGTGGTTGTATTATTGAAAGTTAGGAAGGTGCATCTTATGTTACTG
AAAAAAAAAAA

## COMPLETE AMINO ACID SEQUENCE

MGTMKSYFLLLTICIHWMAIANPVSAQAPRRPPPPPRAPPPPPSIQKTSTLDLNQPTTYRTFITDVR
NKLAEKDSKGNYILKCTMQKISKVVPPEGFMYVELIASSSKTITIALDLPNAYVIGLSRKVKNKRSCF
LLQRVLCKTKCYIKCVPDATGKENRLDLGFTGRYQDLEKYGDRRSTPLGISKLQSSITDIYGKNIKDI
QKGQRKFLLSAVQMVSEAARFTYISDKIPQDAHDTLTVDAKVLALENGWDPLSTAVYKSKTSTTAT
KCALTGPVSLTLDQGKQWTFNTVEDIKQVIGILKAEGTTLFTGNDVEFGKTANNIGPYGNLITCNKE
WLYY

**Fig 12. Complete nucleotide and amino acid sequence of *bdavp* gene in pBDAVP clone**

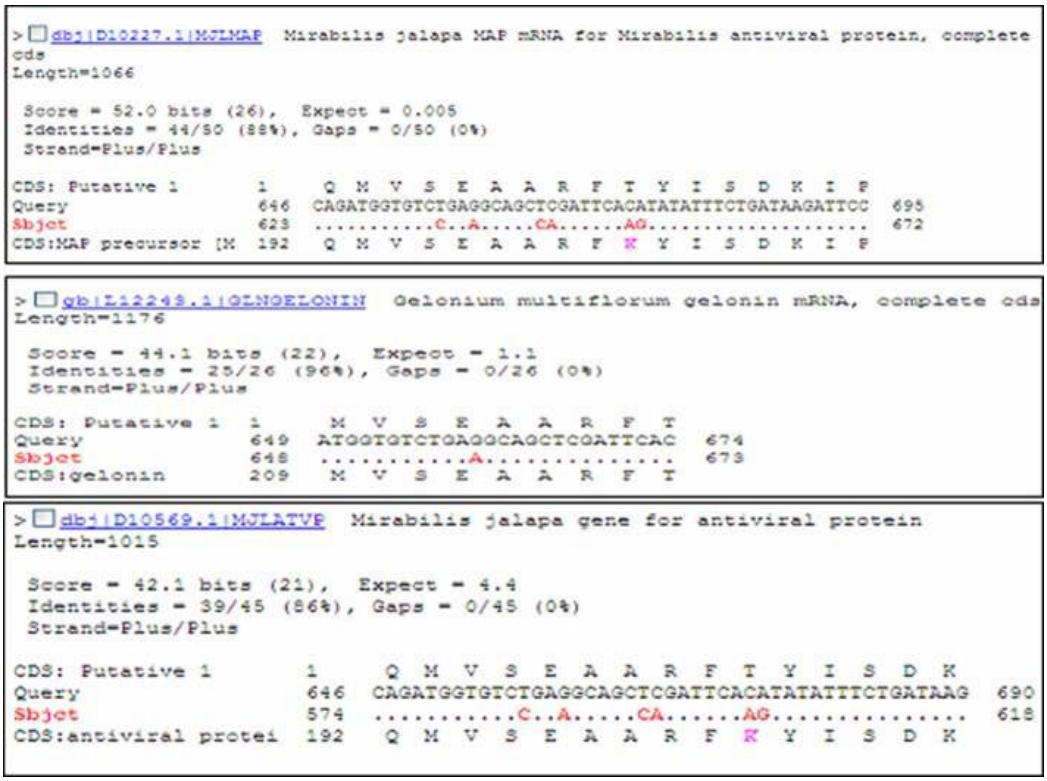


**Fig 13. Nucleotide Blast result of *bdavp* gene with NCBI database**

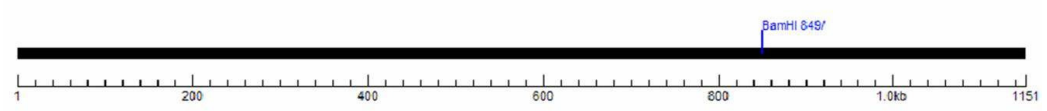


**Fig 14. Restriction map of *bdavp* gene in pBDAVP**

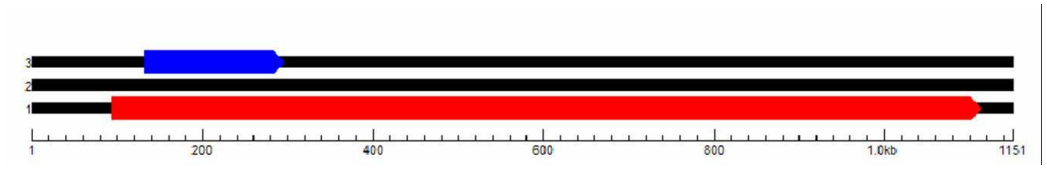


**Fig 15. Open reading frame of *bdavp* gene in pBDAVP**

```
Sequences producing significant alignments:                          Score (Bits)  E Value

gi|132595|sp|P21326|RIPP_MIRJA  Antiviral protein MAP precurso...     223     9e-57
gi|217956|dbj|BAA01079.1|  MAP precursor [Mirabilis jalapa]           215     6e-55
gi|25136301|gb|AAN65450.1|  ribosome-inactivating protein ME1 [Mi     216     2e-54
gi|2088838|gb|AAA72218.1|  Mirabilis antiviral protein                207     5e-52
gi|114325706|gb|ABI64066.1|  ribosome inactivating protein [Bouga     121     5e-26
gi|40890058|gb|AAR97871.1|  antiviral protein [Bougainvillea spec     119     2e-25
gi|17149254|gb|AAL35962.1|AF445416_1  bouganin [Bougainvillea spe     119     2e-25
gi|85549885|pdb|1WUC|A  Chain A, Crystal Structure Of The Type 1      117     7e-25
gi|18149181|dbj|BAB53507.1|  ribosome-inactivating protein [Spina     107     1e-21
gi|1405841|emb|CAA59952.1|  Betavulgin [Beta vulgaris subsp. vulg     105     3e-21
gi|45511452|gb|AAS67266.1|  ribosome-inactivating protein BE27...     102     5e-20
gi|417650|sp|Q03464|RIPA_PHYAN  Antiviral protein alpha precur...     99.0    3e-19
gi|493813|pdb|1APA|  Chain , X-Ray Structure Of A Pokeweed A...       96.7    2e-18
gi|4894969|gb|AAD32679.1|AF141381_1  ribosome-inactivating protei     94.7    6e-18
gi|68342548|gb|AAY34283.2|  antiviral protein 1 [Bougainvillea x      94.4    9e-18
gi|145286528|gb|ABP52093.1|  hIL15/PAP fusion protein [synthetic      94.0    1e-17
gi|42412852|gb|AAS15568.1|  gynostemmin [Gynostemma pentaphyllum]     93.2    2e-17
gi|45826467|gb|AAS77872.1|  antiviral protein [Phytolacca america     92.0    4e-17
gi|417654|sp|P10297|RIP1_PHYAN  Antiviral protein I precursor ...     92.0    4e-17
gi|22947826|gb|AAN05104.1|  ribosome-inactivating protein gyno...     91.7    5e-17
gi|737474|prf||1922356A  pokeweed antiviral protein                   91.7    5e-17
gi|23343925|gb|AAN16076.1|  anti-virus protein [Phytolacca americ     91.3    8e-17
gi|99646720|emb|CAK22418.1|  ribosome-inactivating protein [Beta      90.9    8e-17
gi|16356655|gb|AAL15442.1|  anti-viral protein PAP [Phytolacca ac     90.9    9e-17
```

```
>gi|132595|sp|P21326|RIPP_MIRJA  Antiviral protein MAP precursor (MAP-S) (Ribosome-inactivating
protein) (rRNA N-glycosidase)
 gi|217954|dbj|BAA01425.1|  antiviral protein [Mirabilis jalapa]
Length=278

 Score =  223 bits (569),  Expect = 9e-57, Method: Composition-based stats.
 Identities = 144/307 (46%), Positives = 191/307 (62%), Gaps = 34/307 (11%)

Query  1    MGTMKSYFLLLTICIHWMAIANPVSAQAFRRPPPPPRAPPPPPSIQKTSTLDLNQPTTYR  60
Sbjct  2    LT.T.VF.....TW.T.Y..V..Q.RA..--------------TLETIAS....N....L  47

Query  61   TFITDVRNKLAEKDSKGNYILKCTMQKISKVVPPEGFMYVELIASSSKTITIALDLPNAY  120
Sbjct  48   S...NI.T.V.D.TEQ------..I.....TFT-QRYS.ID..V..TQK..L.I.MADL.  100

Query  121  VIGLSRKVKNKRSCFLLQRVLCKTKCYIKCVPDATGKENRLDLGFTGRVQDLEKYGD-RR  179
Sbjct  101  .L.Y.DIAN..GRA.FFKD.--TEAVANNFF.G...T-..IK.T...S.G....N.GL.K  157

Query  180  STPLGISKLQSSITDIVGKNIKDIQKGQRKFLLSAVQMVSEAARFTYISDKIPQDAHDTL  239
Sbjct  158  DN....FR.EN..VN....-AG.VK.-.A..F.L.I.........K.......SEKYEEV  215

Query  240  TVDAKVLALENGWDPLSIAVYKSKIS-TIATKGAL-TGPVSLTLDQGKQWTFNIVEDIKQ  297
Sbjct  216  ...EYNT....N.AK......N..P.T......Q.A.S..TIS-----P.I.K...E..L  270

Query  298  VIGILKA  304
Sbjct  271  .M.L..S  277
```

**Fig 16. Blast result of Bdavp protein with NCBI database**

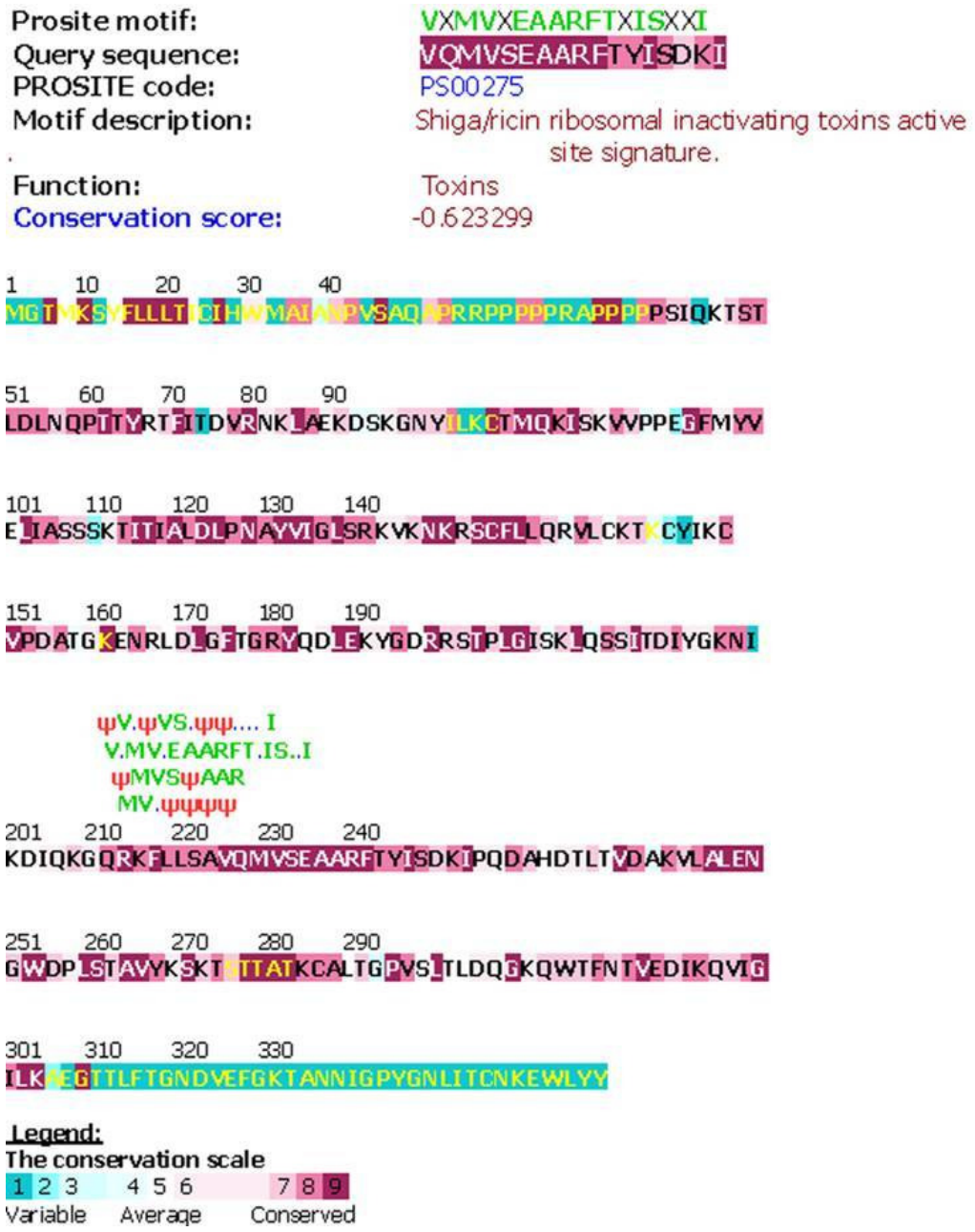


**Fig 17. Quasi Motifinder domain analysis of Bdavp protein**

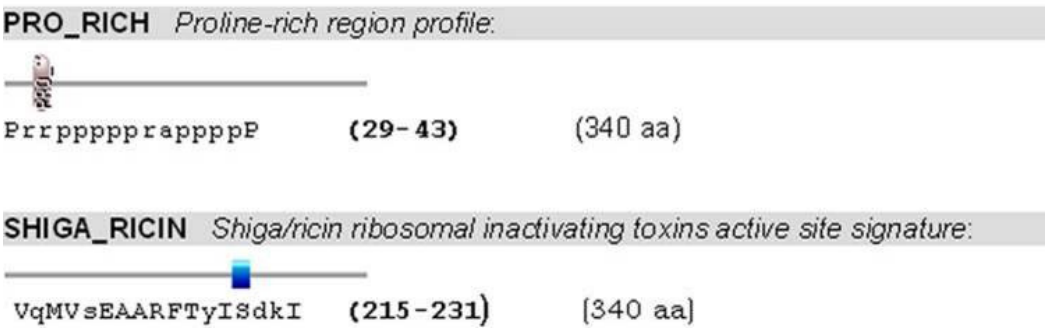


**Fig 18. ScanProsite domain analysis of Bdavp protein**

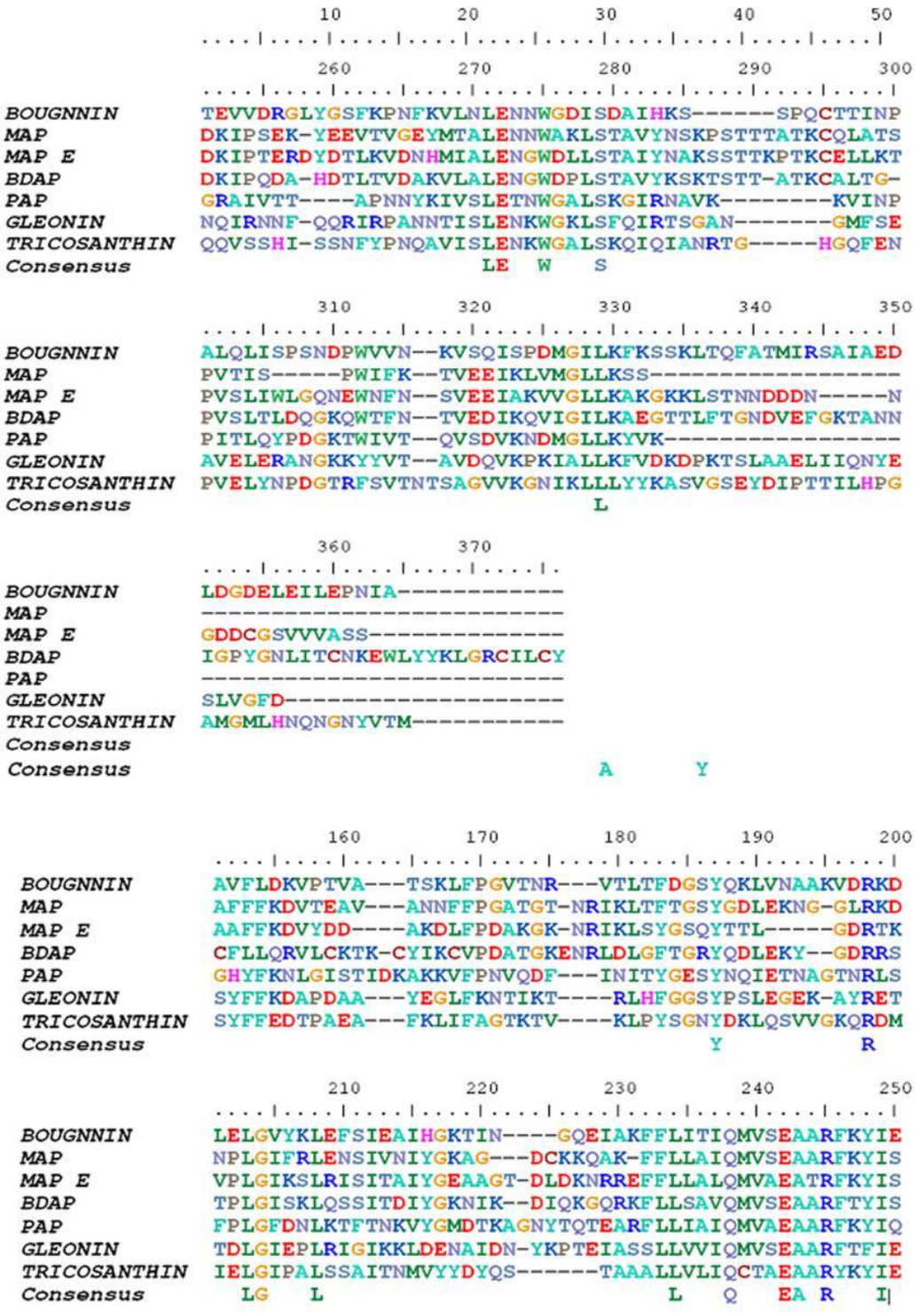


**Fig 19. Alignment of amino acid of Bdavp with other known RIPs**

**Table 2: Similarity Matrix of Boevrhaavin with other known RIPs**

| | Boevrhaavin | ME | MAP | BOUGNIN | PAP | GLEONIN | MOMARDIN | TRICOSANTHIN | JATROPA | RICIN |
|---|---|---|---|---|---|---|---|---|---|---|
| Boevrhaavin | | 48 | 40 | 30 | 24 | 21 | 21 | 18 | 21 | 20 |
| ME (E) | | | 50 | 32 | 29 | 24 | 22 | 23 | 22 | 24 |
| MAP (J) | | | | 29 | 26 | 21 | 25 | 22 | 22 | 22 |
| BOUGANIN | | | | | 26 | 26 | 28 | 25 | 27 | 28 |
| PAP | | | | | | 24 | 27 | 25 | 24 | 26 |
| GLEONIN | | | | | | | 33 | 30 | 30 | 29 |
| MOMARDIN | | | | | | | | 55 | 30 | 31 |
| TRICOSANTHIN | | | | | | | | | 32 | 31 |
| JATROPA | | | | | | | | | | 32 |
| RICIN | | | | | | | | | | |

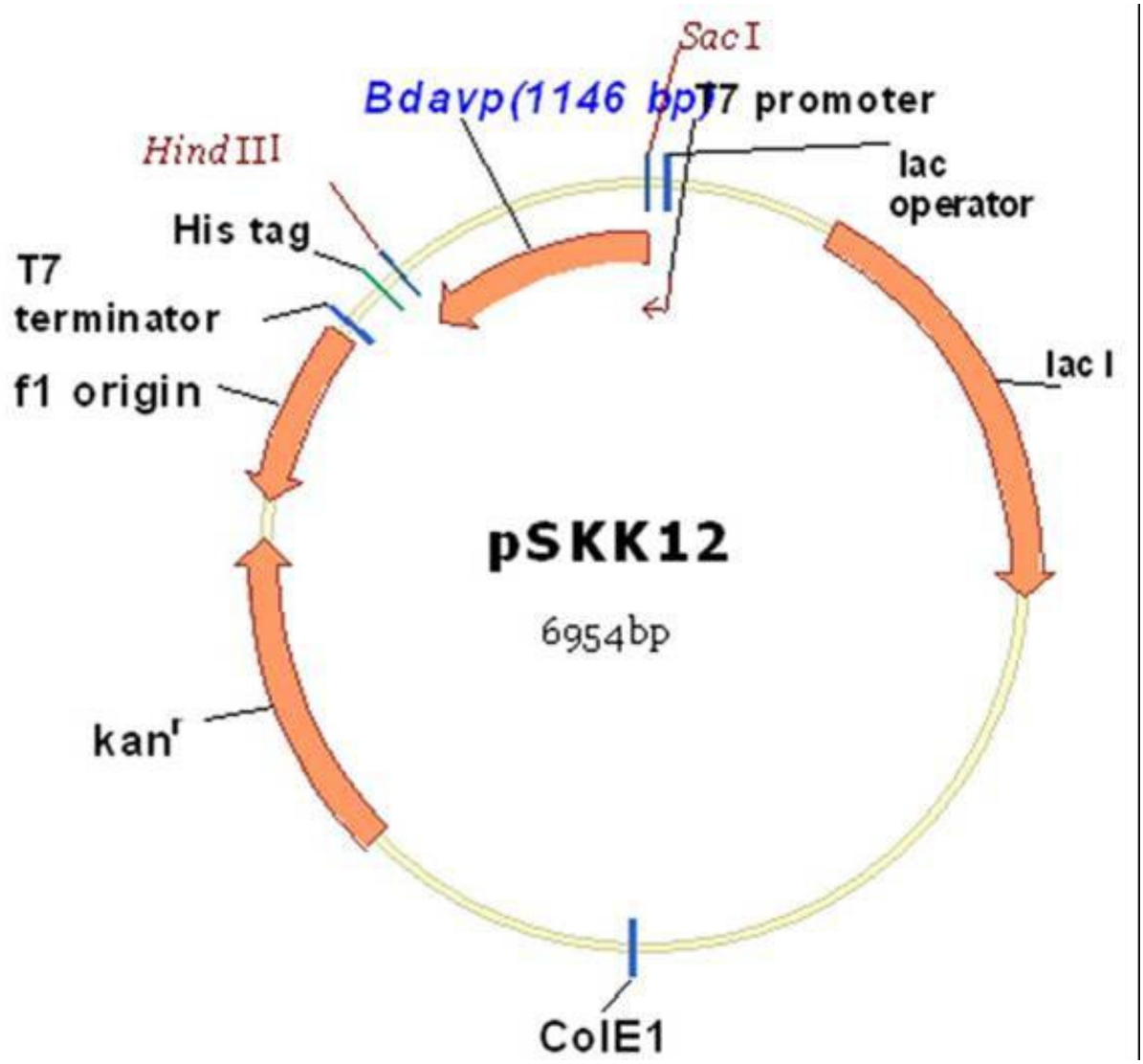


**Fig 20. Restriction map of construct pSKK12 containing full length *bgn* gene in pET28 (a+)**

**Plate 15: Expression of *bdavp* in *E. coli* BL21**

M : Protein marker

1. Induced BL21 (pET28a (+))

2. BL21

3. Uninduced B21 (pET28a (+))

4. Uninduced pSKK12

5. Uninduced pSKK12

6. Induced pSKK12 A

7 Induced pSKK12 B

8 Induced pSKK12 C

9 Induced pSKK12 D

10. Induced pSKK12 E

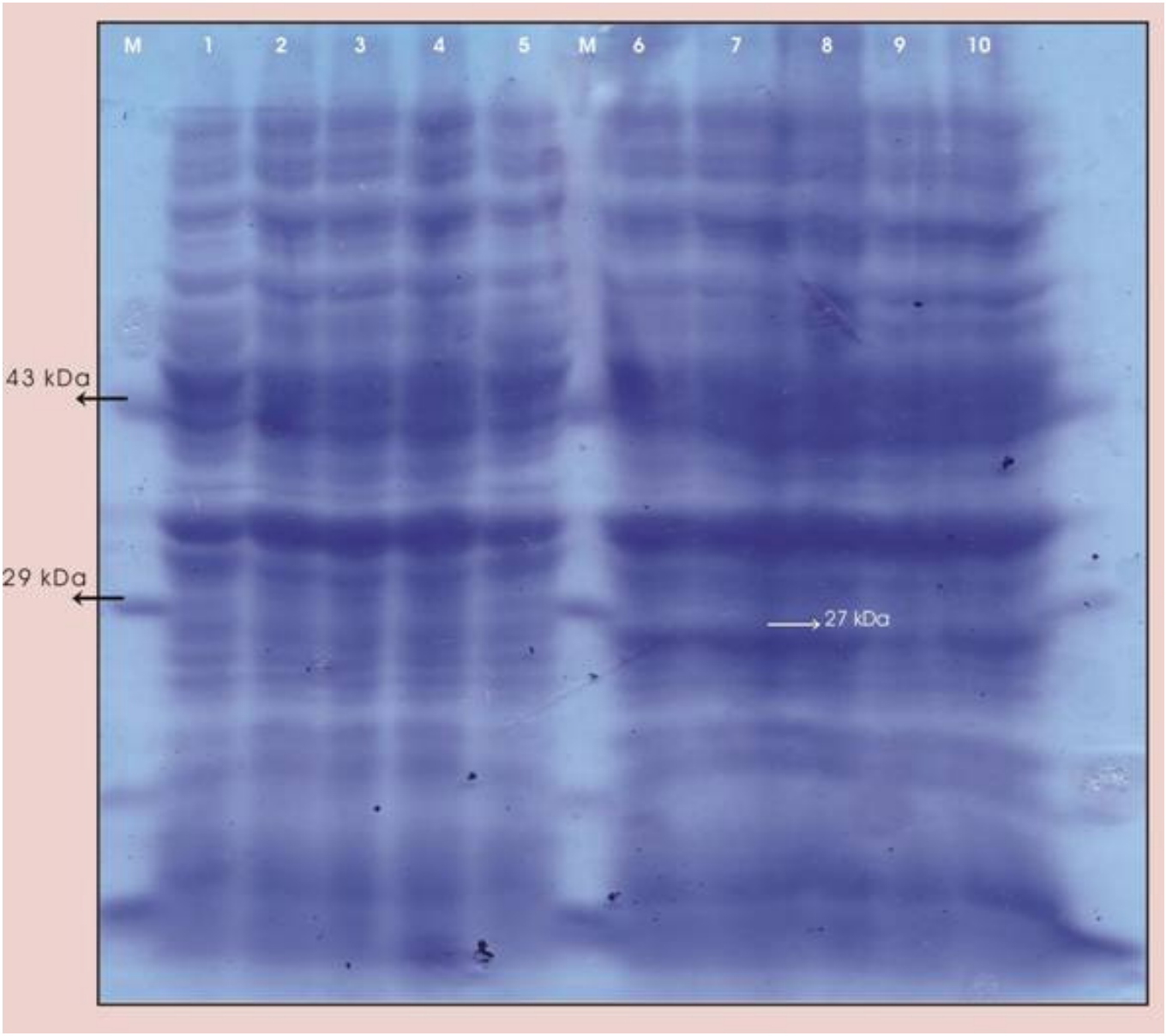


**Plate 15: Expression of *bdavp* in *E. coli* BL21**

# V. DISCUSSION

A vast number of plant pathogens cause diseases in our crops. Their effect ranges from mild symptoms to catastrophes in which large areas planted to food crops are destroyed. Plant pathogens are difficult to control because their populations are highly variable in time, space and the genotype they colonize. About 10 per cent of global food production is lost to plant diseases.

Genes for resistance to diseases are available in primary, secondary and tertiary germplasm for many diseases. Recent development in recombinant DNA technology has let the entire biological resources be considered as one gene pool, permitting transfer of genes across organisms. During the last decade, many genes, whose products are capable of interacting with invading pathogens have been identified and cloned (Takken *et al*., 2000)

Ribosome-inactivating proteins (RIPs) are proteins having an N-glycosidase domain, which can inhibit polypeptide chain elongation by inactivating the ribosomes (Stripe *et al*., 1992). Cloning and characterization of RIP genes has facilitated our understanding on the mode of action of RIPs, their medical and therapeutical applications and their use as antiviral compounds in plant protection.

Bouganin is a type-I RIP that was recently purified from leaves of *Bougainvillea spectabilis* Wild by Bolognesi *et al.* (1997). A cDNA coding for bouganin was cloned and expressed by Marcel et *al*. (2002). In the present study, attempts were made to isolate and characterize a RIP *Bougainvillea spectabilis (RIP)* gene, encoding Ribosome-inactivating protein. Using the sequence information available in database, specific primers were designed for bouganin. About 934bp amplicon was picked up and cloned into pTZ57R/T and transformants having recombinants were isolated through blue-white assay. The white colonies were confirmed as recombinants by gene specific PCR and through restriction analysis. The insert in clone pSKK3680 were sequenced which yielded 934bp.

The comparative analysis showed that the nucleotide sequence of cloned *bgn* gene has 97 per cent homology with published *B. spectabilis (RIP) gene* (DQ98945.1), and *in silico* translated BLAST results also showed 97 per cent amino acid homology with it.

The Bgn amino acid sequence had conserved domain similar to a RIP domain. 934 bp clone having a ORF of 927 bp in pTZ57R subcloned into a prokaryotic expression vector pET28a (+), to study its expression in *E. coli* BL 21 (PlysS). The confirmed *E. coli* containing pSKK3690 were subjected to SDS-PAGE. A 27 KDa protein band corresponding to Bsavp was observed, indicating expression of the cloned gene. Supernatant of pSKK3690 clone inhibited the mycilial growth of plant pathogenic *Sclerotium rolfsii*

Further, in this study an attempt was have been made to clone a novel RIP through the RACE PCR approach from *Boevrhaavia diffusa*. Majority RIPs have been shown to contain conserved amino acid sequences at the active site, which offer opportunities to isolate RIP genes in plant species. RACE-PCR was considered to be most suitable strategy to get at the 5' and 3' ends of the gene starting from the conserved sequences. Using total RNA isolated from leaves of *Boerhaavia diffusa*, 3' RACE was conducted using the degenerate primer designed to conserved amino acid sequences, giving an amplicon of 450bp as expected from blast analysis. This 3' RACE amplicons was cloned into pTZ57R/T and transformants having recombinants were isolated through blue-white assay, confirmed the recombinants by gene specific PCR before sequencing them. In case of 5'RACE, a specific primer was designed using the sequence information from 3' RACE, more than one clone was sequenced to elucidate maximum possible 5'UTR sequence. A 5' segment (650bp) and 3'segment (450bp) of *bdavp* were obtained from RACE-PCR. Subsequently, a new reverse primer designed specifically for 3'end of cDNA, which along with UPM primer could amplify the full-length cDNA of *bdavp*. The cloned *bdavp* gene has 93 bp 5'untranslated region, an open reading frame of 1023 bp, encoding a predicted polypeptide of 340 amino acid residues and a 30 bp 3' untranslated region. The ORF contains initiation codon at position 94 and the ORF is predicted to encode a complete RIP. Sequence comparision indicated that the Bdavp protein is structurally related to type I RIPs from other species, Bdavp has 26 amino acid residues consisting mostly of hydrophobic amino acids at its N -terminal. Computational analysis revealed it as a signal peptide. It has been suggested that the signal peptide is

important for compartmentalization of RIPs (Hartley and Lord, 1993). The database sequence VqMVsEAARFTyISdkI (215-231) of Bdavp protein matches the relatively well conserved region assigned to be the active site of RIPs and the two amino acids Glu $^{177}$ and Arg $^{180}$ (Kim and Robertus, 1992), which are responsible for activity of ricin are also found to be conserved (Glu $^{220}$ and Arg $^{223}$) in the sequence under investigation. The analysis also revealed the presences of Proline-Rich region (29-43). Which is characteristically different from the other reported RIPs, and which might be responsible for bringing proteins together in such a confirmation that favour enhanced antiviral activity (Michael, 1994).

Thus in this study we have isolated a novel RIP gene from *Boerhaavia diffusa*. ~1200bp clone having a ORF of 1023 bp in pTZ57R subcloned into a prokaryotic expression vector pET28a (+), to study its expression in *E. coli* BL 21 (PlysS). The confirmed *E. coli* containing pSKK12 were subjected to SDS-PAGE. 27 KDa protein band corresponding to Bdavp was observed, indicating expression of the cloned gene. However there is a need to study the activity of Bdavp protein against the viruses through in vitro assays and following plant transformation.

# VI. SUMMARY

Several Ribosome inactivating proteins (RIPs) have *been* extensively studied for their antiviral and antitumor activity. RIPs have attracted considerable interest for their possible use in virus resistant transgenic crops and their use in the constuction of immunotoxins. In the present study an attempt was made to clone full length cDNA encoding Ribosome inactivating protein from *Bougainvillea spectabilis* and *Boevrhaavia diffusa* and to study their expression in *E.coli*. The results obtained are summarized below.

1. The *bgn* gene was amplified from *B.spectabilis* using specific primers and the amplicon was cloned in pTZ57R/T vector and transferred to *E.coli* DH5α.
2. Recombinant plasmid pSKK3680 had a full length *bgn* 938 bp.
3. Sequence analysis of *bgn* showed 97 per cent homology with published *bgn* sequence and showed the presence of a RIP domain.
4. *bgn* amplicon from pSKK3680 was cloned into pET 28 (a+), a prokaryotic expression vector, and the resulting clones were named as pSKK3690. SDS-PAGE analysis of the recombinant clones pSKK3690 showed the expression of ~27-kDa protein.
5. To clone a novel full length cDNA encoding Ribosome inactivating protein from *Boevrhaavia diffusa*, RACE-PCR was employed.
6. 3' RACE-PCR with Universal Primer Mix and a degenerate primer designed for the study generated a 450 bp 3' end fragment of the cDNA encoding Bdavp, which was then cloned in pTZ57R/T vector, transferred to *E.coli* DH5α and sequenced the recombinant clone p3bd1 showed homology with known RIPs for this region.
7. A specific antisense primer designed for 3' end, along with Universal Primer Mix in PCR yielded a 650 bp 5' end of the *bdavp* cDNA. The amplicon was cloned in pTZ57R/T vector and transferred to *E.coli* DH5α, and the recombinant clone p5bd1 was sequenced which showed homology with known RIPs.
8. By aligning the sequences of 3' and 5' RACE products, a composite 1167 bp cDNA sequence of *bdavp* was deduced. Further, the complete 1167 bp CDS was obtained using Universal Primer Mix and a sequence specific reverse primer. Full length *bdavp* CDS was cloned in pTZ57R/T vector and transferred to *E.coli* DH5α. Sequencing of recombinant pbdavp, revealed a 1023 bp open reading frame (ORF), 94 bp 5' UTR upstream from start codon and 30 bp 3' UTR down stream of the stop codon.
9. bdavp showed vary low similarity to known RIPs at nucleotide level but at protein level it showed a moderate similarity. Using Quasi MotiFinder software and ScanProsite, major motif of RIP was identified. *bdavp* amplicon from pbdavp was cloned into pET 28 (a+), a prokaryotic expression vector, and the resulting clones were named as pSKK12. SDS-PAGE analysis of the recombinant clones pSKK12 showed the expression of ~27-kDa protein. There is a need to functionally validate the role of *bdavp* gene by in vitro assay and after plant transformation.

# VII. REFERENCES


ALEGRE, C., IGLESIAS, R., FERRERAS, J. M., CITORES, L. AND GIRBES, T., 1996, Sensitivity of ribosomes from *Agrobacterium tumefaciens* to the ribosome-inactivating protein crotin 2 depending on translational state. *Cellular and Molecular Biology.,* **42** : 151-158.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. AND LIPMAN, D. J., 1990, Basic local alignment search tool. Journal of Molecular Biology, **215** : 403–410.

ALTSCHUL, S. F., MADDEN, T. L., SCHAFFER, A. A., ZHANG, J., ZHANG, Z., MILLER, W. AND LIPMAN, D. J., 1997, Gapped BLAST and PSI-BLAST: anew generation of protein database search programs. *Nucleic Acid Research*, **25** : 3389–3402.

ARGON. G. AND IRVIN, J. D., 1980, Inhibition of her-pes simplex virus multiplication by the pokeweed antiviral protein. *Antimicrob Agents Chemother.,* **7** : 1032–33.

BAILEY-SERRES J., 1998, Cytoplasmic ribosomes of higher plants. In *A look beyond transcription: mechanisms determining mRNA stability and translation in plants*, ed. J. Bailey-Serres, D. R. Gallie. pp. 125-144. Vol. 19. Rockville, MD: *American Society of Plant Physiologists*.

BAILEY-SERRES, J., VANGALA, S., SZICK, K. AND LEE, C. H., 1997, Acidic phosphoprotein complex of the 60S ribosomal subunit of maize seedling roots. Components and changes in response to flooding. *Plant Physiology.,* **114** : 1293-1305.

BARBIERI, L., BATTELLI, MG. AND STIRPE, F., 1993, Ribosome-inactivating proteins from plants. *Biochimica Biophysica Acta.,* **1154** : 237-282.

BASS, H. W., WEBSTER, C., OBRIAN, G. R., ROBERTS, J. K. M. AND BOSTON, R. S., 1992, A maize ribosome-inactivating protein is controlled by the transcriptional activator Opaque-2. *Plant Cell,* **4** : 225-234.

BEACHY, R. N., LOESCH-FRIES, S. AND TUMER, N. E., 1990, Coat protein-mediated resistance against virus infection. *Annu. Rev. Phytopathol.,* **28** : 451-474.

BENATTI, L., NITTI, G., SOLINAS, M., VALSASINA, B. AND VITALE, A., 1991, A Saporin-6 CDNA containing a precursor sequence coding for a carboxyl-terminal extension. *FEBS Letters.,* **291** : 285-288.

BIRNBOIN, H. C. AND DOLY, J., 1979, A rapid alkaline procedure for screening recombinant plasmid DNA. *Nucleic Acid Research*, **7** : 1513-1523.

BOLOGNESI, A., POLITO, L., OLIVIERI, F., VALBONESI, P., BARBIERI, L., GIULIA, B. M., VITTORIA, C. M., BENVENUTO, E., DEL VECCHIO BLANCO, F. AND DI MARO, A., 1997, New ribosome- inactivating proteins with polynucleotide: adenosine glycosidase and antiviral activities from *Basella rubra* L. and *B. spectabilis Willd*. *Planta,* **203** : 422-429.

BROEKAERT, W. F., CAMMUE, B. P. A., DE BOLLE, M. F. C., THEVISSEN, K., DE SAMBLANX, G. W. AND OSBORN, R. W., 1997, Antimicrobial peptides from plants. *Critical Reviews in Plant Sciences.,* **16** : 297-323.

BRULAND, O. S., FODSTAD, O., STENWIG, A. E. AND PIHL, A., 1988, Expression and characteristics of a novel human osteosarcoma-associated cell surface antigen. *Cancer Res.,* **48** : 5302–5309.

BUTTERWORTH, A. G. AND LORD, J. M., 1983, Ricin and *Ricinus communis* agglutinin subunits are all derived from a single-size polypeptide precursor. *European Journal of Biochemistry,* **137** : 57-65.

CARZANIGA, R., SINCLAIR, L., FORDHAM-SKELTON, A. P., HARRIS, N., AND CROY, R. D. R., 1994, Cellular and subcellular distribution of saporins, type-1 ribosome-inactivating proteins, in soapwort (*Saponaria officinalis* L.). *Planta,* **194** : 461-470.

CAWLEY, D. B., HEDBLOM, M. L., HOFFMA, E. J. AND HOUSTON L. L., 1977, Differential sensitivity of rat liver and wheat germ ribosomes to polyuridylic acid translation. *Archives in Biochemistry Biophysics,* **182** : 690-695.

CHADDOCK, J. A., MONZINGO, A. F., ROBERTUS, J. D., LORD, J. M. AND ROBERTS, L. M., 1996, Major structural differences between pokeweed antiviral protein and ricin A-chain do not account for their differing ribosome specificity. *Euopean Journal of Biochemistry.,* **235** : 159-166.

CHOW, L. P., CHOU, M. H., HO, C., CHUANG, C. C. AND PAN, F. M., 1999, Purification, characterization and molecular cloning of trichoanguin, a novel type I ribosome-inactivating protein from the seeds of *Trichosanthes anguina*. *Biochemistry Journal,* **338** : 211-219.

CHRISTOPHER, G. W., CIESLAK, T. J. Z., PAVLIN, J. A. AND EITZEN, E. M. J. R., 1997, Biological warfare, A historical perspective. *Jama.,* **278** : 412-417.

THOMPSON, J. D., HIGGINS, D. G. AND T J GIBSON, 1994, CLUSTAL W - Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research,* November 11, **22**(22) : 4673–4680.

CORRELL, C. C., MUNISHKIN, A., CHAN, Y. L., REN, Z., WOOL, I. G., AND STEITZ, T. A., 1998, Crystal structure of the ribosomal RNA domain essential for binding elongation factors. *Proceedings of the National Academy of Science U S A.,* **95** : 13436- 41.

DOWD, P. F., MEHTA, A. D. AND BOSTON, R. S., 1998, Relative toxicity of the maize endosperm ribosome-inactivating protein to insects. *Journal of agricultural and food chemistry* (J. Agric. Food Chem.), **46** : 3775-3779.

DUGGAR, B. M., AND ARMSTRONG, J. K., 1925, The effect of treating virus of tobacco mosaic with juice of various plants. *Ann. Mol. Bot. Gard.,* **12** : 359–365.

ENDO, Y., GLUCK, A. AND WOOL, I. G., 1991, Ribosomal RNA identity elements for ricin A-chain recognition and catalysis. *Journal of Molecular Biology.,* **221** : 193-207.

ENDO, Y., MITSUI, K., MOTIZUKI, M. AND TSURUGI, K., 1987, The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes: the site and the characteristics of the modification in 28S ribosomal RNA caused by the toxins. *Journal of Biological Chemistry.,* **262** : 5908-5912.

ENDO, Y., TSURUGI, K., 1987, RNA N-glycosidase activity of ricin A-chain: mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *Journal of Biological Chemistry,* **262** : 8128-8130.

FRANKEL, A. E., FITZGERALD, D., SIEGALL, C., PRESS, O. W., 1996, Advances in immunotoxin biology and therapy: a summary of the Fourth International Symposium on Immunotoxins. *Cancer Research.,* **56** : 926-32.

FRIGERIO, L., VITALE, A., LORD, J. M., CERIOTTI, A. AND ROBERTS, L. M., 1998, Free ricin A chain, proricin, and native toxin have different cellular fates when expressed in tobacco protoplasts. *Journal of Biological Chemistry.,* **273** :14194-9.

GASPERI-CAMPANI, A., BARBIERI, L., BATTELLI, M. G., STIRPE, F., 1985, On the distribution of ribosome-inactivating proteins amongst plants. *Journal of Natural Products.,* **48** : 446-54.

GATEHOUSE, A. M. R., BARBIERI, L., STIRPE, F. AND CROY R. R. D., 1990, Effects of ribosome inactivating proteins on insect development and differences between Lepidoptera and Coleoptera. *Entomologia Experimentalis et Applicata.,* **54** : 43-51.

GHOLIZADEH, A., BAGHBAN, K. B., SANTHA, I. M., LODHA, M. L. AND KAPOOR, H. C., 2005, Cloning and Expression of Small cDNA Fragment Encoding Strong Antiviral Peptide from *Celosia cristata* in *Escherichia coli. Biochemistry Moscow.,* **70** : 1005-1010.

GOLDBACH, R., 1986, Molecular evolution of plant RNA viruses. *Annu. Rev. Phy-topathol,* **24** : 289–310.

GOLEMBOSKI, D. B., LOMONOSSOFF, G. P. AND ZAITLIN, M., 1990, Plants transformed with a tobacco mosaic nonstructural gene sequence are resistant to the virus. *Proc. Natl. Acad. Sci. USA*., **87** : 6311-6315.

GUO-QING, W., REN-SHUI, L., QIONG, W. AND WANG-YI, L., 2004, Toxicity of two type II ribosome-inactivating proteins (cinnamomin and ricin) to domestic silkworm larvae. *Archives of Insect Biochemistry and Physiology.,* **57** : 160-165.

GUTELL, R. R., GRAY, M. W. AND SCHNARE, M. N., 1993, A compilation of large subunit (23S and 23S-like) ribosomal RNA structures. *Nucleic Acids Research.,* **21** : 3055-74.

HARTLEY, M. R. AND LORD, J. M., 1993, Structure, function and applications of ricin and related cytotoxic proteins. In *Biosynthesis and Manipulation of Plant Products*, ed. D. Griesson. pp. 210-239, New York: Chapman and Hall.

HARTLEY, M. R., CHADDOCK, J. A. AND BONNESS, M. S., 1996, The structure and function of ribosome-inactivating proteins. *Trends in Plant Science.,* **1** : 254-260.

HARTLEY, S. M. AND BEEVERS, H., 1982, Ricin inhibition of *in vitro* protein synthesis by plant ribosomes. *Proceedings of the National Academy of Science USA.,* **79** : 5935-5938.

HOLMBERG, L. AND NYGARD, O., 1996, Depurination of A4256 in 28S rRNA by the ribosome-inactivating proteins from barley and ricin results in different ribosome conformations. *Journal of Molecular Biology.,* **259** : 81-94.

HUDAK, K. A., WANG, P. AND TUMER, N. E., 2000, A novel mechanism of inhibition of translation by pokeweed antiviral protein: depurination of the capped RNA template. *RNA.,* **6** : 369-80.

HUSAIN, J., TICKLE, I. J. AND WOOD, S. P., 1994, Crystal structure of momordin, a type I ribosome inactivating protein from the seeds of *Momordica charantia*. *FEBS Letters.,* **342** : 154-8.

JACKMAN, M. R., ELLIS, J. A., GRAY, S. R., SHURETY, W. AND LUZIO, J. P., 1999, Cell polarization is required for ricin sensitivity in a Caco-2 cell line selected for ricin resistance. *Biochemistry Journal,* **341** : 323-7.

JEAN-LUC, P., JEAN, R. AND HOEVELER., 1994, Isolation and characterization of a cDNA clone encoding the pokeweed antiviral protein II from *Phytolacca Americana* and its expression in *E. coli. FEBS Letters.,* **347** : 268-272.

JENNIFER, K., LODGE, WOJCIECH, K., KANIEWSKI. AND NILGUN, E. TUMER., 1993, Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein Proc. Natl. *Acad. Sci. USA,* **90** : 7089-7093.

JIRO, K. S., NORIYUKI, H., MASAHIRO, F., MASASHI, M., YOICHI, T. AND AKIRA, K., 1991, DNA Sequence of *Mirabilis* Antiviral Protein (MAP), a Ribosome-inactivating Protein with an Antiviral Property, from *Mirabilis jalapa* L. and Its Expression in *Escherichia coli THE JOURNAL OF BIOLOGICAL CHEMISTRY.,* **266** : 8426-8430.

JOUG-SUG, P., DUK- JU, H., SI-MYUNG, L., YEONG-TAE, K., SANG-BONG, C. AND KANG-JIN, C., 2003, Ribosome-inactivating Activity and cDNA Cloning of Antiviral Protein Isoform of *Chenopodium album. Mol. Cells*, **17** : 73-80.

JU-KON, KIM., XIAOLAN, D., RAY, W., SOON, J. S. AND REBECCA, S. B., 1999, In-Cheol Jang, Moo-Young Eun and Baek Hie Nahm Molecular and genetic analysis of transgenic rice plants expressing the maize ribosome-inactivating protein b-32 gene and the herbicide resistance *bar* gene. *Molecular Breeding.,* **5** : 85–94.

KATZIN, B. J., COLLINS, E. J. AND ROBERTUS, J. D., 1991, Structure of ricin A-chain at 2.5 Å. *Proteins,* **10** : 251-259.

KIM, Y. AND ROBERTUS, J., 1992, Analysis of several key active site residues of ricin A chain by mutagenesis and X-ray crystallography *Protein Engineering.,* **5** : 775-779.

KRAWETZ, J. E. AND BOSTON, R. S., 2000, Substrate specificity of a maize ribosome-inactivating protein differs across diverse taxa. *European Journal of Biochemistry.,* **267** : 1966-74.

KREITMAN, R. J., 1999, Immunotoxins in cancer therapy. *Curr Opin Immunol.,* **11** : 570- 8.

KUMAR, M. A., TIMM, D. E., NEET, K. E., OWEN, W. G., PEUMANS, W. J., AND RAO, A. G., 1993, Characterization of the lectin from the bulbs of *Eranthis hyemalis* (winter aconite) as an inhibitor of protein synthesis. *J. Biol. Chem,* **268** : 25176–25183.

LAMB, F. I., ROBERTS, L. M. AND LORD, J. M., 1985, Nucleotide sequence of cloned cDNA coding for preproricin. *European Journal of Biochemistry,* **148** : 265-70.

LEAH, R., TOMMERUP, H., SVENDSEN, I. AND MUNDY, J., 1991, Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *Journal of Biological Chemistry.,* **266** : 1564-1573.

LEE-HUANG, S., HUANG, P. L., HUANG, P. L., BOURINBAIAR, A. S., CHEN, H. C., AND KUNG, H. F., 1995, Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31. *Proc. Natl. Acad. Sci. USA.,* **92** : 8818–8822.

LIN, J., YAN, F., TANG, L. AND CHEN, F., 2003, Antitumor effects of curcin from seeds of *Jatropha curcas. Acta Pharmacol Sin.,* **24** : 241-246.

LODGE, J. K., KANIEWSKI, W. K. AND TUMER, N. E., 1993, Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proceedings of the National Academy of Science U S A,* **90** : 7089-7093.

LOGEMANN, J., JACH, G., TOMMERUP, H., MUNDY, J. AND SCHELL, J., 1992, Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. *Biological-Technology,* **10** : 305-308.

LORD, J. M., 1985, Precursors of ricin and *Ricinus communis* agglutinin. Glycosylation and processing during synthesis and intracellular transport. *European Journal of Biochemistry.,* **146** : 411-6.

MADIN, K., SAWASAKI, T., OGASAWARA, T. AND ENDO, Y., 2000, A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes, *Proceedings of the Natinal Academy of Science U S A.,* **97** : 559-64.

MARCEL, T., DEN, H., CHIARA, L., LOUIS, B., SIJMIE, H., ANTONIO, P. O., BUIJSSE., MARK, D. E. B., AND FIORENZO, S., 2002, Cloning and expression of cDNA coding for bouganin A type-I ribosome-inactivating protein from Bougainvillea spectabilis Wild. *Eur. J. Biochem,* **269** : 1772-1779.

MARIA, G. B., ENZO, LORENZONI., FIORENZO, STIRPE., RINO, C. AND BRUNO, P., 2005, Differential Effect of Ribosome-Inactivating Proteins on Plant Ribosome Activity and Plant Cells Growth. *Journal of Experimental Botany.,* **35** : 882-889.

MEHTA, A. D. AND BOSTON, R. S., 1998, Ribosome-inactivating proteins, In *A look beyond transcription: mechanisms determining mRNA stability and translation in plants*, ed. J. Bailey-Serres, D. R. Gallie. pp. 145-152. Vol. 19. Rockville, MD: American Society of Plant Physiologists.

MICHAEL, P., WILLIAMSON, 1994, The structure and function of proline-rich regions in proteins. *Biochem. J.*, **297** : 249-260.

MLSNA, D., MONZINGO, A. F., KATZIN, B. J., ERNST, S. AND ROBERTUS, J. D., 1993, Structure of recombinant ricin A chain at 2.3 A. *Protein Science.,* **2** : 429-35.

MONTFORT, W., VILLAFRANCA, J. E., MONZINGO, A. F., ERNEST, S. R., KATZIN, B., RUTENBER, E., XUONG, N. H., HAMLIN, R. AND ROBERTUS, J. D., 1987, The three-dimensional structure of ricin at 2.8 Å. *Journal of Biological Chemistry.,* **262** : 5398-5403.

MONZINGO, A. F., COLLINS, E. J., ERNST, S. R., IRVIN, J. D. AND ROBERTUS, J. D., 1993, The 2.5 Å structure of pokeweed antiviral protein. *Journal of Molecular Biology.,* **233** : 705-715.

MORRIS, K. N., WOOL, I. G., 1994, Analysis of the contribution of an amphiphilic α-helix to the structure and to the function of ricin A chain. *Proceedings of the National Academy of Science U S A.,* **91** : 7530-7533.

MUNDY, J., LEAH, R., BOSTON, R., ENDO, Y. AND STIRPE, F., 1994, Genes encoding ribosome- inactivating proteins. *Plant Molecular Biology Report.,* **12** : 560-562.

MUNISHKIN, A. AND WOOL, I. G., 1995, Systematic Deletion analysis of ricin A-chain function, single amino acid deletions. *Journal of Biological Chemistry.,* **270** : 30581-30587.

NEUHAUS, J. M. AND ROGERS, J. C., 1998, Sorting of proteins to vacuoles in plant cells. *Plant Molecular Biology.,* **38** : 127-44.

NIELSEN, K. AND BOSTON, R. S. 2001, RIPs: a plant perspective. *Annual Review of Plant Physiology and Plant Molecular Biology,* **52** : 785-816.

NILGUN, E., TUMER, DUK-JU HWANG. AND MAUREEN. BONNESS, 1997, C-terminal deletion mutant of pokeweed antiviral protein inhibits viral infection but does not depurinate host ribosomes. *Proc. Natl. Acad. Sci.,* **94** : 3866-3871.

NILSSON, L., ASANO, K., SVENSSON, B., POULSEN, F. M. AND NYGARD, O., 1986, Reduced turnover of the elongation factor EF-1 X ribosome complex after treatment with the protein synthesis inhibitor II from barley seeds. *Biochimica Biophysica Acta.,* **868** : 62-70.

O'HARE, M., ROBERTS, L. M., THORPE, P. E., WATSON, G. J., PRIOR, B., AND LORD, J. M., 1987, Expression of ricin A chain in *Escherichia coli. FEBS LetI.,* **216** : 73-78.

ODA, T., KOMATSU, N. AND MURAMATSU, T., 1997, Cell lysis induced by ricin D and ricin E in various cell lines. *Biosci. Biotechnol. Biochem.,* **61** : 291–297.

OLSNES, S. AND PIHL, A., 1982, Chimeric toxins, *Pharmaceutical Therapy.,* **15** : 355-381.

ORITA, M., NISHIKAWA F., SHIMAYAMA, T., TAIRA, K., ENDO, Y. AND NISHIKAWA, S., 1993, High- resolution NMR study of a synthetic oligoribonucleotide with a tetranucleotide GAGA loop that is a substrate for the cytotoxic protein, ricin. *Nucleic Acids Research.,* **21** : 5670-8.

PAROMA, GHOSH. AND JANENDRA, K. BATRA., 2006, The differential catalytic activity of ribosome inactivating protein saporin 5 and 6 is due to a single substitution at position 162**.** *Biochemical Journal Immediate Publication. manuscript BJ20060895.*

PASTON, I. AND FITZGERALD, D., 1991, Recombinant toxins for cancer treatment. *Science.,* **254** : 1173-1176.

PEUMANS, W. J., HAO, Q. AND VANDAMME, E. J. M., 2001, Ribosome-inactivating proteins from plants: more than RNA N-glyco-sidases? *FASEB J.*, **15** : 1493-1506.

POYET, J. L., HOEVELER, A. AND JORGENEEL, C. V., 1998, Analysis of active site residues of the antiviral protein from summer leaves from *Phytolacca americana* by site-directed mutagenesis. *Biochemical and Biophysical Research Communications,* **253** : 582-587.

RAMAKRISHNAN, S. AND HOUSTON, L. L., 1984, Inhibition of human acute lymphoblastic leukemia cells by immuno toxins : potentiation by chloroquine. *Science.,* **223** : 58–61.

READY, M. P., BROWN, D. T., ROBERTUS, J. D., 1986, Extracellular localization of pokeweed antiviral protein. *Proceedings of the National Academy of Science U S A,* **83** : 5053-6.

REINBOTHE, S., MOLLENHAUER, B. AND REINBOTHE, C., 1994a, JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *Plant Cell.,* **6** : 1197-209.

REINBOTHE, S., REINBOTHE, C., LEHMANN, J., BECKER, W., APEL, K. AND PARTHIER, B., 1994b, JIP60, a methyl jasmonate-induced ribosome-inactivating protein involved in plant stress reactions. *Proceedings of the National Academy of Science U S A,* **91** : 7012-7016.

REZZONICO, E., N. FLURY, F. MEINS, J. R., AND R. BEFFA, 1998, Transcriptional down-regulation by abscisic acid of pathogenesis-related β-1,3-glucanase genes in tobacco cell cultures. *Plant Physiol*., **117** : 585–592.

RICH, B. E. AND STEITZ, J. A., 1987, Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. *Molecular and Cellular Biology,* **7** : 4065-4074.

ROBERTS, L. M., LAMB, E., PAPPIN, D. J. C. AND LORD, J. M., 1985, The primary sequence of *Ricznus communis* agglutinin: comparison with ricin. *J. Biol. Chem.,* **260** : 15682-15686.

RUTENBERS, E., KATZIN, B. J., COLLINS, E. J., MLSNA, D., ERNST, S., READY, M. P., AND ROBERSUS, J. D., 1991, The crystallographic refinement of ricin at 2.5A resolution. *Proteins.,* **10** : 240-250.

SAMBROOK, J., AND RUSSEL, D. W., 2001, Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, New York. Pp. A8, 52-55.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T. H., 1989, *Molecular Cloning - A Laboratory Manual*, 2nd Edition. Cold Spring Habour Laboratory Press, New York.

SAVINO, C., FEDERICI, L., IPPOLITI, R., LENDARO, E. AND TSERNOGLOU, D., 2000. The crystal stucture of saporin SO6 from *Saponaria officinalis* and its interaction with the ribosome. *FEBS Letters,* **470** : 239-243.

SEHNKE, P. C. AND FERL, R. J., 1999, Processing of preproricin in transgenic tobacco. *Protein Expression and Purification,* **15** : 188-95.

SMIRNOV, S., SHUALEV, V., AND TUMER, N. E., 1997, Expression of pokeweed antiviral protein in transgenic plants induces virus resistance in grafted wild-type plants independently of salicylic acid accumulation and pathogenesis-related protein synthesis. *Plant Physiol.,* **114** : 1113–1121.

SONG, S. K., CHOI, Y., MOON, Y. H., KIM, S. G., CHOI, Y. D. AND LEE, J. S., 2000, Systemic induction of a *Phytolacca insularis* antiviral protein gene by mechanical wounding, jasmonic acid, and abscisic acid. *Plant Molecular Biology.,* **43** : 439-50.

SPINA, C. A., MYERS, D. E., KUEBELBECK, V., LEDBETTER, J. A. AND UCKUN, F. M., 1990, Inhibition of HIV replication by pokeweed antiviral protein targeted to CD41 cells by monoclonal antibodies. *Nature.,* **347** : 92–95.

SPOONER, R. A. AND LORD, J. M., 1990, Immunotoxins: status and prospects. *Trends in Biotechnology,* **8** :189-193.

STIRPE, F., BARBIERI, L., BATTELLI, M. G., SORIA, M. AND LAPPI, D. A., 1992, Ribosome-inactivating proteins from plants: present status and future prospects. *Biotechnology,* **10** : 405-412.

STRAUSS, J. H., STRAUSS, E. G., 1988, Evolution of RNA viruses. *Annu. Rev. Microbiol.,* **42** : 657–83.

SUROLIA, N. AND MISQUITH, S., 1996, Cell surface receptor directed targeting of toxin to human malaria parasite, *Plasmodium falciparum*. *FEBS Lett*., **396** : 57–61.

SZEWCZAK, A. A, AND MOORE, P. B., 1995, The sarcin/ricin loop, a modular RNA. *Journal of Molecular Biology.,* **247** : 81-98.

SZEWCZAK, A. A., CHAN, Y. L., MOORE, P. B. AND WOOL, I. G., 1991, On the conformation of the alpha sarchin stem-loop of 28S rRNA. *Biochemie,* **73** : 871-877.

TAKKEN, F. L. W., LUDERER, R., GABRIELS, S., WESTERINK, N., LU, R., DE, WIT. P. J. G. M. AND JOOSTEN, MHAJ, 2000, A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J.*, **24** : 275.

THROPE., EDWARDS, D. C., DAVIES, A. J. S., ROSS, W. C. J., 1982, Monoclonal antibody-toxin conjugates: aiming the magnetic bullet. In *Monoclonal Antibodies in Clin-ical Medicine*, ed. *J Fabre,* 67–201.

TSANN-HUEI, WU., LU-PING, C. AND JUNG-YAW, LIN., 1998, Sechiumin, a ribosome-inactivating protein from the edible gourd, *Sechium edule* Swartz Purification, characterization, molecular cloning and expression. *Eur. J. Biochem.*, **255** : 400-408.

UCHIUMI, T., WAHBA, A. J. AND TRAUT, R. R., 1987, Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. *Proceedings of the National Academy of Science U S A.,* **84** : 5580-5584.

VANDENBUSSCHE, F., PEUMANS, W. J., DESMYTER, S. PROOST, C., MARIALIBERA. AND VAN, D. E. J. M., 2004, The type-1 and type-2 ribosome-inactivating proteins from Iris confer transgenic tobacco plants local but not systemic protection against viruses. *Planta.,* **220** : 211-221.

VATER, C. A., BARTLE, L. M., LESZYK, J. D., LAMBERT, J. M. AND GOLDMACHER, V. S., 1995, Ricin A chain can be chemically cross-linked to the mammalian ribosomal proteins L9 and L10e. *Journal of Biological Chemistry.,* **270** : 12933-12940.

VITALE, A. AND RAIKHEL, N. V., 1999, What do proteins need to reach different vacuoles? *Trends in Plant Science.,* **4** : 149-155.

VIVANCO, J. M., SAVARY, B. J., FLORES, H. E., 1999, Characterization of two novel type I ribosome-inactivating proteins from the storage roots of the andean crop *Mirabilis expansa*. *Plant Physiology,* **119** : 1447-1456.

WALSH, T. A., MORGAN, A. E. AND HEY, T. D., 1991, Characterization and molecular cloning of a proenzyme form of a ribosome-inactivating protein from maize: Novel mechanism of proenzyme activation by proteolytic removal of a 2.8- kilodalton internal peptide segment. *Journal of Biological Chemistry,* **266** : 23422-23427.

WANG, P., HUDAK, K. A. AND TUMER, N. E., 2000, A novel mechanism of inhibition of translation by pokeweed antiviral protein: depurination of the capped RNA template. *RNA.,* **6** : 369-80.

WANG, P., ZOUBENKO, O., AND TUMER, N. E., 1998, Reduced toxicity and broad spectrum resistance to viral and fungal infection in transgenic plants expressing pokeweed antiviral protein II. *Plant Mol. Biol..,* **38** : 957–964.

WANG, Y. X., NEAMATI, N., JACOB, J., PALMER, I. AND STAHL, S. J., 1999, Solution structure of anti-HIV-1 and anti-tumor protein MAP30: structural insights into its multiple functions. *Cell,* **99** : 433-42.

WATANABE, K., HONJO, E., TSUKAMOTO, T. AND FUNATSU, G., 1992, Fluorescence studies on the interaction of adenine with ricin A-chain. *FEBS Letters.,* **304** : 249-251.

WIENER, S. L., 1996, Strategies for the prevention of a successful biological warfare aerosol attack. *Military Medicine,* **161** : 251-256.

WU, T. H., CHOW, L. P. AND LIN, J. Y., 1998, Sechiumin, a ribosome-inactivating protein from the edible gourd, *Sechium edule* Swartz--purification, characterization, molecular cloning and expression. *European Journal of Biochemistry.,* **255** : 400-8.

XIA, X. F., AND S. F. SUI., 2000, The membrane insertion of Trichosanthin is membrane-surface-pH dependent. *Biochem. J.*, **349** : 835–841.

ZARLING, J. M., MORAN, P. A., HAFFAR, O., SIAS, J., RICHMAN, D. D., SPINA, C. A., MYERS, D. E., KUEBELBECK, V., LEDBETTER, J. AND UCKUN, F. M., 1990, Inhibition of HIV replication by pokeweed antiviral protein targeted to CD 41 cells by monoclonal antibodies. *Nature*, **347** : 92-95.

ZHANG, C. Y., GONG, Y. X., MA, H., AND CHEN, D. Y., 2000, Trichosanthin induced calcium-dependent generation of reactive oxygen species in human choriocarcinoma cells. *Analyst* **125** : 1539–42.

ZHANG, G. P., SHI, Y. L., WANG, W. P. AND LIU, W. Y., 1999, Cation channel formed at lipid bilayer by Cinnamomin, a new type II ribosome-inactivating protein. *Toxicon,* **37** : 1313–1322.

ZHOU, K., FU, Z., CHEN, M., LIN, Y. AND PAN, K., 1994, Structure of tricosanthin at 1.88 Å resolution. *Proteins,* **19** : 4-13.

ZHOU, X., LIX, D., YUAN, J. Z., TANG, Z. H. AND WANG, Y. L., 2000, Toxicity of Cinnomomin- a new type II ribosome- inactivating protein to bollworm and mosquito. *Insect Biochemistry and Molecular Biology*., **30** : 259-264.

ZHU, R. H., NG, T. B., YEUNG, H. W. AND SHAW, P. C., 1992, High level synthesis of biologically active recombinant trichosanthin in *Escherichia coli*, *Int. J. Peptide Protein Res.,* **39** : 77-81.

ZOUBENKO, O., HUDAK, K., AND TUMER, N. E., 2000, A non-toxic pokeweed antiviral protein mutant inhibits pathogen infection via a novel salicylic acid-independent pathway. *Plant Mol. Biol.,* **44** : 219–229.

ZOUBENKO, O., UCKUN, F., HUR, Y., CHET, I., AND TUMER, N., 1997, Plant resistance to fungal infection induced by nontoxic pokeweed antiviral protein mutants. *Nat. Biol. Technol.,* **15** : 992–996.

**APPENDIX I. Master mix for *bgn* amplification**

| **Components** | **Concentration** | **Volume (µl)** |
|---|---|---|
| dNTPs | 2.5 mM/ µl | 1.0 |
| Taq assay buffer | 1X/ µl | 2.5 |
| Forward primer | 40 pM/ µl | 1.0 |
| Reverse primer | 40 pM/ µl | 1.0 |
| Taq DNA polymerase | 1 U | 0.33 |
| RT product cDNA | 1 µg/µl | 5.0 |
| RNAse free water | - | 13.5 |
| Total | - | 25.0 |

## APPENDIX II. Agarose gel electrophoresis

**A ) Loading dye composition(6X)**

0.25% Bromophenol blue (BPB)

0.25% xylene cyanol (optional)

30% glycerol in water

**B) Recipe for 1 per cent Agarose gel (40 ml)**

| | |
|---|---|
| Agarose | 400 mg |
| 1x TAE | 40 ml |
| EtBr (10 mg/ml) | 2 µl |

**C) 50x TAE composition**

| | |
|---|---|
| Tris base | 242 g |
| Glacial acetic acid | 57.1 ml |
| 0.5 M EDTA (pH 8.0) | 100 ml |

Total volume 1000 ml with double distilled water.

**D) Luria agar**

| Ingredients | Concentration (g/l) |
|---|---|
| Tryptone | 10.0 |
| Yeast extract | 5.0 |
| Sodium chloride | 5.0 |
| Agar | 18.0 |
| pH | 7.2 |

## APPENDIX III. Ligation recipes

### A) Conversion table for the amount of a PCR fragment required per ligation reaction

| Length of DNA fragment (bp) | picomoles of ends per 1 µg of DNA | Quantity of PCR fragments for ligation reacting in µg (0.54 pmol ends) |
|---|---|---|
| 100 | 30.0 | 0.018 |
| 300 | 10.0 | 0.054 |
| 500 | 6.0 | 0.090 |
| 1000 | 3.0 | 0.180 |
| 2000 | 1.5 | 0.360 |
| 3000 | 1.0 | 0.540 |

### B) Ligation reaction recipe

| | |
|---|---|
| Plasmid vector pTZ57R/T DNA (0.165 µg, 0.18 pmol ends) | 3.0 µl |
| Purified PCR fragment, (Approx. 0.54 pmol ends) | 10.0 µl |
| 10x ligation buffer | 3.0 µl |
| PEG 4000 solution | 3.0 µl |
| Deionized water | 10.0 µl |
| T4 DNA ligase, 5U | 1.0 µl |
| Total | 30 µl |

### C) Control ligation reaction recipe

| | |
|---|---|
| PTZ57R/T DNA (0.165 µg, 0.18 pmol ends) | 3.0 µl |
| Purified PCR fragment (Approx. 0.54 pmol ends) | 12.3 µl |
| 10x ligation buffer | 3.0 µl |
| PEG 4000 solution | 3.0 µl |
| Deionized water upto | 29 µl |
| T4 DNA ligase, 5 U | 1.0 µl |
| Total | 30 µl |

## APPENDIX IV. Reagents for plasmid isolation

**STET buffer**

| | |
|---|---|
| Tris-Cl (pH 8.0) | 10 mm |
| NaCl | 0.1 M |
| EDTA (pH 8.0) | 1.0 mM |

Autoclaved and stored at 4℃

---

**Alkaline lysis solution I**

| | |
|---|---|
| Glucose | 50 mM |
| Tris-Cl (pH 8.0) | 25 mM |
| EDTA (pH 8.0) | 25 mM |

Autoclaved and stored at 4℃

---

**Alkaline lysis solution II**

| | |
|---|---|
| NaOH | 0.2 N |
| SDS | 1% (w/v) |

(Prepared fresh and used at room temperature)

---

**Alkaline lysis solution III**

| | |
|---|---|
| 5 M potassium acetate | 60 ml |
| Glacial acetic acid | 11.5 ml |
| Double distilled water | 28.5 ml |

Autoclaved and stored at 4℃

---

## APPENDIX V. Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS PAGE)

**A) Acrylamide solution (30 per cent)**

| | |
|---|---|
| Acrylamide | 29 g |
| Bis acrylamide | 1g |

Made up the volume to 100 ml using the distilled water and stored at $4^{O}C$ ( Do not autoclave).

**B) 4X resolving gel buffer (pH 8.8)**

| | |
|---|---|
| Tris Cl (1.5 M) | 182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 8.8 using 1M HCl and final volume was made up to 1000ml. |

**C) 4X stacking gel buffer (pH 6.8)**

| | |
|---|---|
| Tris Cl (1.5 M) | 182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 6.8 using 1M HCl and final volume was made up to 1000ml. |

**D) Ammonium per sulphate (APS) 10%**

(Add the preparation) It has to be prepared fresh, Don't autoclave.

**E) Tank Buffer**

| | |
|---|---|
| Tris Base | 25 mM |
| Glycin pH 8.3 | 250 m M |
| SDS | 0.1 per cent |

**F) SDS PAGE Gel**

| | Resolving gel | Stacking Gel |
|---|---|---|
| | **12 %** | **5 %** |
| Water | 13.2 ml | 6.8 ml |
| 30 % Acrylamide mix | 16.0 ml | 1.7 ml |
| 1.5 M Tris (pH 8.8) | 10.0 ml | 1.25 ml |
| 10% SDS | 0.4 ml | 0.1 ml |
| 10 % APS | 0.4 ml | 0.1 ml |
| TEMED | 0.016 ml | 0.01 ml |
| **Total** | **40 ml** | **10 ml** |

| **G) Sample loading dye for SDS PAGE (for 2 ml)** | |
|---|---|
| Tris-Cl (100mM, pH 6.8) | 200 µl |
| DTT (200mM) | 100 µl |
| Glycerol (20 %) | 400 µl |
| Bromo Phenol Blue | 4 mg |
| SDS (4 %) | 800 µl |
| Deionized water | Made up to 2 ml |

| **H) Staining solution (For one liter)** | |
|---|---|
| Commasine Brilliant blue | 2.5 mg |
| Methenol | 500 ml |
| Acetic acid | 100 ml |
| Distilled water | 400 ml |

| **I) Destainig solution (for one liter)** | |
|---|---|
| Methenol | 300 ml |
| Acetic acid | 100 ml |
| Distilled water | 600 ml |

## APPENDIX VI

**Ligation of *bgn* insert and pET28 (a+)**

| | |
|---|---|
| *bgn* insert | 6 µl |
| Plasmid DNA pET28 | 2 µl |
| $T_4$ DNA Ligase Enzyme (10U) | 1 µl |
| Buffer (10X) | 1 µl |
| Sterile $H_2O$ | 10 µl |
| TOTAL | 20 µl |

**Control Ligation of pET28**

| | |
|---|---|
| Plasmid DNA pET28 | 2 µl |
| $T_4$ DNA Ligase Enzyme (10U) | 1 µl |
| Buffer (10X) | 1 µl |
| Sterile $H_2O$ | 16 µl |
| TOTAL | 20 µl |

# CLONING AND EXPRESSION OF cDNA ENCODING RIBOSOME INACTIVATING PROTEINS

SALAR ABBAS 2007 Dr.P.U. KRISHNARAJ
Major Advisor

## ABSTRACT

Ribosome Inactivating Proteins (RIPs) play a role in plant defence and hence can be exploited in plant protection. The focus of the present study was to clone and express full length cDNA encoding RIP from *Bougainvillea spectabilis* and *Boevrhaavia diffusa*. A 938 bp amplicon obtained on PCR amplification using *bgn* specific primers was cloned into pTZ57R/T. The recombinant clones pSKK3680 obtained were positive for specific amplification of *bgn*. Sequence analysis of bgn showed 97 per cent homology with published *bgn* sequence. Further, the *bgn* was sub cloned into prokaryotic expression vector pET28a (+) and expressed in *E. coli* BL21 plysS. The recombinant clone's pSKK3690 showed expression of ~27 KDa protein, the protein expressed inhibited mycilial growth of *Sclerotium rolfsi*. Based on short sequence information, a degenerate primer was designed using which a 450 bp 3'region amplicon was amplified by 3' Rapid Amplification of cDNA Ends (RACE) technique and 650 bp 5' region amplicon was amplified using 5'RACE.

A complete 1167 bp CDS of *bdavp* was cloned in pTZ57R/T. Sequencing of recombinant pbdavp, revealed a 1023 bp Open Reading Frame (ORF), 94 bp 5'UTR upstream from start codon and 30bp 3' UTR downstream of the stop codon. *bdavp* showed low and moderate similarity at nucleotide and protein level respectively. The *bdavp* amplicon was sub cloned into prokaryotic expression vector pET28a (+) and expressed in E.coli BL21 plysS. Recombinant clones pSKK12 showed the expression of ~27KDa protein.