2. MATERIALS & METHODS

2.1. Plant Materials
The wheat lines Bobwhite, PBW343 and HD2687 were selected for genetic transformation. Bobwhite is a Mexican spring cultivar and has high level of resistance to the fungal pathogens. However, this cultivar is comparatively easier to transform and is often treated as a lab variety for wheat genetic transformation experiments. PBW343 and HD2687 are the Indian wheat lines susceptible to karnal bunt, loose smut, powdery mildew and leaf and stripe rust diseases. These cultivars are grown in the semi-arid region by the marginal farmers in India. The selection has been reviewed by CIMMYT.

2.2. Bacterial Strains and Culture Media

1. DH5α (SupE44lacU169 80lacZ M15 hsdR17 recA1 endA1 gyrA9 thi1)
2. JM101 (SupE thi (lac-proAB) F' (traD36 PROab^+ lacI lacZ ΔM15)

*E. coli* was grown at 37°C on Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, 16 g Agar). The *E. coli* transformants carrying the plasmid vectors were grown on LB agar containing 100 μg per ml of ampicillin.

Bacterial strains were maintained at 4°C as slant or stab cultures on LB agar. Long-term preservation of bacterial strains was done in 20% glycerol solution at -80°C.
2.3. Yeast Cultures and Media

The yeast strain S288c transformed with the plasmid pEG/KT-Ace (Patkar and Chattoo, unpublished) was grown on 1X YNB medium (10X YNB yeast nitrogen base, 1.7 g and ammonium sulphate, 5 g in 100 ml) The yeast culture was grown for two days to an optical density of 0.5 to 0.8 and 4% galactose was added into the medium for induction of the GAL promoter After few hours of induction, the cells were harvested for extraction of total protein.

2.4. Isolation of total protein from yeast cells and purification of Ace-AMP1

Harvested yeast cells were resuspended in 1/100th culture volume of 1X PBS (10X PBS sodium chloride, 8 g, potassium chloride; 0.2 g, Di-sodium hydrogen phosphate, 1.44 g, Potassium di-hydrogen phosphate, 0.24 g in 1 L, pH 7.4) Cells were ruptured using glass beads, centrifuged at 6708 x g for 10 min and the supernatant was collected as the total cellular protein From this total protein extract, the Ace-AMP1 protein was purified using GST bulk purification kit (Amersham Biosciences, Hongkong)

2.5. Fungal Pathogens and Culture Media

_Tilletia indica_ or _Neovossia indica_ (Class - Basidiomycetes, Order - Ustilaginales, Family - Tilletiaceae) is the causal agent of Karnal Bunt disease This culture was maintained on potato dextrose agar (39 g/l) (HiMedia, Mumbai, India) at 20°C _Ustilago tritici_ (Class - Urediniomycetes, Order - Ustilaginales) is the causal agent of loose smut disease This culture was grown at room temperature on YEPD medium (yeast
extract, 10 g, bacteriological peptone, 20 g; glucose, 20 g and solidified with 1.5% agar, in 1 L.

The model test culture *U. maydis* causing corn smut in *Zea mays* was also grown on YEPD medium at room temperature.

### 2.6. Fungal Growth Inhibition Assays

A simple and sensitive method for testing of the antifungal activities of *Ace-AMP1* and GOX was followed. Two hundred microlitre of the test culture was taken and mixed thoroughly with 3 ml of soft agar (0.6%) and overlay of fungal spores (Koltin and Dey 1975) was made on the respective growth media. The antifungal proteins were applied on the filter discs placed or wells created on the fungal growth medium. The concentrations of GOX (1 unit every 2.94 µg of the enzyme) applied varied from $10^{-10}$ unit/µl to 2.0 unit/µl and the amount of *Ace-AMP1* taken were varied from 2 µg/ml to 12 µg/ml.

### 2.7. Media for Plant Tissue Culture

**SMS Medium for Somatic Embryogenesis**

SMS medium which is MS medium (Murashige and Skoog 1962) that consisted of MS salts with vitamins (4.6 g/l) (full MS), casein hydrolysate (100 mg/l), maltose (30 g/l i.e. 0.3%) and 2, 4 D (1.5 mg/l). All these ingredients were mixed together and the pH was adjusted between 5.5 and 5.8 with dropwise addition of 1 N NaOH. Agarose was added to a final concentration of 0.6% for solidification of the medium.
SMS 20 Medium for Plasmolysis

SMS 20 medium was the same as SMS with 20 % (i.e. 200 g/l) maltose in contrast to 3 % as in case of SMS medium.

Selection or Regeneration Medium

This medium consisted of MS salts and vitamins (4.6 g/l) (full MS), maltose (20 g/l i.e. 2 %), 1 - Naphthalene Acetic Acid i.e. NAA (1 mg/l, dissolved in 1 N NaOH), 6 - Benzylamino purine i.e. BAP (2 mg/l, dissolved in 1 N NaOH), L-Phosphinothricin or Glufosinate Ammonium i.e. PPT (5 mg/l, dissolved in water) and gelarite (2 g/l i.e. 0.2 %) for solidification of the medium. All these ingredients were mixed together and the pH was adjusted between 5.5 and 5.8 with dropwise addition of 1 N NaOH.

Root Induction Medium

This medium consisted of MS salts and vitamins (2.3 g/l) (half MS), maltose (10 g/l i.e. 1 %), L-Phosphinothricin or Glufosinate Ammonium i.e. PPT (3 mg/l, dissolved in water) and gelarite (2 g/l i.e. 0.2 %) for solidification of the medium. All these ingredients were mixed together and the pH was adjusted between 5.5 and 5.8 with dropwise addition of 1 N NaOH.

2.8. Plasmid Constructs

pCl (Cone et al., 1986), pBperu (Goff et al., 1990) (both the genes are driven by CaMV 35S promoter) and pAct::GUS (Jefferson et al., 1987) constructs (Figure 2a) were used in the transient transformation experiments. The Cl and Bperu genes code for
transcription factors in the anthocyanin biosynthetic pathway. Antifungal genes used in the genetic transformation of wheat were GOX *Aspergillus niger* and Ace-AMP1 from *Allium cepa*. Both the genes were placed under either constitutive maize ubiquitin promoter and pathogen and wound inducible rice phenylalanine ammonia lyase (PAL) promoter to make the constructs pUbI::GOX and pPAL::GOX (Kachroo et al., 2003) and pUbI::Ace-AMP1 and pPAL::Ace-AMP1 (Patkar and Chattoo, unpublished), respectively (Figure 2b). Phosphinothricin acetyl transferase (*bar*) that acetylates phosphinothricin, the active ingredient in the herbicide bialaphos or basta, was used a selection marker and was cloned under rice actin 1 (Act) promoter to make the construct pAct::BAR (Figure 2b) (Clausen et al., 2000) The constructs pUbI::GOX, pPAL::GOX, pUbI::Ace-AMP1, pPAL::Ace-AMP1, and pAct::BAR were used in the stable transformation experiments.

### 2.9. Preparation of Competent Cells

A single, well-isolated colony from a fresh Luria-Bertani (LB) agar plate (containing carbenicillin) was inoculated into 3 ml LB medium (containing the same antibiotic). The culture medium was incubated overnight (12-16 h) at 37°C in a shaker-incubator. Next day 100 ml of LB was inoculated with 1% inoculum in one litre flask. The culture was grown at 37°C for 2 h and 15 min in a shaking incubator. The cells (50 ml each) were harvested by centrifugation at 503 x g for 3 min at 4°C. The pellet was resuspended in 20 ml of ice-chilled 0.1 M CaCl₂. The cells were then incubated on ice for 30 min at 4°C. The cells were reharvested from the CaCl₂ solution by centrifugation and resuspended in 2 ml of 0.1 M CaCl₂ containing 20% glycerol. The competent cells were stored at -80°C.
Figure 2: Plasmid constructs used for transient (a) and stable (b) genetic transformation experiments in wheat. The expression of the genes for Ace-AMP1 and GOX was driven by constitutive maize ubiquitin promoter (I, IV) and rice wound/pathogen-inducible phenylalanine ammonia lyase (PAL) promoter (II, V), expression of the selectable marker bar is driven by rice actin promoter (III). For the screenable markers, expression of the transcription factors C1 and Bperu was driven by CaMV35S (VI) and maize ubiquitin promoter (VII), respectively. The expression of the gene for gus driven by rice actin promoter (VIII).
2.10. *E. coli* Transformations

Competent cells of *E. coli* were transformed with plasmid vectors using the calcium chloride method (Sambrook and Russel, 2001). The cells were grown in LB broth to an exponential phase ($\text{OD}_{600} = 0.3$ to $0.4$), harvested by centrifugation at $4^\circ$C and resuspended in an ice-cold 0.1 M calcium chloride. The cells were incubated on ice for 30 min, centrifuged and again resuspended in an ice-cold 20 % glycerol with 0.1 M CaCl$_2$ solution. About 0.2 ml of cell suspension was used for transformation of plasmid DNA (usually 50 ng) and incubated at $4^\circ$C for 15 min. Next, cells were subjected to heat shock at $42^\circ$C for 90 sec, followed by 5 min incubation on ice. One ml of LB broth was added to the above suspension and incubated at $37^\circ$C for 30 min. About 0.1 ml aliquot of transformation mixture was plated on LB agar medium containing ampicillin (100 mg / ml).

2.11. Plasmid DNA Miniprep

Plasmid DNA minipreps were done using Wizard$^R$ Plus SV Minipreps DNA Purification System (Promega, Wallisellen, Switzerland) was used. A single, well-isolated colony from a fresh Luria-Bertani (LB) agar plate (containing carbenicillin) was inoculated into 3ml LB medium (containing the same antibiotic). The culture medium was incubated overnight (12-16 h) at $37^\circ$C in a shaker-incubator. In each eppendorf tube 1.5 ml of bacterial culture was harvested by centrifugation for 30 sec at 13148 x g on a table-top centrifuge. The supernatant was poured off and the inverted tube was blotted on a tissue paper to remove the excess media. Cell resuspension solution (250 $\mu$l) was added in the tube and cell pellet was resuspended completely by vortexing. Then, 250 $\mu$l of cell lysis
solution was added and mixed by inverting the tube 4 times. It was incubated until the cell suspension cleared in approximately 1-5 min. At this step, 10 µl of alkaline protease solution was added and mixed by inverting the tube 4 times and incubated for 5 min. Subsequently, 350 µl of Wizard® Plus SV neutralisation solution was added and mixed by inverting the tube 4 times. Bacterial lysate was centrifuged at 13148 x g, for 10 min at room temperature. Plasmid DNA purification unit was prepared by inserting one Spin Column into a 2 ml Collection Tube for each sample. The cleared lysate (approximately 850 µl) was transferred to the prepared column by decanting. The supernatant was centrifuged at 13148 x g for 1 min at room temperature. The Spin Column was removed from the Collection Tube and the flowthrough was discarded from the Collection Tube. The Spin Column was reinserted into the Collection Tube. Next, 750 µl of Column Wash Solution (previously diluted with 95% ethanol) was added to the Spin Column and centrifuged at 13148 x g for 1 min at room temperature. The Spin Column was removed from the tube and flowthrough was discarded. The Spin Column was reinserted into the Collection Tube. The washing step was repeated using 250 µl Column Wash Solution. The assembly was centrifuged at 13148 x g for 1 min at room temperature. At this step, the Spin Column was transferred to a new, sterile 1.5 ml microcentrifuge tube, taking care not to transfer any of the Column Wash Solution with the Spin Column. Plasmid DNA was eluted by adding 100 µl Nuclease-Free Water to the Spin Column. The assembly was centrifuged at 13148 x g for 1 min at room temperature. After eluting the DNA, the assembly was removed from the 1.5 ml microcentrifuge tube and the Spin Column was discarded. Purified plasmid DNA was stored at -20°C.
2.12. Plasmid DNA Maxiprep

Plasmid DNA maxipreps were made using HiSpeed Plasmid Maxi Kit (Qiagen, Hombrechtikon, Switzerland). A single colony was picked from a freshly streaked selective plate and a starter culture of 3 ml LB medium containing the appropriate selective antibiotic was inoculated and incubated for ~8 h at 37°C with vigorous shaking (~300 rpm). The starter culture was diluted 1000 times into selective LB medium and 100 ml medium was inoculated and grown at 37°C for 12-16 h with vigorous shaking (~300 rpm). Bacterial cells were harvested by centrifugation at 2415 x g for 15 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 10 ml of buffer P1. 10 ml of buffer P2 was added into it and mixed gently but thoroughly by inverting 4-6 times and incubated at room temperature for 5 min. 10 ml of buffer P3 was added into it and mixed immediately but gently by inverting 4-6 times and was incubated on ice for 15 or 20 min. The mixture was then centrifuged at 11337 x g for 30 min at 4°C. The supernatant containing the plasmid DNA was removed promptly. The supernatant was recentrifuged at 11337 x g for 15 min at 4°C. The supernatant containing the plasmid DNA was removed promptly. QIAGEN-tip 500 was equilibrated by applying 10 ml of buffer and the column was allowed to be empty by gravity flow. The plasmid supernatant was applied to the QIAGEN-tip and it was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 30 ml buffer QC and DNA was eluted with 15 ml buffer QF. DNA was precipitated by adding 10.5 ml (0.7 volume) room temperature isopropanol to the eluted DNA. It was mixed and centrifuged immediately at 8117 x g for 30 min at 4°C. The DNA pellet was washed with 5 ml room temperature 70 % ethanol and centrifuged at 8117 x g for 10 min. The supernatant was decanted carefully without
disturbing the pellet. The pellet was air-dried for few minutes and DNA was redissolved in a suitable volume of buffer (e.g., TE, pH 8.0 or 10 mM Tris Cl, pH 8.5) or water.

2.13. Purification of DNA fragments

Plasmid DNA constructs were purified using QIAquick Gel Extraction Kit (Qiagen, Hombrechtikon, Switzerland). The DNA fragment of interest was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colourless tube. 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 µl). The tube was incubated at 50°C for 10 min or until the gel slice had got completely dissolved. To help dissolution of the gel, the sample tube was vortexed every 2-3 min during the incubation. After the gel slice had got dissolved completely, colour of the mixture remained yellow. Subsequently, 1 gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in a 2-ml collection tube. To bind DNA in QIAquick spin column, the sample was applied to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and QIAquick column was placed back in the same collection tube. 0.5 ml of buffer QG was added to the QIAquick column and centrifuged for 1 min. PE buffer (0.75 ml) was added to the QIAquick column to wash and centrifuged for 1 min. The flow-through was discarded and QIAquick column was centrifuged for an additional 1 min at 11337 x g. The QIAquick column was placed in a clean 1.5 ml centrifuge tube. To elute the DNA, 50 µl of buffer EB (10 mM Tris Cl, pH 8.5) or water was added to the centre of the QIAquick membrane and the column was centrifuged for 1 min at maximum speed.
2.14. Plant tissue culture and transformation of wheat

Isolation and culture of immature embryos of wheat

The ears obtained from approximately two month old wheat plants were surface sterilised (in 70 % ethanol for 1 to 2 min) The best to bombard embryos were obtained 10 – 15 days post anthesis (dpa) During anthesis the anther turns yellow and it comes out of the spikelet Embryos from developing seeds that are white-greenish in colour with semi-liquid endosperm are considered ideal for tissue culture The kernels were taken out with the help of forceps in a petriplate The kernels were placed, one by one, under a binocular and the immature embryos were taken out with the help of sterile needles The isolated embryos were immediately placed and incubated on tissue culture media (SMS medium) for somatic embryogenesis, putting the embryogenic axis down in contact with the medium and the scutellum side facing up About 50 immature embryos could be put on a culture plate The embryos were cultured on SMS medium for one week at 22 °C, in the dark

Transfer of cultured immature embryos onto SMS20 medium for osmotic treatment

Tissues were bombarded under plasmolytic conditions in order to reduce the deleterious effect of particle penetration The one-week-cultured embryos were transferred to SMS20 medium About 25 embryos were placed in the centre of the petridish, on an area of about 2.5 cm in diameter The embryos incubated for hours on SMS20 medium, in the light, until bombardment, at room temperature
Preparation of Microprojectiles

50 mg of gold particles (15 – 30 μm) were weighed in microfuge tube and 1 ml of sterile water was added to it. It was vortexed for 2-3 min and then sonicated for 1 min. The gold particles were briefly spun down (at 13148 x g, for 1 min) and the supernatant was removed. Again, 1 ml of water was added into the tube and vortexing, sonication and centrifugation were repeated. The water was removed and 1 ml of pure ethanol was added. The sample was vortexed and sonicated as before. It was centrifuged and ethanol was completely removed from the tube. The pellet was resuspended in 1 ml of sterile 50% glycerol.

Binding of DNA molecules onto the gold particles

Three microgram of each plasmid DNA or constructs were mixed. Twenty five microlitre of gold particles were added into it in a tube and vortexed briefly. Next, 25 μl of 2.5 M CaCl₂ was added and mixed by vortexing. In the following step, 10 μl of 0.1 M spermidine was added and vortexed for 1 min. Following this, 300 μl of 96% ethanol was added and vortexed to make the final mix. The prepared DNA sample was incubated at -20°C for a maximum of 60 min, when required. The DNA sample was centrifuged at 11337 x g for 1 min and ethanol was removed without allowing the sample to dry up. The DNA sample was resuspended in 25 μl of water. Ten microlitre of the sample was used (per shot) to bombard the embryos. After bombardment, the embryos were allowed to recover on the SMS20 medium, overnight at 22°C, in the dark.
Ballistic gene delivery using Particle Inflow Gun (PIG)

The particle holder and the blaffle were air-dried. The firing box device was plugged in and the vacuum pump was turned on. The helium tank valve was opened until the left manometer reached the maximum pressure (this indicates the pressure in the tank). Then, the outlet valve was opened. The working pressure of the manometer was regulated with a reduction valve. The baffle grid (500 μm mesh-sized plastic or metallic grid) was placed at the desired distance. The target support stage was placed at a distance of 6-10 cm from the particle holder. The target dish, without its lid, was placed in the centre of the target support stage. The gold particles were resuspended by vortexing. When required, a brief sonication of less than 1 sec was given. Ten microlitre of the particle-DNA suspension was pipetted out absolutely vertically, in the centre of the particle holder and the particle holder was placed inside the Particle Inflow Gun (PIG) (Figure 3). The plexiglass door was fixed and vacuum was connected. When the chamber manometer showed a pressure of -0.8 bar the firing button was pressed once. The vacuum of the chamber was released and the target dish was removed and covered with the lid. The particle holder was removed.

Transfer of the bombarded embryos onto SMS medium

The day after bombardment the embryos were transferred onto SMS medium and incubated for a week at 22°C, in the dark.
Figure 3: Particle Inflow Gun (PIG) used for DNA delivery to wheat calli (Finer et al., 1992)
Selection and regeneration of the bombarded embryos

After a week’s incubation on SMS medium, the embryos were transferred onto selection or regeneration medium containing the herbicide phosphinothricin. The incubation period varied from 4 to 8 weeks at 22°C, in the light, depending upon the response of the bombarded embryos.

Root induction in the putative transgenic regenerants

When there was a desired growth of putative transgenic regenerants (almost 2 cm), they were transferred onto root induction medium. The incubation period varied from 2 to 4 weeks at 22°C, in the light, depending upon the response of the regenerants.

2.15. Growth of wheat transformants

Putative transgenic plantlets were transferred from rooting media to the pot mixture (soil Optima Fomaperlite, 1 : 1) and acclimatised to lower humidity (45-70%) at less than 20°C with a 16 h photoperiod. After two weeks, the plants were transferred to the greenhouse. These were the primary transformants i.e. the T₀ plants. The first generation progeny of these plants are the T₁ plants.

2.16. Histochemical GUS assay

Two days after bombardment, GUS expression was examined by immersing explants in X-gluc buffer containing 1 mM X-gluc, 100 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% Triton-x-100. Blue staining was assessed after incubation overnight at 37°C or 2 days at 26°C.
2.17. Chlorophenol red assay

Chlorophenol red assay to analyse the expression of bar was carried out as described previously (Bieri et al. 2000, Kramer et al. 1993). The green and healthy leaf tissues were taken and more than two cut sites were made since the protons from the leaf needed to diffuse into the media. The leaf pieces were placed with up to four cut sites into the 96-well microtiter plate containing 200 µl of the different chlorophenol red assay solutions (solutions without PPT, with 10 and 100 µg PPT etc., respectively). The leaf pieces were kept floating on the surface of the solution. The microtiter plate was incubated in the culture room until the positive controls turned yellow, and for evaluation, daily observation was necessary. The colour change of the dye could be scored after 2 to 3 days.

2.18. Leaf paint assay

Leaf paint assay was done to examine tolerance of the transformants to the herbicide bialaphos according to published procedures (Anand et al. 2003). To examine the expression of the transgene bar in the transgenic plants, a freshly made aqueous solution containing 1 mg/l of the herbicide bialaphos was used to paint on the mid lamina portion of the second-youngest leaf using a cotton bud. The painted area (~2.5 cm long) was marked using a marker pen and observed for damage in the following week.

2.19. Isolation of genomic DNA from wheat plants

Five gram of leaf tissue was taken from 3 week old plants, frozen in liquid nitrogen and ground into a fine powder in a prechilled mortar and pestle. The powder was transferred
to a 50 ml centrifuge tube and 15 ml of extraction buffer (0.1 M Tris, pH 8.0, 0.05 M EDTA, pH 8.0, 0.5 M NaCl, 0.01 M β-mercaptoethanol) was added. To this mixture, 1 ml of 20% SDS was added, mixed and the tubes were incubated at 65°C for 10 min. Thereafter, 5 ml of 5 M potassium acetate was added, the tubes were shaken gently and were incubated at 0°C for 20 min. The tubes were then spun at 2012 x g for 30 min on a centrifuge. The supernatant was collected in a clean 50 ml centrifuge tube containing 10 ml of isopropanol. It was mixed and the tubes were incubated at -20°C for 30 min. The DNA was pelleted down at 2012 x g for 30 min and the supernatant was gently poured off. Liquid was removed by keeping the tubes inverted and the pellets were then air dried. The DNA was redissolved in 700 µl of sterile distilled water. The solutions were transferred to 1.5 ml microfuge tube and centrifuged to remove all the insoluble debris. One µl of RNase (10 mg/ml) was added to the DNA solution and was incubated at 37°C for 10 min. Further, 400 µl of chloroform:isoamylalcohol (24:1) was added to these tubes, mixed well and spun for 1 min in microcentrifuge. The top aqueous phase was transferred to another 1.5 ml tube. To this solution, 75 µl of 3 M sodium acetate, pH 5.2 and 500 µl of isopropanol was added, mixed well and DNA was pelleted by centrifugation in a microfuge. The pellet was washed with 70% ethanol, dried and redissolved in 100 µl of sterile distilled water. Quality of DNA isolated was determined by electrophoresis on agarose gels and quantified by UV spectrophotometry.

2.20. Polymerase Chain Reaction (PCR)

PCR amplifications were carried out in a thermal cycler (Perkin Elmer 2400 System, MD, USA) in 0.2 ml microcentrifuge tubes, using Taq DNA polymerase (Bangalore Genei,
Bangalore, India) Reaction system (50 µl) included 20 pmol of the primers, 5 mM dNTPs and 3 mM Mg$^{2+}$ The following primers were used for the amplification of different genes (Table 3)

Table 3: Primer sequences with corresponding annealing temperatures and molecular sizes for GOX, Ace-AMP1 and bar.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (F: forward; R: reverse)</th>
<th>Annealing temperature</th>
<th>Size of the PCR product</th>
</tr>
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| GOX  | F 5'-ATCTGCCCATCATGCAGACT-3'  
R: 5'-ATACCACTCACTGCATGGAA-3' | 50°C | 1.892 kb |
| Ace-AMP1 | F 5'-ATGGGTTCGCGTTGTATCTTTTAC-3'  
R 5'-TCAGTTAATCCTGCCGCATTG-3' | 52°C | 363 bp |
| bar  | F 5'-GTCTGCACCATCGTCAACC-3'  
R: 5'-GAAGTCCAGCTGCCAGAAAC-3' | 52°C | 444 bp |

The PCR program profile for Ace-AMP1 and bar was as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C. At the end, one additional elongation step was performed for 5 min at 72°C. GOX was amplified at 72°C for 1 min in the elongation step. The amplification products were run on 1% agarose gel and visualised by ethidium bromide staining.
2.21. Southern blot analysis

Genomic DNA (25 μg) was digested to completion with restriction endonuclease (HindIII) as recommended by the supplier and fractionated on 0.8% agarose gels. The fractionated DNA was denatured with 0.5 M NaOH for 30 min, neutralised with 25 mM sodium phosphate, pH 6.5 and transferred to nylon membrane (Hybond N⁺, Amersham Biosciences, Hongkong). The transferred DNA was fixed to the membrane by baking at 80°C for 2 h. followed by ultraviolet irradiation (12 x 10⁴ μJ / cm²) using UV cross-linker (Spectrolinker, Spectronics Corporation, USA). DNA from T₁ and T₂ transgenic plants, null segregants and untransformed control, was hybridised to the 363 bp AceAMP₁ and 444 bp bar coding sequences, respectively. The probes were labeled and hybridising fragments were detected using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer’s instructions (Amersham Biosciences, Hongkong).

2.22. Isolation of total RNA and Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Leaf material (0.5 g) was collected from 3 week old plants and total cellular RNA was isolated using Trizol Reagent as per manufacturer’s instructions (Trizol Reagent, Invitrogen life technologies, California, USA). Quality of RNA isolated was determined by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry.

RT-PCR was carried out to determine the expression of the gene for AceAMP₁. 5μg of total RNA was used to synthesise the first strand cDNA using MuMLV reverse
transcriptase (Fermentas GmBH, St Leon-Rot, Germany) and oligo (dT)$_{12}$ in 20 µl reaction system. Five microlitre of this RT product was used to carry out the PCR.

### 2.23. Northern blot analysis

In the northern analysis, 20 µg of RNA was dried under vacuum, dissolved in 2.2 µl of buffer A {10X MOPS buffer (0.5 M MOPS, pH 7.0, 0.01 M EDTA, pH 7.5) 294 µl and water 796 µl} and 4.8 µl of formaldehyde / formamide (37 % formaldehyde 89 µl, deionised formamide 250 µl) was added to the RNA sample. After denaturation at 70°C for 10 min and quenching on ice, the RNA was mixed with 1.5 µl gel loading buffer (buffer A 322 µl, xylene cyanol 5 mg, bromophenol blue 5 mg, sucrose 400 mg, formaldehyde 12.3 M, formamide 500 µl) and loaded on a 1 % agarose gel in 1X MOPS buffer containing 2.2 M formaldehyde. Electrophoresis was carried out at 60 V for 2-3 h in 1X MOPS buffer. Following electrophoresis, the gels were washed in distilled water for 30 min and RNA was transferred to nylon membrane (Hybond N+, Amersham Biosciences, Hongkong). RNA from different transgenic lines and untransformed control, was hybridised to the 363 bp Ace-AMP1 and 444 bp bar coding sequences, respectively. The probes were labeled and hybridising fragments were detected using Gene Images AlkPhos Direct Labelling and Detection System as per manufacturer’s instructions (Amersham Biosciences, Hongkong). The fold difference in the levels of various transcripts detected was measured using ImageQuant Version 2.0 for Windows (ImageQuant, Molecular Dynamics, Amersham Biosciences, HongKong).
2.24. Isolation of total protein and Western blot analysis

In order to extract total protein, 0.5 g of leaf tissue was ground to a fine powder in liquid N₂ and then resuspended in phosphate buffered saline (PBS), pH 7.4. After mixing the ground tissue with the buffer for 30 min at 4°C, the cell debris was separated by centrifugation at 11337 x g for 10 min at 4°C. Clarified extract was stored in aliquots. To prevent degradation of proteins by proteases, phenylmethyl sulfonyl fluoride (PMSF) was used to a final concentration of 1 mM.

Total protein concentration in the samples was determined by a standard protocol (Bradford, 1976). Protein samples were electrophoresed on SDS-PAGE according to Sambrook and Russel (2001). After running, the protein samples were visualised using Coomassie Brilliant Blue R250 stain (0.25 g CBB R250 in 90 ml of 50 % methanol and 10 ml of glacial acetic acid).

During western blot analysis, proteins separated on SDS-PAGE were transferred to nitrocellulose membrane (Amersham Biosciences, HongKong) using a fabricated transfer unit for mini gels (8 cm x 7 cm). Prior to transfer the gel was equilibrated in buffer for 5-10 min. Semi-dry transfer of proteins was carried out at 50 mA current for 70 min. Subsequent to transfer, the membrane was stained with Ponceau S for 10-15 min to ensure efficient transfer of proteins and to mark positions of the molecular weight marker bands. After destaining, the membrane was incubated in the blocking solution (3 % BSA, 0.02 % NaN₃, 0.02 % Tween 20 and 1X PBS) for 2 h. The blocking solution was thrown off and the membrane was incubated with primary antibody (Rabbit anti-AceAMP1 antibody) in 1
. 500 dilution for 2 h and washed 5 times (20 min each) with the blocking solution. Then, the membrane was incubated with the alkaline phosphatase labeled secondary antibody (Goat anti-rabbit antibody, Bangalore Genei, Bangalore) in 1 1000 dilution in the secondary solution (0.15 M NaCl, 0.05 M Tris, 0.002% Tween 20, 0.001% NaN3 and 0.05% skim milk powder) for 1 h and the blot was washed 4-5 times (20 min each) in the secondary solution. Secondary antibody bound bands were developed by adding alkaline phosphate buffer (0.5 M NaCl; 5 mM MgCl2; 0.1 M Tris, pH 9.5) containing 66 µl of (10 mg / ml) nitroblue tetrazolium (NBT) and 33 µl of (10 mg / ml) 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

2.25. Enzyme-linked immunosorbent assay (ELISA)

Protein samples from transgenic lines and untransformed control were taken in 96-well ELISA plate and incubated for 1-2 h followed by 5 times wash with 1X PBS containing 0.2% Tween 20. After blocking the wells with 3% BSA for 2 h another wash of 1X PBS was given. Primary antibody (Rabbit anti-AceAMP1 antibody) was diluted (1 500) in blocking solution and applied into the wells for an incubation of 1 h followed by 5 times wash with 1X PBS. Secondary antibody (Goat anti-rabbit antibody, Bangalore Genei, Bangalore) was diluted to 1 1000 and applied into the wells for incubation for another 1 h. To detect the secondary antibody bound molecules 50 µl of 1X substrate tetramethyl benzidine (TMB) / hydrogen peroxide (H2O2) was added to the wells and the reaction was stopped by the addition of 1 N H2SO4. Final reaction system was diluted to 1 ml and absorbance was measured at A450.
2.26. Detached leaf assay using *Blumeria graminis* f. sp. *tritici*

Freshly harvested spores of *Blumeria graminis* f. sp. *tritici* were used for detached leaf assay according to the standard protocol (Limpert *et al.*, 1987). For both transgenic wheat lines and the untransformed control when the fourth leaf was emerging the 2nd/3rd leaf was cut into sections of 3 cm and placed on watery agar containing benzimidazole (30 mg/l) with the adaxial side up and the leaf pieces were inoculated with spores in a settling tower. Then the whole unit was placed on the lab work-bench for about a week. The fungal colonies were counted under a stereomicroscope (C-DSLS, Nikon, Japan).

2.27. Karnal bunt pathogen assay

Karnal bunt (*Neovossta indica*) spores were collected from the culture tubes, sieved through a muslin cloth and a suspension of $10^5$ spores/ml was made. This spore suspension was injected into the spikes of both the transgenics and null segregants, at the booting stage. Inoculated wheat spikes were harvested at ½ h, 24 h, 48 h and 72 h after inoculation (hai) for the isolation of total RNA.

2.28. Extraction and estimation of salicylic acid from wheat leaf tissues

Salicylic acid was extracted from wheat leaf tissues and estimated according to the protocol as described with a few modifications (Verberne *et al.*, 2002). The leaf material was collected and frozen in liquid nitrogen. Prior to extraction all material was pulverised in liquid nitrogen using mortar and pestle. Samples of 0.5 g were further homogenised using liquid nitrogen and transferred to 1.5 ml microfuge tube. One millilitre of 90% methanol was added. This extraction mixture was vortexed for 1 min and
samples were sonicated for 5 min and centrifuged for 5 min at maximum speed in an Eppendorf bench-top centrifuge. The supernatant was collected in a 2 ml microfuge tube. The pellet was re-suspended in 0.5 ml of 100% methanol, and the sonication and centrifugation steps were repeated. The supernatants were combined and centrifuged again, and the methanol-water mixtures were evaporated in a SpeedVac concentrator at high drying speed. To the residue, 250 µl of trichloroacetic acid (TCA 5% solution in water) was added and the mixture was mixed vortexed. Partitioning with 800 µl of ethyl acetate-cyclohexane (1:1, v/v) resulted in the separation of an upper organic solvent with free SA and lower aqueous phase with salicylic acid glucoside (SAG). This partitioning was carried out twice. The combined upper layers containing free SA were evaporated to dryness in a SpeedVac concentrator, using medium drying speed. The aqueous phase with SAG was subjected to acid hydrolysis by adding approximately 300 µl of 8 M hydrochloric acid to the remaining TCA fraction and heating the sample at 80°C for 1 h (Meuwly and Metraux, 1993). After this step, the acid fraction was partitioned again with ethyl acetate-cyclohexane mixture and the procedure was continued as described above. Through acid hydrolysis, SAG in the aqueous phase is converted to SA. These samples were dried in a Savant (Farmingdale, NY, USA) SpeedVac Plus SC 110A concentrator using a high drying rate. After drying, the SA recovered was dissolved in 600 µl of the HPLC eluent and the sample composition of the residues was analysed by HPLC.

Known amount of SA (5, 10, 20 and 30 µg) was taken as standard, dissolved in 50% ethanol, and mixed with 800 µl of the ethyl acetate-cyclohexane solution. These samples were dried in the SpeedVac concentrator using medium drying rate. After removal from
the concentrator, 600 μl of the HPLC eluent was added to each tube and the contents were analysed by HPLC

**HPLC analysis**

Analysis of SA was performed using a Phenomenex (Torrance, CA, USA) column, type LUNA 3μC18(2) (150 x 4.60 mm d, 3 μm) with a Phenomenex SecurityGuard pre-column. The eluent was 0.2 M sodium acetate buffer pH 5.5 (90 %) with methanol (10 %) at a flow rate of 0.80 ml / min. SA was detected with Shimadzu (Tokyo, Japan) model RF-10Axl spectrophotometric detector operated at an emission wavelength of 407 nm and excitation wavelength of 305 nm. Samples of SA recovered from the input material in various experiments were dissolved in 600 μl of the HPLC eluent. The efficiency of recovery was determined by comparing peak areas of the recovered SA with the peak areas of the eluent samples of the known standards.

**2.29. Measurement of agronomic parameters**

Morphological characters like plant height, number of tillers per plant, days to 50 % flowering, flag leaf length and panicle length of the untransformed control and transgenics were measured.

**2.30. Statistical analysis**

Morphological characteristics of the wheat plants were statistically analysed using GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, California, USA) ANOVA for detached leaf assay infection scores and MANOVA for agronomic
parameters were done using SPSS version 10.0 for Windows (SPSS Software, Seattle, WA, USA)