Summary

In the present study, cDNA encoding Ace-AMP1 preprotein from onion seeds was synthesised and used for transformation of commercially important rice cultivar Pusa Basmati 1. Ace-AMP1 was chosen as a candidate gene for rice transformation, since it was seen to inhibit two important rice fungal pathogens, *M. grisea*, *R. solani*, and a Gram-negative bacterium *X. oryzae* in *in vitro* assays. Transgenic rice plants expressing Ace-AMP1 were tested for enhanced resistance to blast, sheath blight, and bacterial leaf blight.

The 363 bp cDNA encoding Ace-AMP1 pre-protein was synthesised from RNA extracted from immature seeds of *Allium cepa*. The Ace-AMP1 coding region was cloned downstream to the rice phenylalanine-ammonia lyase (*PAL*) promoter in pBlueScript KS+ and maize ubiquitin (*Ubi*) promoter with the first intron in pAHC17, to obtain the plasmids pPAL-Ace and pUbi-Ace, respectively. Both these gene cassettes were also cloned separately in pCAMBIA-1305.2 to obtain pPAL-Ace-T and pUbi-Ace-T, respectively. *Agrobacterium tumefaciens* (strain LBA4404 (pSB1) harbouring pUbi-Ace-T, was used to transform rice calli. The plasmid pPAL-Ace, along with pCAMBIA-1200 containing the *hpt* gene was co-transferred into rice calli by particle bombardment. The *hpt* gene driven by Cauliflower mosaic virus (CaMV) 35S promoter was used as a selection marker. Conditions for regeneration of calli into whole plants were optimised by manipulating the regeneration medium composition.
Eighty six out of 170 (51%) and 79 out of 170 (46%) calli were found to be hygromycin resistant when transformed using *A. tumefaciens* and by particle bombardment, respectively. Out of 165 hygromycin-resistant calli, 84 (51%) regenerated into whole plants and 54 (64%) plants set seeds. Total DNA was extracted from a few of the hygromycin resistant calli and analysed by PCR using specific primers for both, *Ace-AMP1* and hpt coding regions. Approximately 80% of the calli tested showed the presence of the transgene.

*Ace-AMP1* cDNA was also cloned translationally 'in frame' with Glutathione-S-Transferase (GST) gene into a yeast expression vector ‘pEG(KT)’. Yeast strain, S288C, was transformed with the recombinant plasmid. Two percent galactose was used to induce expression of GST:*Ace-AMP1* fusion protein in the yeast transformant. Protein extract from the transformed yeast cells was used to purify GST:*Ace-AMP1* fusion protein by affinity chromatography using GST purification module. The purified fraction was used to perform an in vitro assay where it inhibited the phytopathogens *M. grisea*, *R. solani* and *X. oryzae*. The concentration of *Ace-AMP1* completely inhibiting spore germination of *M. grisea* and growth of *X. oryzae* was found to be 4 μg/ml. The purified fusion protein fraction was used to raise polyclonal antibodies in rabbit. In order to study the effect of *Ace-AMP1*, treated and untreated germinating conidia were observed by microscopy using Calcoflour White. The untreated biomass of the fungus showed normal and profuse growth whereas the *Ace-AMP1* treated samples showed densely stained bulbous structure at the tip of hyphae, unlike untreated control.
Presence of *Ace-AMP1* gene was confirmed by PCR in all the 54 plants regenerated from hygromycin resistant calli. The DNA from untransformed plants (control) showed no amplification, while a fragment of 363 bp was amplified in PCR from genomic DNA obtained from all the 54 plants. Integration pattern of the transgene, in four representative lines (*PAL-Ace-4, PAL-Ace-54, Ubi-Ace-T-6 and Ubi-Ace-T-11A*), was studied by Southern blot analysis. Similar hybridisation pattern was observed among the transgenic lines studied. Inheritance of the transgene *Ace-AMP1* was tested using PCR, in the T₁ generation. Stable integration was confirmed by identical banding pattern in Southern analysis in subsequent generation.

Protein extracts from leaf tissues of the 4 transgenic lines were used for the immunodetection of *Ace-AMP1*, which showed positive immunological reaction with a band corresponding to *Ace-AMP1* (9 KDa). Transgenic lines designated as *PAL-Ace-54* and *Ubi-Ace-T-11A* showed higher accumulation of *Ace-AMP1* than *PAL-Ace-4* and *Ubi-Ace-T-6*. The untransformed control plants showed no immunoreactive protein. Western blot analysis was also performed in subsequent T₁ and T₂ generations and identical results were obtained. The average amount of *Ace-AMP1* when its expression was driven by ubiquitin promoter was found to be 3.49 (*Ubi-Ace-T-6*) and 4.58 (*Ubi-Ace-T-11A*) μg/mg of total protein. In case of transgenic plants using *PAL* promoter, upon induction of the promoter by wound treatment, there was 3.4 to 4.5 fold increase in amount of *Ace-AMP1* protein.
Extra-cellular fluid and intracellular protein extract were collected to study subcellular location of Ace-AMP1 expressed in leaf tissue of the transgenic plant. Cytosolic enzyme glucose-6-phosphate dehydrogenase was used as a marker that showed a ratio of 85:15 between intracellular extract and extra-cellular fluid fractions; while 82% of the Ace-AMP1 expressed was found to be present in extra-cellular fluid fraction, indicating its accumulation in the apoplast. Tissue localisation of Ace-AMP1 was also studied by treating sections of both untransformed and transgenic rice leaf tissue with anti-Ace-AMP1 antibody followed by FITC-conjugated goat anti-rabbit IgG antibody. Labeling of vascular bundle and mesophyll cells was evident within the transgenic rice leaf tissue sections. The immunofluorescence was strongly associated with wall material while cytoplasmic content remained unlabelled.

The degree of resistance of Ace-AMP1 expressing transgenic plants against M. grisea, R. solani, and X. oryzae was evaluated in both, in vitro assays and in planta infection assays. In an in vitro assay with M. grisea, protein extract from leaf tissue of transgenic plants either inhibited germination of spores till 24 h or led to altered morphology of hyphae, such as tip ballooning. Incubation with protein from leaf tissue of untransformed plant showed normal and profuse growth of hyphae.

Progress of the blast disease in in planta assays was studied over 10 dpi. The untransformed control plants showed typical susceptible type of lesions after 5-6 dpi. Transgenic plants expressing Ace-AMP1 showed type 1-3 lesions developed after 6-7 dpi. After 10 dpi, severity of infection was high in untransformed plants unlike in transgenics. The four transgenic lines showed % diseased leaf area (% DLA) ranging
from 0.97 to 14.11 %, whereas it was 47.65 % in untransformed plants. Detached leaf assay was also performed where the untransformed leaf tissue sections showed normal and profuse growth of hyphae in 2-3 days of inoculation with spores of blast fungus. While, the leaf tissue sections from inoculated leaves of T\textsubscript{2} transgenic plants showed either no hyphal growth or hyphae with altered morphology.

Similarly, resistance of \textit{Ace-AMP1} transgenic lines to \textit{R. solani} was evaluated over 12 dpi. The untransformed plants started developing disease symptoms on 5\textsuperscript{th} dpi, while no symptoms were seen in transgenic plants till 7 dpi. Untransformed plants, after 12 dpi, showed class III and IV infection, while it was restricted to class 0, I, and II in case of transgenic lines. Untransformed plants showed 62.50 % infection, while plants from transgenic lines showed only 16.67 to 26.67 % infection.

Approximately, 45 day old transgenic and untransformed plants were also inoculated with \textit{X. oryzae}. The untransformed control plants with large diseased area as compared to the transgenic plants indicated enhanced resistance against bacterial leaf blight in transgenics. The length of diseased area was found to be 12 mm in inoculated untransformed plants and that ranged from 1.76 to 2.6 mm in four transgenic lines.

Effect of \textit{Ace-AMP1} on host gene expression was studied using DDRT-PCR. Total four primers (OPA-1, OPA-5, OPD-10, and OPD-17) were used for the reactions. Out of all the polymorphic fragments, 4 clearly differential fragments ranging from 75 bp to 350 bp from both the samples were picked up, and cloned at \textit{Eco RV} site in \textit{pBlueScript KS}\textsuperscript{+}. Sequence analysis was performed with 4 differentially expressed partial cDNAs, where 3 out of 4 were from transgenic sample and 1 was from
untransformed sample. All the sequences analysed showed significant homology to transcript of chilling inducible protein, putative anther ethylene responsive protein ER1, auxin responsive transcription factor, and putative Bowman Birk trypsin inhibitor protein.

Phenotypic characteristics studied among the four representative transgenic lines were found to be similar to the untransformed plants. All the four transgenic individuals showed 100% germination like the untransformed plants. Plant height in transgenics ranged from 67.80 ± 1.70 cm to 89.30 ± 1.39 cm, while it was found to be 84.20 ± 2.02 cm in untransformed plants. Days required for 50% flowering ranged from 116 ± 6.00 to 138 ± 2.00 in case of transgenics, while in untransformed plants it was 110 ± 5.00 days. Test weight of transgenic seeds ranged from 16.20 ± 0.15 to 20.60 ± 0.23 g and was found to be 20.23 ± 0.14 g in case of untransformed plants.

The strategy of genetic transformation with gene for ns-LTP-like protein followed in the present work provides a practical approach to achieve broad-spectrum disease resistance in crop plants.