DISCUSSION
V. DISCUSSION

The development of pathogen resistant traits has become an important target in plant biotechnology, since the traditional approaches to control epidemic spread of diseases are no longer sufficient. Using molecular techniques, various natural disease resistance genes have been isolated during the last few years. There has been an increasing interest in a novel transgenic approach in development of disease resistance. The continuous progress in plant transformation and gene cloning techniques has opened new avenues for augmenting disease resistance in crops. The concept of genetically acquired resistance includes a variety of strategies based on the transgenic expression of genes in plants from different origins. Some of the potentially useful transgenes include several classes of antifungal proteins that involve inhibition of the synthesis of the fungal cell wall or inhibit the fungal proteins or enzyme. This group includes various pathogenesis related (PR) proteins. Chitinase is one of the most important PR protein which is used to improve plant defense against fungal pathogens.

The first attempts to use a transgenic chitinase to enhance plant defense were made by using a bacterial chitinase gene chiA from *Serratia marcescens*, by Jones *et al.* (1986). The chiA from *Serratia marcescens* showed significant reduction in necrosis and chlorosis development due to *Alternaria longipes* in tobacco (Suslow *et al.*, 1988). Similarly the genes encoding chitinase from plant have also been used for transformation with varying rates of success (Broglie *et al.*, 1991; Punja and Raharjo, 1996; Marchant *et al.*, 1998; Yamamoto *et al.*, 2000). However Lorito *et al.* (1998) successfully demonstrated the expression of an endochitinase gene from a biocontrol
agent, *Trichoderma harzianum*, conferring resistance to *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, *Rhizoctonia solani* and *Sclerotia sclerotium* in several crop plants. This study conclusively demonstrated the usefulness of the fungal chitinase gene as a source of disease resistance genes, which apparently overcome the limitations of using plant chitinase genes.

In addition to these, there are also a group of fungi known as entomopathogenic fungi, which are used as biological agents against insect pests. *Metarhizium anisopliae* and *Nomuraea rileyi* are the two well known fungi belonging to this group, which have parasitic or pathogenic association with arthropods. As chitin is the main component of the tough exoskeleton of arthropods, it is expected that these fungi may have very high chitinolytic activity. Although a number of chitinase genes have been isolated from *M. anisopliae* and *N. rileyi* (Inglis *et al.*, 1997; Bogo *et al.*, 1998; Kang *et al.*, 1998; Wattanalai *et al.*, 2004), there are no reports of testing these chitinase genes transgenically in plant to improve disease resistance. Keeping this in view, in the present study the chitinase gene was isolated from *N. rileyi/M. anisopliae* and the results pertaining to the isolation and expression of isolated chitinase gene upon transformation in tobacco are discussed hereunder.

### 5.1 Isolation of chitinase gene from *N. rileyi/M. anisopliae*

There are several methods available for gene isolation. However, the most commonly used are through screening of the libraries (genomic and cDNA libraries) and amplification through polymerase chain reaction. The libraries are screened based on radioactively or fluorescently labeled DNA probe which is complementary or partially complementary to a region of the gene sequence, and which can be used to detect it by
hybridization. The probe sequence might be an oligonucleotide derived from the sequence of the protein product of the gene or by the polymerase chain reaction. The other most widely used approach is through PCR technique which is mainly based on sequence conservation.

5.1.1 RNA isolation

The first step in the isolation of gene involves extraction of good quality RNA or DNA. Isolation of pure and intact RNA is the most critical step involved in all experiments leading to cloning. The isolation of undegraded RNA requires inhibition of high levels of endogenous ribonucleases and efficient deproteinization of the RNA. Major protocols of RNA extraction use guanidium thiocyanate reagent alone (Delye and Corio, 1998; Inglis and Inglis, 1997) or in combination with phenol – chloroform (Chomczynski and Sacchi, 1987). Another protocol given by Datta et al. (1989) involves the phenol-chloroform extraction with β-mercaptoethanol coupled with LiCl precipitation. In the present study, the protocol of Datta et al. (1989) was followed with some modification to isolate the RNA from *N. rileyi* and *M. anisopliae*. The yield of RNA varied ranging from 2-10 µg/gm of fungal mycelia. Denaturing agarose gel resolutions of total RNA extraction revealed a good banding pattern of undegraded 28S and 16S ribosomal bands. The absorbance ratio of A260/A280 was found to be 1.8-2.0 indicating the good quality of RNA.

5.1.2 DNA isolation

The isolation of DNA with high molecular weight and sufficient purity is an essential requirement in cloning experiments. The presence of contaminant such as proteins, phenols, polysaccharides and other secondary metabolites inhibit the enzyme
action in PCR. Purification has to be done to get clear DNA, which is consistently amplifiable in PCR. There are several protocols available for the extraction of DNA (Murray and Thompson, 1980; Tai and Tanksley, 1990). A modified CTAB method of DNA extraction is relatively quick, inexpensive and consistent protocol for leaf samples having large quantities of polyphenols and polysaccharides (Porebski et al., 1997). The same protocol with some modification was followed to isolate the DNA form *M. anisopliae*. The yield of DNA varied ranging from 6-10 μg/gm of fungal tissue. Agarose gel electrophoresis of DNA revealed a good banding pattern. The absorbance ratio of A260/A280 was found to be around 1.8 indicating the purity of the preparation.

5.1.3 Designing primer for chitinase gene amplification

5.1.3.1 Designing degenerate primers of chitinase

In case of PCR based gene isolation technique, successful amplification of a DNA segment of unknown sequence by PCR depends primarily on the sequence of primers. Primers can be degenerate or gene specific.

Degeneracy in primer sequence helps in designing the primers that amplify a specific DNA from heterologous organisms. Three sets of degenerate primers designed from the eight fungal species belonged to the order Moniliales to which the fungus in study belongs. The degenerate primers based on the amino acid sequence of conserved regions have been used to search for members of a gene family (Wilks *et al.*, 1989). Computer programmes have also been developed specifically to design degenerate primers (McGreath *et al.*, 1995; Inglis *et al.*, 1997; Bogo *et al.*, 1998 and Watannalai *et al.*, 2004).
5.1.3 **Isolation of chitinase gene using degenerate primers of chitinase**

5.1.4 **RT-PCR**

The conversion of RNA sequences to first strand cDNA is referred as RT-PCR. The successful cDNA synthesis should yield full length copies of the original population of RNA molecules. First strand synthesis is primed with oligo (dT) linker-primer or sometimes with reverse gene specific primer in the presence of nucleotides and buffer. Two types of enzymes are commonly used and are commercially available (1) Avian myeloblastosis virus (AMV) and Molony murine leukemia virus (MMLV). The RNA of *N. rileyi* and *M. anisopliae* was primed with oligo-dT (23) and the AMV enzyme was used as it has powerful RNase H activity and it works well at 42°C, which is particularly useful when the RNA template has a high degree of secondary structure. The advantage of RT-PCR is that it is easy to obtain the cDNA for sequence determination and subsequent cloning without resorting to construction and screening of cDNA library (Baumforth et al., 1999).

5.1.4.1 **Isolation of chitinase gene from *N. rileyi*/*M. anisopliae M. anisopliae** using degenerate primers of chitinase

The isolation of chitinase gene through the RT-PCR product of *N. rileyi* and *M. anisopliae* was carried out based on the degenerate primers. Among these three sets, only the first set of primer gave an amplification of 240bp in case of *N. rileyi* and no amplification in case of *M. anisopliae*. Therefore, the six combinations of primers arising from the three sets of degenerate primers were used to amplify a chitinase gene from *M. anisopliae*. Out of these six combinations, two sets *viz.* forward Primer (FP1) + reverse Primer (RP2) and forward primer (FP1) + reverse primer (RP3) gave an
amplification of 500 bp and 400 bp products respectively. The obtained sequences were characterized with blast (X) programme of NCBI site. The 240 bp and 400 and 500bp RT-PCR products of *N. rileyi* and *M. anisopliae* obtained from the degenerate primers and their combinations respectively did not show any significant homology to the chitinase gene deposited in NCBI data bank.

5.1.5 Designing gene specific primers of chitinase from *Metarhizium* species

Although the degenerate primers of chitinase have given successful amplification, in several cases PCR amplification of many degenerate primers resulted in the production of only the central position of cDNA or products of no homology. Therefore, it is worthwhile to design the gene specific primers so that the time and effort utilized for initially testing with degenerate primers may be saved. To design gene specific primers of chitinase the available full length gene from *Metarhizium* species were taken from NCBI database. Two sets of primers were designed. The same approach has been used by Hayes *et al.* (1994) to amplify chitinase gene from *T. harzianum*.

5.1.5.1 Isolation of chitinase gene from *M. anisopliae* genomic DNA using gene specific primers of chitinase

The two sets of gene specific primers were tried for amplification *M. anisopliae* genomic DNA. A good amplification of 1.5 kb was observed when the annealing temperature was reduced to 50°C for 1 minute for the first set of primer.
5.2 Cloning and characterization of chitinase gene

5.2.1 Cloning

A successful cloning of the amplified product is the key step for further analysis of the recombinant product. In this experiment T/A cloning was followed, which is a very simple and efficient method. The PCR products with 3’dA overhang without modification by restriction enzymes were directly cloned into a linearized plasmid with complementary 5’dT overhangs (Guo and Bi, 1998). To clone the amplified gene pTZ57R/T was used which is a modified pUC vector.

In the present study, the plasmid vector was chosen for cloning as they are of small size and easy to handle and capable of replicating easily in the host genome. This plasmid vector also has ampicillin as selectable marker, a gene which allows the host cells containing vector to be selected amongst those which do not. Further pTZ57R/T vector also has method of screening based on blue-white selection due to insertional inactivation of lacZ in the multiple cloning site (MCS). Most of the chitinase genes isolated from various species have been cloned in plasmid vectors (Inglis et al., 1997; Kang et al., 1998; Morisette et al., 2003 and Watanalai et al., 2004). The amplified 1.5kb product of M. anisopliae from the genomic DNA using gene specific primers were cloned into the plasmid vector pTZ57R/T vector by T/A cloning.

5.2.1.a. Confirmation of recombinant clones

The process of characterizing a clone begins by obtaining pure preparation of the DNA. Plasmids from recombinants were isolated using small scale preparation of the DNA. The recombinant clones were purified by poly ethylene glycol (PEG)
precipitation method before giving for sequencing and the presence of the insert was confirmed by

a) Comparing the size of recombinant plasmid with that of control vector provided in the cloning kit. The fragments above the control vector were selected as the size of the recombinant plasmid increased because of the presence of the insert.

b) by restriction digestion of the recombinant clone using the Bam HI enzyme present in the MCS of vector. This resulted in the linearization of the cloning vector along with insert.

c) PCR amplification with chitinase gene specific primers of Metarhizium and M13 primers resulted in amplification of 1.5kb insert which also confirmed the presence of insert.

5.2.2. Characterization

The 1.5kb gene sequences obtained from M. anisopliae from genomic DNA amplified using gene specific primers gave matching homology to chitinase gene sequences deposited in NCBI site. The 1525bp sequence showed significant homology to M. anisopliae strain HN1 (99%), M. anisopliae CHIT42 (96%), M. anisopliae CHIT42 (95%), T. harzianum Chit HAR3 (73%), T. viridae (72%), T. hamatum endochitinase (72%) and C. immitis (56%). The two sequences from cDNA as well as genomic DNA were identical containing an ORF of 1525 and interrupted by 3 regions of introns of 101bp, 68bp and 131bp at positions 144bp, 242bp and 373bp respectively, which were comparable to the introns present in homologous gene of M. anisopliae and N. rileyi obtained by Bogo et al. (1998) and Wattanali et al. (2004) respectively.
After editing the 3 regions of introns, the 1275bp full length gene showed remarkable homology with other chitinase gene sequences like *M. anisopliae* (99% identity), *M. anisopliae* bacterial type endochitinase (98% identity), *M. flavoviridae* (95% identity), *M. anisopliae var acridum* (95% identity), *M. anisopliae* CHIT42 (92% identity), *N. rileyi* (77% identity), *Stachybotrys elegans* (71% identity), *Aphanocladium album* (70% identity) and *Trichoderma viridae* (69%) which have already been deposited in NCBI data bank.

The full length chitinase gene encodes 425 amino acids with start codon of ATG and stop codon of TAG. Since it showed matching homology with 42kDa endochitinase of other *M. anisopliae* which are already deposited in the NCBI data bank, it is presumed that the chitinase isolated from *M. anisopliae* is also 42kDa endochitinase encoding protein. The sequence analysis of the chitinase gene from ProtoParam website revealed that it is having theoretical pi of 5.38 and having charges of 5.6 at pH 7 suggesting that it may be an acidic chitinase gene. This is in conformity with earlier reports of St.Leger et al. (1996a) who demonstrated the acidic chitinases are the predominant enzymes secreted by *M. anisopliae* when grown on insect cuticle. Further they also reported that this result contrasts with results with other fungal species, in which basic enzymes are predominating. The potential chitin binding domain SIGG located at 127 aa and the conserved catalytic site FDGIDVDWE is located at 145aa. These regions were found to be conserved amongst chitinase sequences of the different species of *Metarhizium* and amongst various entomopathogenic fungi belonging to class Deuteromycetes from NCBI site by the clustalW alignment.
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The alignment of the chitinase gene sequences by Wattanalai et al. (2004) revealed that the chitin binding domain and the catalytic site is conserved among the class V chitinases and the chitinase gene isolated from *M. anisopliae* suggesting that the isolated chitinase belongs to class V.

5.3. Mobilization of the isolated chitinase gene into binary vector for *Agrobacterium* mediated transformation

In order to utilize the cloned chitinase gene obtained from genomic DNA of *M. anisopliae* for gene transfer through *Agrobacterium* mediated transformation, the gene was cloned to binary vector. The binary vector contains a gene of interest between the T-DNA borders and a helper Ti-plasmid in the *Agrobacterium* that provides the vir gene products to facilitate transfer into plant cells. Dis armed helper Ti-plasmids have been engineered by removing the oncogenic genes while still providing the necessary vir gene products required for transferring the T-DNA to the host plant cell.

5.3.1. Mobilization of the isolated chitinase gene into binary vector by directional cloning

In the present study, binary vector pBIN-mgfp5-ERA was used. To mobilize the chitinase gene from *M. anisopliae*, directional cloning was followed. For this purpose, both the plasmids, namely, binary vector and cloning vector containing chitinase gene were isolated in large quantities and were subjected to restriction digestion with *XbaI* and *BamHI* as the mgfp region is flanked by the two restriction sites of *BamHI* and *XbaI*. Thus the isolated chitinase gene was cloned by replacing mgfp region in 35S – mgfp-ERA with Nos terminator cassette by directional cloning. The presence of chitinase gene was confirmed with amplification of chitinase gene specific primer and
the orientation of the gene was confirmed by using 35S CaMV forward primer and the chitinase reverse primers. Directional cloning was also used by Bolar et al. (2000) and Yamamoto et al. (2000) for developing chitinase gene construct while, Terakawa et al. (1997) introduced R. oligosporus chitinase gene into binary vector pB1-121 by blunt end ligation. In this experiment the directional cloning was convenient since the restriction sites flanking the region to be replaced by the gene of interest were found to be non cutters in the chitinase gene.

5.3.3. Mobilization of binary vector harboring chitinase gene into Agrobacterium by triparental mating technique

The binary vector containing the chitinase gene was mobilized into Agrobacterium by triparental mating technique. The pBIN vector harboring chitinase gene was introduced into Agrobacterium tumefaciens strain LBA4404 strain with the help of pROK 2073 helper plasmid. A. tumefaciens harboring this plasmid was used for transformation studies. The same technique has been used by Bolar et al. (2000) and Yamamoto et al. (2000) to introduce a chitinase containing binary plasmid into A. tumefaciens strain EHA 105 and pB1-121-RCC2 rice chitinase into A. tumefaciens LBA4404 respectively.

5.4 To study the expression of the isolated chitinase gene upon transformation in tobacco

5.4.1 Transformation and regeneration of tobacco

Tobacco has been used routinely as model system to study the effect of genes isolated through transformation. There are several reports on the regeneration in tobacco. Most of the protocols used the growth regulator of BAP 1mg/l. In the present
study, the same treatment gave a very good regeneration response. The regenerated shoots were transferred to the rooting media containing 0.5mg of IAA, which resulted in good rooting response.

The *Agrobacterium* mediated transformation was carried out following the protocol of Horsch *et al.* (1987) with some modifications. The tobacco leaf bits preconditioned for 2 days helped in good regeneration after inoculation with the culture. Culture grown overnight was diluted to two times and inoculated for 10 minutes was found to be optimum as it did not result in overgrowth upon transfer to selection medium. However the leaf bits had to be washed with cefotaxime before transfer to selection medium containing 100mg/l of kanamycin and 500mg/l of cefotaxime. There was a regeneration of putative transformed shoot buds to the extent of 66 percent. These shoot buds gave rise to 36 percent elongated shoots with the increase in the selection pressure to 200mg/l kanamycin. The elongated shoots were then transferred to rooting media containing reduced levels of (50mg/l) kanamycin and 250mg/l of cefotaxime to ensure rooting as high concentration of kanamycin inhibited rooting (Estopa *et al.*, 2001). Rooting to the extent of 83 percent was achieved from the elongated shoots. After 2 weeks the rooted putative transformants were transferred for *ex vitro* establishment.

5.5. Confirmation of the transgene

5.5.1 PCR with NPT-II gene

Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation, however spontaneous variants with increased resistance to chemicals can be readily selected in plant tissue culture. This includes resistance to
kanamycin, which is the most commonly used selection agent for plant transformation. Therefore biochemical and molecular evidence is essential to confirm the expression and integration of transferred gene. PCR analysis gives an indirect indication of presence of transgene. This method can be used as preliminary screening used for analysis of transgene for a large number of samples. Neo, the gene encoding Neomycin phosphotransferase II (NPT II), was originally isolated from transposon TN5. This enzyme specifically phosphorylates aminoglycoside antibiotics of the neomycin family such as kanamycin and G418. The expression of the gene in transgenic plants also confers resistance to Neomycin antibiotics in these plants. Consequently this gene has been widely used as either a reporter gene in studies of plant gene regulation or as a selectable marker in plant genetic engineering studies (Peng et al., 1993). The presence of NPT-II gene under the control of Nos promoter in the binary vector pBIN, enables the use of this gene as selectable marker for selection of putative transformants. PCR analysis of NPT-II gene in 5 randomly selected tobacco samples detected the presence of 750 bp band of NPT-II in 3 samples out of 5 samples and also in plasmid DNA which was used as positive control and was not detected in nontransformed control. Such approach for detecting the presence of transgene has been used by several workers (Bolar et al., 2000; and Yamamoto et al., 2000) to detect the presence of transgene after transformation.

5.5.2 PCR with gene specific primers

To confirm the integration of the gene of interest it is necessary to carry out further analysis with gene specific primers. The presence of the chitinase gene of interest was also confirmed by amplification with the oligonucleotides used for the isolation of
The amplification of NPT-II sequences also shows the homologous sequences of the primers exist in close enough proximity for a product to be made. The results do not indicate whether template DNA or the intended sample material or if contamination. The introgression of *M. anisopliae* chitinase gene into the plant genome was analyzed by southern blot. The non radioactive method was followed to label the probe by random priming method. The two NPT-II positive PCR samples along with nontransformed control were digested with *Xba*I and *BamHI* separately and plasmid DNA as positive control without digestion, showed the positive integration of the chitinase gene when probed with the 1.5 kb PCR product of *Metarhizium* chitinase gene obtained from the plasmid isolated in the tobacco genome. The developed blot showed the positive integration of the chitinase gene. However the copy number could not be detected as the DNA samples were not digested properly and the blot appeared as smear. Terekawa *et al.* (1997) Bolar *et al.* (2000), Yamamoto *et al.* (2000) and Jeffrey *et al.* (2000) have used this method of confirmation of chitinase gene in the transformants.

5.5.4 *In vitro* fungal inhibition assay

The antifungal activity of chitinase from plants and bacteria has been known for a long time (Mauch *et al.*, 1988; Pleban *et al.*, 1997). However in recent years,
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Substantial inhibitory effect of fungal chitinase in several fungal pathogens has been demonstrated (Lorito et al., 1993). Thus chitinases are being exploited from fungus to enhance the disease resistance against several fungal pathogens. In the present study, the inhibitory effect of the crude protein extracts from transgenic plant expressing chitinase of *M. anisopliae* on the growth of fungus *Alternaria alternata* was demonstrated. The growth inhibition to the extent of 43 percent from the transgenic plant extract when compared with non-transformed plant extract and 46 percent with water extract was observed. The presence of introns in the chitinase gene of *M. anisopliae* did not seem to affect the expression of gene in inhibiting the fungal growth. This result is in conformity with that of Lorito et al. (1998) who have reported that the level of transcription obtained with the *Trichoderma* genomic DNA with introns was higher than with the cDNA copy suggesting that the presence of introns may improve gene expression in plants. This shows that chitinase from an entomopathogenic fungus, *M. anisopliae* can effectively be used for transformation studies in other crops for enhanced resistance against pathogens.