CHAPTER 6
SUMMARY

In the present study, plant transformation cassettes with antifungal protein genes, chitinase and lipid transfer protein and cyanamide hydratase marker gene have been constructed. Chitinase gene, Bar2chi was amplified from the cDNA library clone of barley using gene-specific primers. The amplified chitinase showed 1.1 kb size with an ORF, 318 amino acids. The predicted amino acid sequence of Bar2chi has 95.3% homology with chitinase of rye and other plant chitinases. Lipid transfer protein gene, Ltp 3F1 was amplified from the cDNA library clone of wheat using gene-specific primers. The amplified Ltp 3F1 showed 345 bp size with an ORF, 115 amino acids. The predicted amino acid sequence of Ltp 3F1 has 80% homology with barley and other LTPs of monocots.

Bar2chi gene was cloned into pET 28a+ and overexpressed in E. coli. The recombinant chitinase was purified from the inclusion bodies of bacterial pellets. SDS-PAGE and Western blot analyses with an antiserum against barley chitinase confirmed the production of 35 kDa recombinant chitinase in E. coli. Ltp 3F1 gene was cloned into pMAL-p2x and overexpressed in E. coli as soluble protein. SDS-PAGE analysis confirmed the production of 51 kDa LTP fusion protein.

Purified chitinase showed a broad-spectrum antifungal activity even at a concentration of 100 μg (0.42 U) against fungi such as Botrytis cinerea (blight of tobacco), Pestalotia theae (leaf spot of tea), Bipolaris oryzae (brown spot of rice), Alternaria sp. (grain discoloration of rice), Curvularia lunata (leaf spot of clover) and
*Rhizoctonia solani* (sheath blight of rice). SEM image from the zone of inhibition of fungi revealed lysis and fragmentation of mycelia and inhibition of mycelial branching, whereas the control showed highly branched and well developed mycelium. When the mycelia from the periphery of the zone of inhibition were examined under light microscope, the hyphae appeared to have mycelial deformations such as lysis and fragmentation, whereas the control showed normal, well developed and intact mycelia. Purified chitinase (300 µg) also suppressed the germination of sclerotia of *R. solani*.

Purified LTP showed a broad-spectrum antifungal activity against *Alternaria* sp. (grain discoloration of rice), *R. solani* (sheath blight of rice), *C. lunata* (leaf spot of clover), *B. oryzae* (brown spot of rice), *Cylindrocladium scoparium* (root necrosis of banana) and *Sarocladium oryzae* (sheath rot of rice) even at a concentration of 100 µg. When the mycelia from the periphery of the zone of inhibition were examined under light microscope, the hyphae appeared to have lysis and fragmentation, whereas the control showed normal, well developed and intact mycelia without any distortion.

Site-directed mutagenesis was performed and the internal HindIII site was eliminated in order to facilitate the cloning of *cah* gene into plant transformation cassettes. Sequencing data of mutated *cah* indicated the single base substitution, G to A at nucleotide position 81 or T to C at nucleotide position 84 of *cah* gene. The one base substitution does not change the amino acid it encodes since both AAG (wild) and AAA (*cah* MutK mutant) encode lysine and the codons, CTT (wild) and CTC (*cah* MutL mutant) encode leucine.
The *cah* genes (wild-type and mutants) were amplified using gene-specific primers and recombinant plasmids, pQ-*cah* (wild-type), pQ-*cahMutK* and pQ-*cahMutL* (mutant-type) were constructed using *E. coli* expression vector, pQE-60. The *cah* genes were overexpressed by IPTG induction of *E. coli* carrying wild-type pQ-*cah* as well as mutants, pQ-*cahMutK* and pQ-*cahMutL* as His-tag fusion protein and the Cah enzyme was purified using Ni²⁺ column. Crude enzyme preparations of pQ-*cah*, pQ-*cahMutK* and pQ-*cahMutL* showed similar level of cyanamide hydratase activity (1090, 1060 and 1000 U/mg) respectively and purified enzyme exhibited 5-fold higher activity than the crude enzyme preparation. SDS-PAGE and Western blot analyses of the purified enzyme preparations with *cah* antibody (1:1000 dilution) confirmed the presence of 27.7 kDa Cah protein.

The mutated *cah* gene devoid of HindIII site was successfully cloned along with *Ubi1* promoter and *nos* terminator into pCAMBIA 1300 vector and the recombinant plasmid, pCAMBIA-*cah* was constructed. The gene cassettes harboring *cah* marker, chitinase and LTP were constructed by three-fragment ligation of the dephosphorylated vector (pCAMBIA-*cah*) digested with HindIII, *Ubi1* promoter (*HindIII-BamH1* fragment) and *Bar2chi* or *Ltp* 3F1 gene (*BamH1-HindIII* fragment).

Freeze-thaw method facilitated the transformation of pCAMBIA-*cah-Ltp* 3F1 into *Agrobacterium* LBA4404. *Agrobacterium*-mediated gene transfer (leaf-disc method) was followed for gene transformation in tobacco. Untransformed leaf disc when cultured on tobacco shoot selection media with cyanamide failed to regenerate. Co-cultivated leaf discs, produced shoots after 2 weeks on MS medium amended with 5 mM cyanamide and putative transformants formed roots by 2 weeks.
When the genomic DNA of putative transformants was used as template, gene-specific primers amplified a 732 bp cah marker gene, 1.1 kb chitinase gene and 348 bp Ltp 3F1 gene in transformants. Southern blot analysis confirmed the integration of cah, chi and Ltp 3F1 in putative transformants. Conversely, no such band was observed in the untransformed control under identical conditions. Expression of cah, Chi and Ltp in the transformants was further confirmed by Western blot analysis.

Transgenic tobacco plants expressing chitinase or LTP showed resistance against phytopathogenic fungi. The cah marker gene may be considered as a unique marker because of its ability to convert calcium cyanamide into urea, a useful nitrogen fertilizer source to plants. Plant transformation cassettes harboring the antifungal Bar2chi or Ltp 3F1 genes and cah marker reported in this study can be efficiently used for developing transgenic resistance in crop plants against phytopathogenic fungi.