Chapter 6

SUMMARY
Since the beginning of the organized agriculture, phytopathogens have caused major yield losses and have impacted the well being of humans worldwide. With the exception of diseases epidemics that lead to complete crop destruction, global loss because of pathogens is estimated to be 12% of potential crop production. Principal cause for this loss has been identified mostly as fungal diseases. To combat fungal diseases various husbandry techniques and breeding resistant variety have been employed. But both of these have their drawbacks. To overcome the drawbacks and to produce fungus resistant crops now a days transgenic crop production has been started. Many genes that are involved in defense mechanism in plants have been identified, cloned and characterized, and finally transferred into crop plant to assess their effectiveness. In a continuation of these attempts in the present study antifungal gene was isolated, cloned and characterized and ultimately transgenic plants were produced to evaluate the effectiveness of the isolated gene.

Plants are exposed to enormous numbers of pathogens. But the appearances of diseases are rare. This is due to the presence of defense mechanisms. Many antifungal compounds, which are synthesized by plant to combat fungal infections, have been identified from seeds. In this study various plant seeds were screened to identify such antifungal compound.

Screening of antifungal agent was performed against *Pythium aphanidermatum*. Out of six legumes seeds, chickpea (*Cicer arietinum*, L) was found to have inhibitory effect on the test fungus. Isolation and purification was performed in a simple two-step method. Its sequence analysis has shown that it belongs to PR-12 group, which are commonly known as “Plant Defensin” peptide and we termed it as AFP1-Ca. Further characterization revealed that this peptide is highly stable at extreme pH (2-10) and active even after heat treatment at 100°C for 30 mins. The optimum temperature for biological activity of this peptide was also found to be wide ranged.

When the effectiveness of these peptide against various phytopathogenic fungi was assessed it was found that AFP1-Ca was effective against non-chitinase fungus like *Pythium aphanidermatum* and chitin containing fungi like foliar pathogen *Alternaria alternata* and *Alternaria solani* and other important fungi, namely, *Botrytis cinerea*, *Alternaria brassicicola* and *Drechslera bicolor*. All these fungi were effectively inhibited in sub-microgram concentrations ranging from 4-12 μg/ml. But this
inhibitory effect was non-lethal rather than lethal like many other reported antifungal proteins. And a striking observation of this peptide was AFP1-Ca caused less branching hyphae in contrast to hyper branching nature of other plant defensins. Antibacterial activity was not observed in AFP1-Ca. Similarly, toxic effect on insect and human culture cell line and erythrocyte was also absent. Due to broad range of antifungal activity and high efficiency to inhibit fungal growth AFP1-Ca is a potential candidate to explore the effectiveness of this peptide in combating fungus in transgenic plants. Especially its non-toxic nature towards insect and human culture cell line made it more promising candidate as a transgene. Even after being an antifungal agent this peptide was non-lethal. This creates more interest to assess its effectiveness in transgenic plants.

To assess the antifungal activity of AFP1-Ca in unrelated plant species, transgenic tobacco plants were raised by introducing AFP1-Ca gene under the control of a strong constitutive 35S promoter using Agrobacterium-mediated transformation. A total of 162 independent hygromycin resistant plantlets were regenerated. All the putative plantlets were successfully acclimatized into greenhouse. They grow normally and flowered as control plant. No morphological difference was observed in comparison to non-transformed control. Integration of transgene of these putative plants was analyzed by PCR and Southern hybridization. Most of the transgenic plants were found to contain one copy of insert. However, there were few plants with 2 or more numbers of inserts. GUS expression of leaves of these plants showed variable levels, which were categorized into high, moderate and low level of expression. Similarly, Western blot analysis showed variation in expression at AFP1-Ca protein levels. Northern blot analysis supported these variations of expression indicating the position effect of transgene incorporation in the transgenics containing one copy of insert. Fungal bioassay with leaves of these plants demonstrated effective inhibition of Alternaria solani. The effectiveness ranged from complete restriction of the inoculated fungus to reduced fungal growth. The effectiveness was directly correlating with the transcript level observed during Northern hybridization, indicating that the AFP1-Ca mediated fungal resistance is a dose dependent phenomenon. Germination data of seeds of transgenic plants showed Mendelian segregation in presence on hygromycin selection.
As the AFP1-Ca was functional in a heterologous plant (tobacco) system and as this peptide exhibits antifungal activity against *Pythium aphanidermatum, Alternaria solani, A. alternata, A. brassicicola, Botrytis cinerea* and *Drechslera bicolor* we assume that this gene can be used to develop fungal resistant transgenic crop plants.