Integration patterns of the transgene, in four representative lines (PAL-Ace-4, PAL-Ace-54, UbI-Ace-T-6 and UbI-Ace-T-11A), were studied by Southern blot analysis. Genomic DNA from transgenic lines was digested with either Bam HI (cuts only once in the construct but outside the coding region) or Pst I (cuts once within the coding region) and blotted onto nylon membrane. When coding region of Ace-AMP1 was used as a probe, it hybridised to the genomic DNA from the transgenic lines tested. However, no hybridisation was observed to DNA of the untransformed plants (Figure 7). Inheritance of the transgene Ace-AMP1 was tested using PCR, in the T₁ generation (Figure 8). The stable integration of the transgene was confirmed by Southern analysis and identical banding patterns were observed over subsequent generations (Figure 9). The four independent transgenic lines (PAL-Ace-4, PAL-Ace-54, UbI-Ace-T-6 and UbI-Ace-T-11A), confirmed for integration and inheritance of the transgene Ace-AMP1, were analysed further for expression of Ace-AMP1 over next two generations.

3.3 Expression of Ace-AMP1 in transgenic rice

*S. cerevisiae* (S288C) was transformed with the vector pEG(KT)-Ace (Figure 2b). Two percent galactose was used to induce *GAL1* promoter driving expression of GST::Ace-AMP1. The fusion protein expressed in *S. cerevisiae* was purified using glutathione sepharose affinity matrix; and the purified fraction was subjected to CDNB assay (Table 5) where it showed significant GST activity (1.83 ΔA340/ min/ml), compared to undetectable activity in unbound and wash fractions used as controls. The fusion protein fraction was also checked on 10% SDS-PAGE where it
showed a single fragment of 35 KDa (26 KDa of GST + 9 KDa of Ace-AMP1) when stained with Coomasie Blue (Figure 10). Polyclonal antibodies were raised against Ace-AMP1 and the titer for the antibodies was found to be 1600. Protein extracts from leaf tissues of 4 transgenic lines were used for the immunodetection of Ace-AMP1. Equal concentration of protein from each sample was used to analyse expression of the protein. All the 4 lines tested showed positive immunological reaction where a band corresponding to Ace-AMP1 (9 KDa) was detected. Transgenic lines designated as PAL-Ace-54 and Ubi-Ace-T-11A showed higher accumulation of Ace-AMP1 compared to PAL-Ace-4 and Ubi-Ace-T-6. The untransformed control plants showed no immunoreactive protein (Figure 11). Western blot analysis was also performed in T2 generation and a band of expected molecular weight was observed (Figure 12).

### 3.4 Levels of Ace-AMP1 in transgenic rice

Levels of Ace-AMP1 in different T2 transgenic lines were estimated by Indirect ELISA, using the antibodies against Ace-AMP1. The levels of Ace-AMP1 in untransformed and 4 transgenic lines were compared, which showed that there was difference in the level of expression of the protein among transgenic lines (p-value: <0.001) and between untransformed plants and transgenic lines (p-value: <0.001; Figure 13). In the transgenic lines Ubi-Ace-T-6 and Ubi-Ace-T-11A, where expression of the transgene was driven by maize ubiquitin promoter, the amount of protein produced was found to be $3.49 \pm 0.34$ and $4.58 \pm 0.24 \mu g/mg$ of total protein, respectively. In case of transgenic plants where expression of the transgene was driven by PAL promoter, induction of the promoter by wound treatment led to 3.4-
4.5-fold increase in amount of Ace-AMP1 protein produced. The levels of Ace-AMP1 produced in the transgenic line PAL-Ace-4 were found to be $1.03 \pm 0.08$ and $3.58 \pm 0.46$ μg/mg of total protein, before and after wound treatment, respectively; while those in the transgenic line PAL-Ace-54 were found to be $1.12 \pm 0.11$ and $4.98 \pm 0.23$ μg/mg of total protein, respectively (Figure 13).
Figure 3: Putatively transformed rice calli confirmed by ‘callus PCR’ for presence of *Ace-AMP1* gene. Total genomic DNA was extracted from calli and subjected to PCR using specific primers for *Ace-AMP1* coding region. (1) untransformed rice callus; (2) positive control pKS-Ace; (3) to (8) individual transformed calli; (9) 1 Kb marker. The figures on the right side indicate DNA fragment sizes.
**Figure 4**: Regeneration of transformed rice calli and development of whole plants. (1) Transformed calli growing on selection medium; (2) Emerging green shoots from individual transformed calli on regeneration medium; (3) Further growth of the shoot; (4) Development of roots from the growing plantlets on rooting medium; (5) Plantlets emerging from individual transformed calli; (6) Primary hardening of the plantlets in small cups in the plant growth chamber.
Figure 5: *Ace-AMP1* transgenic (*T₀*) rice plants at maturity in the green house.
Figure 6: PCR amplification of gene for Ace-AMP1 (a) and Hpt (b) from genomic DNA of transgenic rice lines in T₀ generation. (a) (1) Positive control (pKS-Ace); (2) Untransformed PB1; (3) 100-bp ladder (pUC 18/Sau 3A I - pUC 18/Taq I digest); (4) PAL-Ace-4; (5) PAL-Ace-42; (6) PAL-Ace-52; (7) PAL-Ace-54; (8) UbI-Ace-T-6; (9) UbI-Ace-T-10; (10) UbI-Ace-T-11A. (b) (1) Untransformed PB1; (2) Positive control (pKS-Ace); (3) 1 Kb ladder; (4) PAL-Ace-4; (5) PAL-Ace-42; (6) PAL-Ace-52; (7) PAL-Ace-54; (8) UbI-Ace-T-6; (9) 1 Kb ladder; (10) UbI-Ace-T-10; (11) UbI-Ace-T-11A. The figures on the left side indicate DNA fragment sizes.
Figure 7: Southern blot analysis using genomic DNA from transgenic rice lines in T₀ generation. (1) Untransformed; (2) PAL-Ace-8; (3) PAL-Ace-42; (4) PAL-Ace-52; (5) PAL-Ace-54; (6) PAL-Ace-A; (7) Ubi-Ace-T-6; (8) Ubi-Ace-T-10; (9) Ubi-Ace-T-11A; (10) Ubi-Ace-T-11B; (11) Ubi-Ace-T-39. Ace-AMP1 coding region used as probe was hybridised to the genomic DNA digested with Bam HI.
Figure 8: PCR amplification of gene for Ace-AMP1 from genomic DNA of transgenic rice lines in T1 generation. PCR was carried out using DNA isolated from representative individuals (3 plants each line) from the transgenic lines PAL-Ace-4 (A), PAL-Ace-54 (B), Ubl-Ace-T-6 (C), and Ubl-Ace-T-11A (D). DNA from untransformed plant and plasmid (pKS-Ace) was used as negative (N) and positive (P) controls, respectively. One Kb ladder (M) was run along side the samples. The figures on the left side indicate DNA fragment sizes.
A M N P
1 0 0 0
7 5 0
5 0 0
2 5 0
3 6 3 b p
PCR product

363 bp
PCR product

363 bp
PCR product
Figure 9: Southern blot analysis using genomic DNA from transgenic rice lines in the T2 generation. (1) Untransformed plant; (2) PAL-Ace-4-1; (3) PAL-Ace-4-3; (4) PAL-Ace-54-5; (5) Ubi-Ace-T-6-6; (6) Ubi-Ace-T-11A-5. Ace-AMP1 coding region used as probe was hybridised to the genomic DNA digested either with Pst I or Bam HI. (P- Genomic DNA digested with Pst I, B- Genomic DNA digested with Bam HI).
Table 5: CDNB assay with purified GST::Ace-AMP1 fusion protein. Presence of GST in the protein fraction was detected using GST detection module and the activity was measured using the formula $\Delta A_{340} / \text{min / ml} = \frac{A_{340} (t2) - A_{340} (t1)}{(t2-t1)} \times \text{ml of sample}$. The wash and unbound fractions were used as controls. Note: 500 ng of protein from each fraction was used for the assay.
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<tr>
<td>Purified fraction</td>
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Figure 10: Purified GST::Ace-AMP1 fusion protein. The fusion protein was purified by affinity chromatography using glutathione-sepharose as affinity matrix. (1) Purified GST::Ace-AMP1; (2) Molecular weight marker; (3) Total protein from yeast (S288C) transformants expressing fusion protein under induced conditions; (4) Total protein from yeast (S288C) transformants expressing only GST under uninduced conditions.
~35 KDa
GST::Ace::AMP1
**Figure 11:** Immunodetection of \textit{Ace-AMP1} in transgenic rice lines in \( T_0 \) generation.

(1) Untransformed PB1; (2) Mol. wt. marker; (3) \textit{PAL-Ace-54}; (4) \textit{PAL-Ace-4}; (5) \textit{Ubi-Ace-T-6}; (6) \textit{Ubi-Ace-T-11A}; (7) Positive control (S288C-pEG(KT)-Ace). All the lanes were run with equal concentration of protein extract (20 \( \mu \)g) from respective lines. Antibodies used were anti-\textit{Ace-AMP1} raised in rabbit, and Alkaline Phosphatase-conjugated anti-rabbit IgG. The figures on the left side indicate molecular weights of proteins.
Figure 12: Immunodetection of Ace-AMPI in transgenic rice lines in T$_2$ generation.

(1) *Ubi-Ace-T-6-6*; (2) *Ubi-Ace-T-11A-5*; (3) *PAL-Ace-4-1*; (4) *PAL-Ace-4-3*; (5) *PAL-Ace-54-5*; (6) Molecular weight marker; (7) Positive control.
3.5 Sub-cellular location of Ace-AMP1 expressed in transgenic plants

Extra-cellular fluid and intracellular protein extract were collected to study the subcellular location of Ace-AMP1 expressed in leaf tissue of the transgenic plant. Cytosolic enzyme glucose-6-phosphate dehydrogenase was used as a marker to detect contamination of extra-cellular fluid with intracellular protein. The levels of glucose-6-phosphate dehydrogenase estimated showed that the extracellular fraction was 85% pure; and 82% of the Ace-AMP1 expressed was found to be present in extra-cellular fluid fraction, indicating its accumulation in the apoplast (Figure 14a). Tissue localisation of Ace-AMP1 was also studied by treating sections of both untransformed and transgenic rice leaf tissue with anti-Ace-AMP1 antibodies followed by FITC-conjugated goat anti-rabbit IgG antibody. Two controls were run along with the test tissue samples from transgenic plant. One of the two control tissues was from untransformed plant and was treated with both anti-Ace-AMP1 antibodies and FITC-conjugated goat anti-rabbit IgG antibody (Figure 14b-1). The other control tissue was from transgenic plant and was exposed only to the FITC-conjugated goat anti-rabbit IgG antibody, in the absence of anti-Ace-AMP1 antibodies (Figure 14b-2). Labeling of vascular bundle and mesophyll cells was evident within the transgenic rice leaf tissue sections treated with both anti-Ace-AMP1 antibody and FITC-conjugated goat anti-rabbit IgG antibody. The immunofluorescence was strongly associated with wall material, while cytoplasmic content remained unlabelled (Figure 14b-3).
Figure 13: Levels of $Ace$-AMP1 in transgenic rice lines. Concentration of $Ace$-AMP1 in the protein extracts from transgenic leaf tissues (using either $PAL$ or $UbI$ promoter) was estimated by plotting the absorbance values on standard graph prepared using known concentrations of purified $Ace$-AMP1. Fold increase in the amount of $Ace$-AMP1 upon induction of $PAL$ promoter by wound treatment, was calculated using protein extracts from uninduced (UI) and wound induced (I) (24 h) leaf tissues from $PAL$-$Ace$ transgenic lines. The data are presented as means ± SEM from three independent estimations.
Figure 14: Sub-cellular location of Ace-AMP1 in transgenic leaf tissue. (a) (i) G-6-PD estimated in different protein fractions showed 85% purity of the extracellular fraction. (ii) Levels of accumulated Ace-AMP1 in different protein fractions were estimated by ‘indirect ELISA’ using antibodies against Ace-AMP1. (b) Indirect immunolocalisation of Ace-AMP1 in transgenic rice leaf tissue. Leaf tissues were sectioned through vascular bundle. The control section 1 was from untransformed leaf tissue, whereas sections 2 and 3 were from transgenic leaf tissue. The control section 2 was treated without anti-Ace-AMP1 antibody but with FITC-conjugated goat anti-rabbit IgG antibody. Sections 1 and 3 were treated with both anti-Ace-AMP1 antibody as well as FITC-conjugated goat anti-rabbit IgG antibody. Notes: VB, vascular bundle. Arrows indicate strong fluorescent labeling and bars correspond to 10 μm.
(a)

(i) Graph showing Sp. activity of G-6-PD.

(ii) Graph showing Ace-AMP (nmol/mg total protein).

(b) Images showing VB (Ventral Body) labeled with different markers:

1. General view of VB.

2. Close-up of VB highlighting specific structures.

3. Detailed view of VB with arrows indicating specific points of interest.
3.6 Inhibitory concentration of Ace-AMP1 against *M. grisea* and *X. oryzae*

*In vitro* growth inhibition assay with the two pathogens showed inhibitory activity of Ace-AMP1. The concentration of purified Ace-AMP1 completely inhibiting spore germination of *M. grisea* (~$1 \times 10^5$ spores/ml) and growth of *X. oryzae* (~$1 \times 10^4$ cells/ml) was found to be 4 μg/ml (Table 6).

3.7 Inhibitory effect of Ace-AMP1 on the blast fungus

Effect of Ace-AMP1 on germinating conidia of *M. grisea* was observed by microscopy using Calcofluor White (CFW). The untreated biomass of the fungus showed uniform distribution of chitin and glucan (Figure 15a). The septa were also distinctly visible. The Ace-AMP1 treated samples showed densely stained bulbous structure at the tip of mycelia, unlike untreated control. When spores of the blast fungus were treated with Ace-AMP1, tip ballooning or filaments with hyperbranching and morphological abnormalities were noticed. The spores, which were not treated with Ace-AMP1 showed normal growth of the hyphae (Figure 15b (i)). The germ tube in case of treated samples appeared normal at 1 h. The tip of the mycelia started swelling 2 h after Ace-AMP1 treatment, followed by a well developed bulbous structure at the tip of fungal filament clearly visible within 4 h. Further exposure led to more bulging of the balloon-like structure at 6 h that apparently ruptured resulting in release of cellular content in the surrounding medium.
Table 6: Antimicrobial activity of Ace-AMP1. (a) *X. oryzae* pv. *oryzae* (*Xoo*) strain BXO479 (10^4 cells/ml) were incubated in sterile Luria Bertani broth containing various amounts of Ace-AMP1 for 48 h. Bacterial growth (%) in Ace-AMP1 containing medium calculated as compared to 100% growth in control medium (medium without Ace-AMP1) was determined by measuring absorbance at 600 nm. (b) The spores of *M. grisea* (10^5 spores/ml) were germinated in sterile yeast extract glucose broth containing various amounts of Ace-AMP1 at 25 °C for 24 h. Percentage spore germination in presence of Ace-AMP1 was compared to 100% germination in medium without Ace-AMP1.
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<td>M. grisea</td>
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Figure 15: Inhibitory effect of Ace-AMP1 on the blast fungus. (a) Samples were stained with CFW. (i) Untreated *M. grisea* (ii) Fungus exposed to Ace-AMP1 (3 µg). It was evident that the inhibitory molecule (Ace-AMP1) leads to bulb formation at the tip of growing hyphae. (b) Fungus was treated with Ace-AMP1 and observations were made at 0 h, 2 h, 6 h, and 10 h. (i) Untreated fungus grew well with normal development of filaments. (ii) Swelling of hyphal tip was visible within 2 h after incubation with Ace-AMP1. Apparent leakage from the bulbous structure was seen after 6 h of incubation (shown by arrow). (iii) Hyperbranching and abnormal development of hyphae was also visible 2 h onwards.
At the end of 10 h, a second emerging filament from the same conidium also started developing a bulbous structure at its tip (Figure 15b (ii)). Another kind of morphological abnormality like hyper branching was also observed (Figure 15b (iii)).

3.8 Electrolyte leakage caused by *Ace-AMP1*

To study the effect of *Ace-AMP1* on the blast fungus, electrolyte leakage (EL) was measured at 12 h and 24 h, using untreated and treated fungal samples. Supernatants from growing cultures of treated and untreated fungal cultures were assayed for EL. The %EL increased within 12 h of exposure to *Ace-AMP1* (Figure 16). The EL for *Ace-AMP1* treated samples was 26.14 ± 0.16 % and 41.89 ± 0.33 % at 12 h and 24 h, respectively. Whereas, for the untreated samples the EL values were 2.97 ± 0.47 % and 1.13 ± 0.12 % at 12 h and 24 h, respectively.
Figure 16: Electrolyte leakage caused in the blast fungus after treatment with Ace-AMP1. Conductivity was measured in the supernatant from treated fungal cultures after 12 h and 24 h of incubation at 25 °C in presence of 5 µg Ace-AMP1. Untreated culture was used as control. Conductivity of autoclaved culture supernatant was considered as absolute conductivity. The percentage EL was calculated by dividing the initial conductivity by the absolute conductivity and it was found to be significantly higher in treated samples.
3.9 Enhanced fungal and bacterial disease tolerance of transgenic rice expressing Ace-AMP1

The transgenic rice lines (PAL-Ace-4, PAL-Ace-54, Ubi-Ace-T-6 and Ubi-Ace-T-11A) analysed for integration and expression of the transgene, were used for in vitro and in planta assays using M. grisea, R. solani, and X. oryzae pv. oryzae, while untransformed plants were used as control.

Enhanced resistance to blast disease

In an in vitro assay with M. grisea, protein extract from leaf tissue of transgenic plants either inhibited germination of spores or led to abnormal development of hyphae, such as tip ballooning. Incubation with protein from leaf tissue of untransformed plant had no effect on the growth and morphology of the fungal hyphae (Figure 17a). For whole plant infection assay, 21 day old plants, in both T1 and T2 generations, were inoculated with blast isolate B157 and severity of the infection was evaluated over a period of 10 dpi. The untransformed control plants (18 out of 20 plants) showed typical susceptible type of lesions (Type 4) after 5-6 dpi. Transgenic lines expressing Ace-AMP1 showed type 1-3 lesions (~14 to 18 out of 20 plants, each from 4 lines) developed 6-7 dpi. Severity of infection 10 dpi was high (spreading of lesions) in untransformed plants unlike in transgenics (Figure 17b). The average % diseased leaf area (% DLA) of untransformed and transgenic lines was compared, which showed a difference among the lines (p-value: <0.001); and between untransformed and the transgenic lines (p-value: <0.001; Figure 17c). The four transgenic lines showed %
DLA ranging from 0.97 ± 0.2 to 14.11 ± 1.82%, whereas it was 47.65 ± 2.97% in untransformed plants. Detached leaf assay was also performed using a concentration of inoculum of 1 x 10⁵ spores/ml. Disease symptoms on control leaves started appearing around 3 dpi whereas in leaves of Ace-AMP1 transgenic plants disease symptoms were absent till 3 dpi. Actively spreading lesions developed on the leaves of control plants. Control leaves were visibly damaged by the fungus 7 dpi, whereas leaves from Ace-AMP1 transgenics appeared to be much healthier. Small necrotic lesions were formed on the leaves of the transgenic lines which were similar to hypersensitive response (HR). To substantiate the results of symptom development in inoculated leaves, tissue sections from these inoculated leaves were stained with Trypan Blue and observed microscopically. Extensive growth of fungal hyphae was seen in the leaf tissue of control leaves. In contrast, only hyphae showing abnormal morphology, namely, short and swollen hyphae were detected at the infected sites of leaves from transgenic lines (Figure 18).

Enhanced resistance to sheath blight disease

To evaluate whether transgenic plants expressing Ace-AMP1 were resistant to another fungal pathogen *R. solani*, 4-5 week old seedlings, in both T₁ and T₂ generations, were challenged with the pathogen in whole plant infection assays and appearance of disease symptoms were observed over 15 dpi.
Figure 17: Enhanced blast disease tolerance of transgenic rice lines expressing gene for *Ace*-AMP1. (a) Total protein from leaf tissue of untransformed Pusa Basmati 1 and transgenic rice plants was extracted and used to check growth of *M. grisea*. The samples were stained with Calcofluor White; and it was evident that protein from transgenic lines inhibited the fungus by leading to development of bulbous structure at the tip of growing hyphae. (b) Challenged transgenic lines showed resistance towards blast disease in whole plant infection assay in green house; (A) Untransformed PB1; (B) PAL-Ace-4; (C) PAL-Ace-54; (D) Ubi-Ac'e-T-6; (E) Ubi-Ace-T-11A. Large spreading lesions on untransformed plants in contrast to hypersensitive response on transgenics are indicated by arrows. (c) Infected leaf area was measured as % DLA after 10 dpi. Data shown are means ± SEM.
Transgenic lines

(a) Untransformed

(b) Transgenic

(c) % DLA
Figure 18: Effect of Ace-AMP1 on the blast fungus, *in vivo*. Detached leaf assay was performed using leaves from untransformed and *PAL-Ace-54* transgenic line. Leaf pieces from untransformed and transgenic line were inoculated with the blast fungal spores (solution contained $1 \times 10^5$ spores/ml and 0.05% Triton X-100). Sections (LS) were cut at the inoculated sites on both the leaf samples. A normal and profuse fungal growth was evident from the sections of the leaf from untransformed plants, whereas those from transgenic plants showed either no growth or abnormal hyphal growth (shown by arrow).
The untransformed plants started developing disease symptoms 5th day onwards following inoculation, while no symptoms were seen in transgenic lines till 7 dpi. Untransformed plants (15 out of 20 plants) 12 dpi, showed class III and IV infection; while it was restricted to class 0, I, and II in case of transgenic lines (~14 to 17 out of 20 plants, each from 4 lines) (Figure 19a). Most of the untransformed plants (10 out of 20 plants), with progressing disease, showed completely infected stems with folding and curling of leaves (class III), and 2 of 20 plants died (class IV) due to infection. However, transgenic plants showed improved level of resistance against the fungus. The average % infection of untransformed and transgenic lines was compared, which showed that there was difference among the lines ($p$-value: <0.001); and between untransformed and the transgenic lines ($p$-value: <0.001; Figure 19b). Untransformed plants showed 62.50 ± 3.58% infection with class ranging from I to IV, while plants from transgenic lines showed only 16.67 ± 4.67 to 26.67 ± 6.21% infection mainly with class I and II.

**Enhanced resistance to bacterial leaf blight disease**

In order to determine whether expression of Ace-AMP1 in transgenics could confer resistance to bacterial pathogens, *in vitro* assay, and *in planta* inoculation assays were performed with *X. oryzae*. The protein extracts from leaf tissue of transgenic plants inhibited growth of the bacterial pathogen *in vitro*. Approximately, 45 day old plants from transgenic lines and untransformed plants were inoculated with *X. oryzae* pv. *oryzae*, for whole plant infection assays.
Figure 19: Enhanced sheath blight disease tolerance of transgenic rice lines expressing gene for Ace-AMP1. (a) Whole plant infection assays in green house showed that transgenic rice lines were resistant to sheath blight disease. (A) Untransformed PB1; (B) PAL-Ace-4; (C) PAL-Ace-54; (D) Ubi-Ace-T-6; (E) Ubi-Ace-T-11A. Class IV infection on untransformed plants as opposed to class I or II on transgenics are shown by arrows. Response to the sheath blight infection was scored in terms of % infection (b) of plants from individual transgenic lines and untransformed plants. Data represent means ± SEM.
(a) Untransformed percentage infection.

(b) Bar graph showing infection rates for different transgenic lines.
Figure 20: Enhanced bacterial leaf blight disease tolerance of transgenic rice lines expressing Ace-AMP1. (a) Whole plant infection assay showed reduced area of infection in transgenic rice lines unlike untransformed PB1; (A) Untransformed PB1; (B) PAL-Ace-4; (C) PAL-Ace-54; (D) UbI-Ace-T-6; (E) UbI-Ace-T-11A. Length of arrow on left and right side indicates area of infection in untransformed and transgenics, respectively. (b) Infected area was measured in terms of length (mm) of enlarged lesion after 10 dpi. Values are means ± SEM.
Transgenic lines

(a)

(b)

Length of diseased area (mm)

Untransformed  PAL-Ace-4  PAL-Ace-54  Ubi-Ace-T-6  Ubi-Ace-T-11A

Transgenic lines
The plants were checked for water soaked areas in the inoculated leaves within 48-72 h and length of enlarged lesions was measured from the inoculated ends after 12 dpi. The untransformed plants with large diseased area as compared to the transgenic lines indicated enhanced resistance against bacterial leaf blight in transgenics (Figure 20a). The average length of diseased area of leaves from untransformed and transgenic lines was compared, which showed that there was difference among the lines (p-value: <0.001); and between untransformed and the transgenic lines (p-value: <0.001; Figure 20b). The length of diseased area was found to be 12 ± 0.62 mm in inoculated untransformed plants, whereas it ranged from 1.76 ± 0.16 to 2.6 ± 0.19 mm in the four transgenic lines.

Although, levels of expression of Ace-AMP1 varied among the four transgenic lines, there was a correlation between the amount of protein produced and degree of enhanced tolerance to fungal or bacterial disease in the corresponding line. Among the 4 transgenic lines studied, UbI-Ace-T-11A with higher level of expression of Ace-AMP1 was found to be most tolerant to all three diseases than other transgenic lines.

3.10 Differential display of RNA from wound induced untransformed and Ace-AMP1 transgenic rice

The Differential Display using Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) was optimised and carried out using silver staining detection method. Total four primers (OPA-1, OPA-5, OPD-10, and OPD-17) were used for the reactions. The primers were used either singly or in combination (OPA-1 + OPA-5, and OPD-10 + OPD-17). Different primer combinations yielded polymorphic partial
cDNA fragments in both transgenic as well as untransformed sample. The combination of OPD-10 and OPD-17 for the PCR reaction showed maximum polymorphic cDNA fragments in both the samples. Out of all the polymorphic bands, 4 noticeably differential fragments from both the samples were picked up (Figure 21a). The fragments picked up from transgenic samples were designated as t1, t2, t3, whereas that from the untransformed sample was labeled as c1. Re-amplification from these fragments showed different molecular sizes ranging from 75 bp to 350 bp on 2% agarose gel (Figure 21b).

3.11 Nucleotide sequence of the differentially expressed cDNA fragments

The 4 polymorphic cDNA fragments were cloned at Eco RV site in pBlueScript KS". The recombinant plasmids were again used for the amplification using same primers to confirm cloning of right fragments. Amplification showed similar molecular sizes as in earlier re-amplification. These recombinant plasmids were used for sequencing using universal primers and the sequences obtained (Figure 22) were analysed using BLAST to identify differentially expressed genes with their putative role (Table 7). Sequences analysed showed significant similarities to *Oryza sativa* mRNA for chilling inducible protein, putative anther ethylene responsive protein ER1, auxin responsive transcription factor, and putative Bowman Birk trypsin inhibitor. Northern blot analysis was performed using the cloned partial cDNA fragment of chilling inducible protein gene to validate the DDRT-PCR results. Northern blot analysis detected variation in the levels of the transcripts of the ‘Chilling inducible protein’ in wound induced *PAL-Ace-54* transgenic line at 24 h of wound treatment (Figure 23).
The increase in the level of transcript was 3.2-fold in the transgenic line as compared to the untransformed.

3.12 Agronomic characteristics of transgenic rice expressing Ace-AMP1

Phenotypic characteristics studied among the four representative transgenic lines were found to be similar to the untransformed plants (Figure 24). All the four transgenic lines showed 100% germination like 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.
Figure 21: Differential display of RNA from untransformed and Ace-AMP1 transgenic rice. Total RNA was extracted from wound induced (24 h) untransformed and PAL-Ace-54 transgenic line, and subjected to DDRT-PCR using arbitrary but defined primers. (a) The DDRT-PCR products are run on 6% sequencing gel. From left to right- lane (1) and (2) transgenic and untransformed, respectively, using OPD-10 and OPD-17 primers; lane (3) molecular weight marker (100 bp ladder); lane (4) and (5) transgenic and untransformed, respectively, using OPA-1 and OPA-5 primers; lane (6) molecular weight marker (1 Kb ladder). The polymorphic partial cDNA fragments picked up from the gel are marked with arrows and respective designations. (b) Re-amplification of the differentially expressed partial cDNA fragments using the same respective primers and PCR profile. The PCR products are electrophoresed on 2% agarose gel.
Figure 22: Nucleotide sequences of the differentially expressed partial cDNA fragments. The re-amplified PCR products were cloned in pBlueScript KS⁺ and sequenced.
t1 Sequence
5'
AGCGCGCGTATAACGACTCATATATGGGCGAATGCCAAGCGCGCAATTA
ACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCG
3'

t2 Sequence
5'
CCGCGGTGGCGGCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATCTCCATCCAATGTCTCATCCATGCCACGATACACTGAGATCATTCCCTTCAGAAGCTGCTTCA
3'

t3 Sequence
5'
AATACGACTCAT ATTGGGGCAATTGGAGCTCCACCGCGG CGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATTAGTT
AGACTAGAGGGTGTAGACCATCAAGCTTATCGATACCGTCGACCTCGAGG
GGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGT
3'
c1 Sequence
5'
GCGCGTATAACGACTCATATATGGGCAATTGGAGCTCCACCGCGG CGTGGCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATTAGTT
AGACTAGAGGGTGTAGACCATCAAGCTTATCGATACCGGTCCGACCTCGAGG
GGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGT
3'
Table 7: Differentially expressed genes with their putative role in Ace-AMP1 transgenics.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Size of the amplicon</th>
<th>Sequence homology</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1</td>
<td>~150 bp</td>
<td><em>O. sativa</em> mRNA for chilling-inducible protein (98%)</td>
<td>Cold stress tolerance</td>
<td>Binh and Oono, 1992. <em>Plant Physiology</em></td>
</tr>
<tr>
<td>t2</td>
<td>~250 bp</td>
<td><em>O. sativa</em> mRNA for putative anther ethylene-upregulated protein ‘ER1’ (99%)</td>
<td>Calmodulin binding protein-involved in signalling</td>
<td>Direct submission to NCBI (01-Jul-2004)</td>
</tr>
<tr>
<td>t3</td>
<td>~150 bp</td>
<td><em>O. sativa</em> mRNA for putative auxin response transcription factor (ARF6)</td>
<td>Transcription factor (activator) that binds to TGTCTC auxin response elements in promoters of early auxin response genes</td>
<td>Tiwari et al., 2003. <em>The Plant Cell</em></td>
</tr>
<tr>
<td>e1</td>
<td>~200 bp</td>
<td><em>O. sativa</em> rbbi2-3 gene for putative Bowman Birk trypsin inhibitor (100%)</td>
<td>Induced by wound and jasmonic acid</td>
<td>Qu et al., 2003. <em>Plant Physiology</em></td>
</tr>
</tbody>
</table>
Figure 23: Differential expression of ‘chilling inducible protein’ in the wound induced transgenic rice leaves. Northern blot analysis was performed using total RNA from wound induced (24 h) untransformed and PAL-Ace-54 transgenic line. Differentially expressed partial cDNA of chilling inducible protein was used as a probe. Fold increase in the transcript level of ‘chilling inducible protein’ was found to be 3.2. Hybridisation using 18S rDNA as probe was used as an internal control.
Chilling-inducible protein
Ace-AMP1
rRNA
rRNA on agarose gel
Untransformed
PAL-Ace-54
Figure 24: Agronomic characteristics of transgenic rice expressing Ace-AMP1. Parameters like percentage germination (a), height of plant (cm) (b), days required for 50% flowering (c), and test weight (g) (d), were considered as per breeder’s standard procedures.