4. DISCUSSION

In the recent years, a number of transgenic rice plants showing enhanced resistance to fungal pathogens have been generated, using genes coding for different antimicrobial proteins (Krishnamurthy et al., 2001, Iwai et al., 2002, Kanzaki et al., 2002, Itoh et al., 2003, Nishizawa et al., 2003, Coca et al., 2004). In the present study, a gene encoding ns-LTP-like protein Ace-AMP1 from Allium cepa has been used to engineer increased resistance to both fungal and bacterial diseases.

LTPs are ubiquitous plant proteins able to load and transfer hydrophobic molecules such as fatty acids or phospholipids to membranes. Among them, LTPs 1 (type 1 LTPs) constitute a multigenic family of secreted plant lipid binding proteins that are constitutively expressed in specific tissues and/or induced in response to biotic and abiotic stress (Buhot et al., 2001). Their biological function is still unclear, even though some experimental evidence indicates the role of these proteins in the assembly of extracellular hydrophobic polymers (i.e. cutin and suberin) and/or in plant defence against fungal pathogens (Hoh et al., 2004).

Broad-spectrum antimicrobial activity in vitro, and stability in the presence of cations make Ace-AMP1 a potential candidate gene for transformation of important crop plants. Ornamental plants like Scented geranium (Pelargonium sp. cv. Frensham) (Bi et al., 1999), and Rose (Rosa hybrida cv. Carefree Beauty) (Li et al., 2003) have been transformed with the gene for Ace-AMP1 and shown to be resistant to fungal pathogens Botrytis cinerea and Sphaerotheca pannosa, respectively. Tissue culture
media for rice varieties Pusa Basmati 1, CO39, and BPT 5204, were optimized and a significant response in terms of callus induction (≥ 90%), and regeneration (≥ 60%) was observed in all the 3 varieties studied. In the present work, a commercially important rice cultivar Pusa Basmati 1 has been transformed using the gene for Ace-AMP1. The putative transformants were selected based on their resistance to hygromycin used in the medium. Transformation efficiency was found to be 46 - 51%, which is comparable to the published reports on genetic transformation of *indica* rice varieties (Sridevi et al., 2005). The putative rice transformants were confirmed by PCR for the presence of *hpt* gene in plants regenerated from hygromycin resistant calli. In total, 54 transgenic Pusa Basmati 1 rice plants (27 plants each with *PAL* and *Ubi* promoter) were obtained that set seeds and were found to be PCR-positive for the presence of Ace-AMP1 gene. PCR positive lines were subjected to Southern blot analysis, to ascertain the number of integrations of the transgene in the genome. Similar hybridisation pattern was observed in 4 transgenic rice lines studied, suggesting that all the lines were from a single transformation event. Similar Southern profile has also been reported in independent transgenic rice lines expressing *tms2* gene (Upadhayaya et al., 2000). This might be due to presence of integration hot-spots in the genome (Kohli et al., 1999) where either a 19-bp palindromic sequence surrounding the TATA box of the CaMV 35S promoter is often involved in recombination events in the rice genome or naturally-occurring DNA breaks may act as hot spots with the recruitment of DNA repair complexes to sites of DNA damage (Kohli et al., 1998). Alternatively, the exogenous DNA may interact with a group of local replication forks. In each case, this would result in a dense
cluster of free genomic DNA ends and thus, the transgene might get integrated at same locus.

Western blot analysis of leaf protein from four transgenic lines (PAL-Ace-4, PAL-Ace-54, UbI-Ace-T-6, and UbI-Ace-T-11A) detected a 9 kDa protein. The relative amounts of Ace-AMP produced in the 4 transgenic lines varied from 3.5 to 5 μg/mg total protein. The variation in the expression of the protein might be due to position effects of the transgene, the transgene copy number, and the influence of DNA methylation (Meyer, 1998). The PAL promoter has been reported to show a 4- to 8-fold induction in transgenic rice (Kachroo et al., 2003) as well as tobacco plants upon wounding (Zhu et al., 1995). The present study showed that wound induction led to 3.4- to 4.5-fold increase in levels of Ace-AMP1, upon induction of PAL promoter, in the transgenics as compared to uninduced condition. Specific activity of glucose oxidase enzyme was higher when its expression was driven by UbI promoter than when it was driven by PAL promoter in transgenic rice expressing Aspergillus niger GOX gene (Kachroo et al., 2003). However, levels of Ace-AMP1, in the transgenics were comparable when its expression was driven either by activated PAL or UbI promoter. Further, results showed that levels of Ace-AMP1 in the transgenic rice plants were sufficient to enhance resistance against all the three phytopathogens tested. Interestingly, there was a positive correlation between the levels of Ace-AMP1 and the degree of disease resistance in these lines, with transgenic line UbI-Ace-T-11A demonstrating highest level of resistance to bacterial and fungal pathogens.
In plants, innate immune response confers an essential first line of defense against invading pathogens. Interaction between plants and various pathogenic microorganisms occurs in extracellular space during the onset of the infection process. Transgenic plants, secreting protein like AMPs in the apoplastic region, are therefore desirable for effective defense against phytopathogens. Ace-AMP1 contains an N-terminal signal peptide (Cammue et al., 1995) that presumably directs the molecule to the extracellular space in its native host Allium cepa. Hence, to study the localisation of Ace-AMP1 in transgenic rice leaf tissue, antibodies against Ace-AMP1 were generated, and immunolocalisation as well as studies on different protein fractions from cytosol and extracellular spaces were carried out. Accumulation of Ace-AMP1 was found predominantly (82%) in the extracellular space, in transgenic rice plants.

Ace-AMP1 (an ns-LTP-like protein) is a highly basic and low-molecular-weight protein structurally related to plant antifungal proteins, namely defensins. Plant defensins that show characteristic features (positively charged, and in most cases four disulfide bonds that stabilise each protein in solution) similar to ns-LTPs, have been well characterised. Based on their biological activities, defensins are classified into two groups. Group I defensins cause morphological changes in susceptible fungi and are known as morphogenic defensins, whereas group II defensins inhibit fungal growth but do not cause morphological changes (non-morphogenic) (Broekaert et al., 1997). In vitro studies, on the inhibitory effect of Ace-AMP1 against blast fungus showed that the protein alters morphology of the growing hyphae of rice blast fungus which is similar to the effect caused by Rs-AFP2 and Hs-AFP1 (Thevissen et al., 1999).
The aberrant morphology, due to binding-site-mediated insertion in plasma
membranes, has previously been proposed to explain the antimicrobial activity of a
number of proteins. Interaction of plant defensins with such a binding site
subsequently enables their insertion into the plasma membrane, causing a structural
disruption and alteration of membrane permeability (Thevissen et al., 1999). In order
to have a better understanding of the inhibitory effect of Ace-AMP1, M. grisea was
treated with the protein and morphological alterations were monitored over a period
of 10 h. Swelling of hyphae was observed within 2 h of treatment and bulbs were
formed within 4 h at the hyphal tips. The increased hyphal width in the treated fungus
might be a result of delay in the establishment of polarity, which may also cause
altered branching patterns (Harris et al., 1994; 1997). The result of the current work is
similar to what was reported earlier (Osborn et al., 1995). It was shown that seed
derived plant defensins from families Asteraceae, and Fabaceae, caused germ tube
and hyphae of Fusarium culmorum to swell and form multiple hyphal buds.
Furthermore, swelling and subsequent rupture of bulbous tips of the fungal hyphae, in
presence of Ace-AMP1 indicates that the protein disturbed the osmotic balance,
leading to the release of cellular content. To substantiate the observation, electrolyte
leakage was assayed at 12 and 24 h after treating the blast fungus with Ace-AMP1.
The assay showed significantly higher conductivity in treated sample compared to
untreated sample, indicating leakage of electrolytes due to presence of Ace-AMP1.

Several LTPs in maize, barley and pepper leaves were induced by pathogen infection
(Molina and Garcia-Olmedo, 1993; Molina et al., 1993) and some LTP isoforms in
radish and sugar beet were also demonstrated to inhibit the growth of bacterial and fungal pathogens in vitro (Terras et al., 1992; Nielsen et al., 1996). In addition, high accumulation of LTPs in cuticular wax has also been reported (Thoma et al., 1993; Kader, 1996). These observations are consistent with the role of LTPs in plant defence. In the present study, transgenic lines expressing Ace-AMP1 showed enhanced resistance to blast disease, with ~86% reduction in symptoms. Both constitutive and inducible expression of the Ace-AMP1 gene in transgenic rice resulted in enhanced resistance against *M. grisea*. The degree of resistance of the transgenic lines was studied both by monitoring the blast reaction in planta, and performing Trypan Blue staining followed by microscopic observations for the fungus in the inoculated rice leaves. A consistent difference in the severity of tissue damage, between untransformed and Ace-AMP1 transgenic lines was observed. Accumulation of Ace-AMP1 in the extracellular space in transgenics indicated the possibility of the protein causing similar effects on morphology of fungal hyphae in vivo as in in vitro assays. Swelling and abnormal growth of the hyphae in the leaf tissue sections from transgenic line was seen. In contrast, the leaves of the fungus-infected untransformed plants showed profuse fungal growth in vivo. Morphological alterations of the fungal hyphae on leaf tissue of Ace-AMP1 transgenic rice, suggested that the enhanced resistance was due to the inhibitory activity of the protein. Similarly, transgenic lines showed resistance against another important rice fungal pathogen *R. solani*, with 67% reduction in disease severity.

Ace-AMP1 has previously been proved to be effective against only two Gram-positive bacteria (*B. megaterium* and *S. lutea*) tested; whereas ns-LTPs from radish and maize
were either not or less effective (~20 to 60 times lesser with respect to IC\textsubscript{50}) against bacteria (Cammue et al., 1995). \textit{In vitro} assays, in the present study, showed ability of Ace-AMP\textsubscript{1} to inhibit a Gram-negative bacterial pathogen \textit{Xanthomonas oryzae} pv. \textit{oryzae}. Surprisingly, Ace-AMP\textsubscript{1} did not prove effective for use against any of the three Gram-negative bacteria (\textit{E. coli}, \textit{P. syringae}, and \textit{A. tumefaciens}) tested earlier \textit{in vitro} (Cammue et al., 1995). Pusa Basmati 1 is a highly susceptible \textit{indica} variety to bacterial leaf blight disease caused by \textit{X. oryzae}. Under favourable conditions, a complete yield loss from Pusa Basmati 1 due to bacterial blight is also reported (Singh et al., 1977). Notably, transgenic lines exhibited increased tolerance to bacterial leaf blight pathogen in both \textit{in vitro} and \textit{in planta} assays. The spread of the disease lesions was reduced by \~82\% in transgenic lines.

Active inhibition of the fungal pathogen \textit{in vivo} in the present study was in agreement with the findings of Cammue et al. (1995) that the activity of Ace-AMP\textsubscript{1} unlike some other AMPs like \textit{Mj}-AMP2 (De Bolle et al., 1996) is not affected by physiological concentrations of cations. The antifungal activity of plant defensins, whether morphogenic or not, is affected by physiological concentrations of inorganic cations.

Ns-LTPs show some structural similarities to small cysteine-rich proteins called elicitins secreted by \textit{Phytophthora}. In plants, elicitins trigger a hypersensitive response which is associated with the induction of non-specific systemic resistance (Keller et al., 1996). Related early steps in the signaling pathways involve the specific recognition of elicitins by high affinity plasma membrane proteins, a calcium signal, several protein phosphorylation steps and changes in membrane permeability to ions.
leading to the production of active oxygen species (Ponchet et al., 1999).

A lipid transfer protein (wheat LTP1) has been reported to share binding sites with an elicitin on tobacco membranes (Buhot et al., 2001), suggesting that plant-encoded antimicrobial proteins can directly affect host gene expression. DIR1, a putative apoplastic lipid transfer protein has been shown to interact with a lipid-derived molecule to promote long distance signaling in plant defense (Maldonado et al., 2002). In this context, it is important to elucidate the possible downstream role that Ace-AMP1, accumulated in the apoplastic space, might be playing for enhanced disease resistance in transgenic rice.

Differentially expressed transcriptomes of wound induced untransformed and Ace-AMP1 transgenic plants were analysed where four clearly polymorphic partial cDNA fragments were seen on 6% denaturing polyacrylamide gel. These fragments were isolated, cloned, and sequenced. The DNA sequences obtained from transgenic sample showed significant homology to ‘chilling inducible protein’, ‘putative anther ethylene upregulated protein ER1’, and ‘putative auxin response transcription factor (ARF6)’. The DNA sequence obtained from untransformed sample showed homology to putative ‘Bowman Birk trypsin inhibitor protein’.

Gene expression studies of plants in response to biotic or abiotic stress found that disease resistance-related genes could be up-regulated by abiotic stresses and vice versa. Some stress-related proteins have been reported to not only confer stress-tolerance, but also enhanced disease resistance (Chen et al., 2004). Transgenic tobacco plants over-expressing a Brassica juncea glyoxalase I showed enhanced tolerance to methylglyoxal and high salt (Veena et al., 1999). Further investigation
suggests a direct role for glyoxalase I in corn resistance against aflatoxin accumulation through the removal of its aflatoxin inducing substrate, methylglyoxal (Chen et al., 2004). Chilling inducible protein, which was differentially up-regulated in transgenic rice plants expressing Ace-AMP1, has an important role in cold stress tolerance. Higher levels of such stress-related proteins in resistant lines may prove to be helpful as compared to susceptible ones in the ability to synthesise proteins and defend against pathogens while under stress. Similarly, anther ethylene upregulated protein ER1, a calmodulin binding protein, is involved in signaling. Calmodulin is a calcium binding protein that may have a pivotal role in stress tolerance via Ca\(^{2+}\) signaling. Calcium is a ubiquitous second messenger in eukaryotic signal transduction cascades and the intracellular Ca\(^{2+}\) levels, in plants, are modulated in response to pathogen elicitors (Rudd and Franklin-Tong, 2001). Calmodulins and calcium-dependent protein kinases (CDPKs) are some of the several known classes of Ca\(^{2+}\)-binding sensory proteins. Extensive studies in various plant/pathogen systems have demonstrated that a cytosolic calcium influx is a crucial early step for the activation of pathogen-induced signal transduction cascades (Rudd and Franklin-Tong, 2001). Pathogen response pathways are often activated by the interaction between a pathogen-encoded elicitor (such as the *Cladosporium fulvum* Avr9 peptide) and a corresponding plant-encoded receptor (such as the tomato Cf-9 resistance protein). Recently, a CDPK activated *in vivo* after a Cf-9/Avr9 gene-for-gene interaction has been identified in Cf-9 transgenic tobacco, suggesting that CDPKs are important calcium sensors in inducible defense responses (Romeis et al., 2001). Similar results are reported for the Cf-4/Avr4 gene-for-gene interaction, indicating a more general role for CDPKs in elicitor signaling events. Additionally, calmodulin has been shown
to mediate a significant amount of cross talk between ROS and calcium, both of which are signaling molecules facilitating cross tolerance to a variety of stresses (Bowler and Fluhr, 2000). Recently, it has been reported that auxin response transcription factor ARF 6 promotes jasmonic acid production and flower maturation (Nagpal et al., 2005). Salicylic acid (SA), ethylene, and jasmonic acid (JA) are important signaling molecules in plant defense response to biotic stress. Results from the present study suggest that accumulation of Ace-AMP1 might modulate the expression of a subset of genes in rice. In a very recent study, induction of PR genes (PR-2, PR-3, and PR-5) upon inoculation with karnal bunt fungal pathogen has been reported in transgenic wheat expressing Ace-AMP1 (Roy-Barman et al., unpublished). However, the mechanism of action of Ace-AMP1 in conferring disease resistance needs to be elucidated further.

Transgenic lines were comparable to the untransformed Pusa Basmati 1 plants with respect to agronomic parameters critical from a breeder's point of view. The transgenic rice plants generated may be useful as breeding materials since both constitutive and inducible expressions of Ace-AMP1 showed accumulation of the protein in the apoplast and the levels sufficient to restrict the economically important phytopathogens.

The high antimicrobial potency of Ace-AMP1 and enhanced resistance of transgenic rice against an array of pathogens makes Ace-AMP1 a promising candidate for genetic engineering of crop plants for improved disease resistance.