MATERIALS & METHODS
3. MATERIALS AND METHODS.

3.1 MATERIALS

3.1.1 Chemicals and reagents

All the chemicals and reagents used in molecular biology experiments were obtained from US Biochemical’s (distributors-Amersham Pharmacia). Restriction enzymes and T4 DNA Ligase was obtained from New England BioLabs. Klenow fragment. Taq polymerase and RNaseA were obtained from MBI Fermentas. DNA labeling kit and radio labeled αP32 dCTP were obtained from BARC India. Nylon membrane Hybond N+ was purchased from Amersham Pharmacia. Oligo nucleotide primers of HPLC grade purity were synthesized from MWG, Germany.

All the inorganic salts and organic supplements used in preparation of culture media were obtained from Hi-Media, India, and are of tissue culture grade purity. Biochemical’s used for microbiological studies were also obtained from Hi-Media. All phytohormones and antibiotics except hygromycin-B were obtained from Sigma Chemicals, USA. Antibiotic hygromycin-B was obtained from Calbiochem, La Jolla, USA. Filter purified “RO” grade water obtained from Fristream Multipure system was used for media preparation.

All the glassware used in routine tissue culture practice such as 50 ml test-tubes, 90 mm petri-plates, conical flasks and beakers were obtained from Borosil, India. Glass culture bottles 7 mm X 11 mm (diameter x length) with autoclavable screw caps, used for growth and hardening of tissue culture raised plants were obtained from local manufacturer. Glass petri plates (10cm diameter x 2.5 cm height) were specifically used
for *in vitro* regeneration experiments.

### 3.1.2 Plant genotypes

Mature seeds of *indica* genotypes “Manasarovar”, “Pusa basmathi” and "Nagina22W" were obtained from Directorate of Rice Research, Rajendra Nagar, Hyderabad, India. Seeds of genotype "Nootripattu" were obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. These cultivars were grown in 18” clay pots containing black soil enriched with organic compost. The plants were grown till maturity in green house.

### 3.1.3 Bacterial strains

*E. coli* strain DH5a obtained from “Bangalore Genie” was used for general cloning and clone maintenance. *Agrobacterium tumefaciens* strain EHA105 used for rice transformation was obtained from Dr. Gelvin, Purdue University, USA.

### 3.1.4 Plasmid vectors

Binary vector pCAM1A1305.1 obtained from GAMBIA, Australia, was used in optimizing rice transformation protocol.

Plasmid “pMAPX1” harboring the “apxi” cDNA encoding maize cytosolic ascorbate peroxidase, was obtained from Dr. Van Montagu (Belgium).

Super promoter driven plant expression vector “pE1805” was obtained from Dr. Gelvin (Purdue University).
3.2 METHODS

3.2.1. Tissue culture and plant transformation methods

3.2.1.1. Seed pretreatment

After complete seed filling, mature seeds were harvested and sun dried for three days in the mid day sunlight from 11 a.m. to 3 p.m. and then incubated at 42°C for 16 h to break their dormancy.

3.2.1.2. Seed surface sterilization

Mature seeds were manually dehusked and healthy seeds were selected. For each experiment involving callus induction, 100 seeds were dehusked and thoroughly washed in a 100 ml glass beaker with ample quantities of sterile double distilled water before rinsing them for two min in 25 ml of 70% ethanol. The seeds were washed three times with sufficient quantities of sterile distilled water to remove traces of ethanol. Finally, the seeds were rinsed in 25 ml of 0.1% HgCl₂ for 10 min with occasional swirling, this was followed by five times washing with ample quantities of sterile double distilled water. These surface sterilized seeds were blotted dry on sterile whatman # 1 filter papers before placing them on callus induction medium.

3.2.1.3. Callus Induction

Callus induction medium (NBC1) was prepared by adding all the constituents stated in Table 1. The NBCJ medium containing 2.25 mg/L of 2,4-D was autoclaved in conical flasks for 15 min at 15 lbs pressure on liquid cycle. The autoclaved media was
poured into 9 mm sterile glass petri-plates under laminar hood and allowed to solidify for 30 min. About 25 ml of medium was added to each petri-plate.

Scutellar callus was initiated from the surface sterilized mature rice seeds by placing them on the callus induction medium. Ten seeds were placed in each plate containing about 25 ml of NBCI medium and the plates were sealed with parafilm. The cultures were maintained in dark at 27 ± 1°C.

3.2.1.4. In vitro plant regeneration

Regeneration medium (NBRE) was prepared by adding 3 mg/L of BA to NB (N₆ macro nutrients + B₄ micro nutrients) basal medium (Table 2). All the plant hormones, except 2,4-D, were dissolved in DMSO and added to media after autoclaving. The 18 d old embryogenic callus was cut to about 3 mm size and placed over NBRE medium for in vitro regeneration. For regeneration from callus cultures, about 20 calli were transferred to each 10 cm diameter glass petri-plate containing about 40 ml of NBRE regeneration medium. Calli on regeneration medium were initially given a dark treatment for one week and later transferred to continuous light at 27 ± 1°C. The illumination was from four fluorescent tubes each of 40 W capacity.

3.2.1.5. Callus cocultivation

Cocultivation media (NBCC; Table 1) was prepared by adding 100 µM of filter sterilized acetosyringone to NBRE medium (Table 1). Eighteen day old scutellar embryogenic callus was cut to about 2-3 mm in size and were infected with 1.0 O.D.
Agrobacterial culture, strain EHA105. A 200 ml Agrobacterial culture was grown to an O.D.₆₀₀ ~ 0.6 and centrifuged at 5000 rpm for 5 min at room temperature. The obtained bacterial pellet was resuspended in 100 ml of NBCC medium so as to get a final O.D.₆₀₀ ~ 1.0, and used for infecting the rice calli. For cocultivation, the calli after infecting for 10 min in Agrobacterium culture were blotted dry on whatman # 1 filter papers and transferred onto filter paper discs (5 mm diameter, whatman # 1) wetted with 700 µl of liquid cocultivation medium. Cocultivation was performed in petri-plates containing only filter papers discs wetted with 700 µl of liquid NBCC medium (without any semi-solid medium) and the calli were incubated in dark for 2.5 d at 25 °C. Cocultivation was also performed as reported by Rashid et al., (1996) where the explants were placed on filter papers overlayed on semi-solid medium containing 100 µM acetosyringone.

After cocultivation, the calli were washed thoroughly in sterile distilled water till the bacteria was removed. Final wash was with cefotaxim solution (500 mg/L) and dried on whatman # 1 filter paper for 5 min. before transferring to the selective regeneration medium (NBSR; Table 1).

3.2.1.6. Selective regeneration of transgenic plants

NBSR medium consists of NB basal medium supplemented with 3 mg/L BA, 300 mg/L cefotaxim and 50 mg/L hygromycin-B. The above cocultivated calli were transferred onto NBSR medium and incubated in dark for the initial one week at 25°C. later the cocultivated calli were transferred to continuous light. After two weeks, the transformed calli that survived the selection pressure and proliferating on NBSR
medium were sub-cultured onto NBRE medium containing 350 mg/L cefotaxim and maintained under the same growth conditions for next 30 d (Kumaria et al., 2001). The number of plants regenerated after 30 d on NBRE medium was scored and the frequency of plant regeneration was calculated as stated by Rubulo et al., (1984) and Khanna and Raina (1998).

3.2.1.7. Rooting and acclimatization

The *in vitro* regenerated shoots after attaining a height of about 2 cm were transferred to NB basal medium for rooting. The cultivars “Nootripattu” and “Pusa basmati-1” were rooted successfully when transferred to NB basal medium. Cultivar “Manasarovar” failed to root on NB basal medium. Rooting was induced by transferring the shoots to "M" shaped filter paper boats placed in 5 ml of liquid NB medium fortified with 1 mg/L NAA, in 50 ml glass tubes capped with paper plugs. The shoots were given a 16/8 light/dark regime to enhance rooting.

3.2.2. Vector construction: Gene cloning and analysis

3.2.2.1 Plasmid DNA isolation

Plasmid DNA was isolated following the standard protocols (Sambrook et al., 1989). The *E. coli* culture (DH5a) harboring the plasmid pMAPX1 was grown overnight at 37°C in 5 ml liquid LB medium containing 50 μg/ml of ampicillin, on an orbital shaker maintained at 220 rpm. The culture was raised in 50 ml Borosil tubes capped with paper plugs. A 1.5 ml of the overnight grown culture was transferred into
1.8 ml microfuge tubes and centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was discarded and the bacterial pellet was suspended by vortexing in 100 μl of ice cold Solution I and briefly incubated on ice for 5 min. The cell suspension was lysed by adding 200 μl of freshly prepared Solution II followed by a brief incubation at room temperature for 5 min. Finally, 150 μl of ice cold Solution III was added and mixed by slow inversion. The lysate was centrifuged at 5000 rpm for 5 min at 4°C and the supernatant was purified by extracting once with Phenol-Chloroform (1:1) mix. After centrifugation at 5000 rpm for 5 min at room temperature, the top aqueous phase was collected and the plasmid DNA was precipitated by adding two volumes of ice-cold absolute ethanol with a brief incubation on ice for 10 min. The DNA pellet was obtained by centrifuging at 5000 rpm for 5 min at 4°C and the plasmid DNA pellet was washed once with 70% ethanol. The DNA pellet was air dried and dissolved in 50 μl of TE buffer or in sterile double distilled water and stored at 4°C. RNA that was co-precipitated with plasmid DNA was removed by treating the sample with 1 μl of 20 mg/ml RNaseA and incubating it at 37°C for 2 h. The plasmid DNA was purified again using Phenol-Chloroform mix and the DNA was precipitated by adding 1/10 volume of Ammonium acetate (pH-5.2) and 2.5 volumes of absolute ethanol. The DNA was pelleted by centrifugation at 5000 rpm for 5 min at 4°C, washed with 100 μl of 70% ethanol, air dried and dissolved in 50 μl TE buffer or in sterile double distilled water. Plasmid DNA was quantified using spectrophotometer by taking the O.D values corresponding to 260 nm and 280 nm absorbance.
Reagents:

Solution I: 50 mM Glucose, 25 mM Tris (pH -8.0). 10 mM EDTA (pH -8.0)

Solution II: 0.2 N NaOH. 1% SDS.

Solution III: 10 ml solution contains 6 ml of 5 M Potassium acetate. 1.15 ml of Glacial acetic acid and 2.85 ml H₂O.

TE Buffer: 10 mM Tris (pH -8.0) and 1 mM EDTA (pH -8.0)

RNase A: 10 mg/ml RNase A in 10 mM Tris-HCl (pH -7.5) and 15 mM NaCl

3.2.2.2 Restriction digestion

In a 30 ul reaction volume, 1 µg of the plasmid DNA (pMAPX1) was double digested with Xho I and XbaI restriction enzymes (10U each and NEB buffer 2) to release a 1.1 kb fragment corresponding to the full length cDNA coding for maize cytosolic ascorbate peroxidase. The sample was incubated at 37°C overnight and the digested sample was fractionated on 0.8% agarose gel.

Reaction setup:

Plasmid DNA pMAPX1 (1 µg)       x µl
NEB2 buffer (10X)                3 µl
Xho I enzyme (10U)               1 µl
XbaI enzyme (10U)                1 µl
Sterile water                    y µl
Total volume                     30 µl

(Note: x + y = 25 µl)
Similarly 400 ng of the binary pEI805 was digested with *SacI* restriction enzyme by incubating the sample at 25°C for 4 h. After digestion the sample was purified using Phenol-Chloroform mix and then precipitated using ammonium acetate and ethanol.

Reaction setup:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid pE1805 (400 ng)</td>
<td>a</td>
</tr>
<tr>
<td>NEB4 buffer (10X)</td>
<td>2</td>
</tr>
<tr>
<td><em>SacI</em> enzyme (10U)</td>
<td>1</td>
</tr>
<tr>
<td>Sterile water</td>
<td>b</td>
</tr>
<tr>
<td>Total volume</td>
<td>20</td>
</tr>
</tbody>
</table>

(Note: a + b = 17 μl)

3.2.2.3 *Fragment elution*

The digested plasmid DNA was run on 1% agarose gel casted in 0.5X TBE and the gel was cut to about 5 mm x 10 mm dimensions having the band of interest. The cut gel slice was placed inside pretreated dialysis tubing (Sigma D9777, MW 12,400, cellulose membrane dialysis tubing with a 16 mm average diameter) sealed at one end using clips. The dialysis bag was then filled with nearly 400 μl of 0.5 X TAE buffer and sealed the other end with clips, ensuring no air bubbles inside. The sealed dialysis tube was placed in an electrophoresis tank in such a way that the DNA band was parallel to the electrodes. The electrophoresis was carried out in 0.5 X TBE buffer by powering the apparatus with a 5 V/cm electric field for 30 min. The electric field was reversed for 30 seconds and the buffer in the dialysis tubing was transferred to a 1.5 ml microfuge tube.
The sample was purified by extracting once with Phenol-Chloroform and the DNA was precipitated with ethanol. The DNA pellet was washed once with 70% ethanol and then dissolved in 10 µl of sterile water. Finally the amount of DNA present was quantified using spectrophotometer.

3.2.2.4 End filling

The 1.1 kb Xho I-Xba I fragment corresponding to the cDNA sequence encoding maize cytosolic ascorbate peroxidase was gel eluted using dialysis tubing method and then end filled using Klenow fragment (MBI Fermentas). About 500 ng of insert DNA was end filled using Klenow fragment (E.coli DNA polymerase 1, large fragment). The reaction mix containing 20 µl of DNA solution. 5 µl of 10 X reaction buffer. 10 µl of 0.25 mM dNTP mix. 1 ul of Klenow fragment (10U) and 14 ul of sterile water with a total reaction volume of 50 µl was incubated at 37°C for 10 min and then heat inactivated at 75°C for 10 min. The end filled DNA was purified by Phenol-Chloroform extraction and the DNA was precipitated using ethanol. The DNA pellet was washed in 70% ethanol and dissolved in 10 µl of sterile water.

3.2.2.5 Ligation

Plasmid DNA ligations were carried out in a 20 µl reaction volume containing 200 ng of combined DNA concentrations of vector and insert, in a molar ratio of 1:10. The ligation mix containing, 2 µl of 10 X reaction buffer. 1 µl (400U) of T4 DNA ligase (NEB), 85 ng of end filled insert DNA and 115 ng of vector DNA digested with Smal
was incubated overnight at room temperature.

Calculating DNA concentrations based on the molar ratios of vector to insert

Formula: \( I = \frac{(S_i \times T)}{[(S_v/R_{IV})+S_i]} \) & \( V = \frac{T}{[(1+(S_i*R_{IV}/S_v)]}; \) (Gruenwald and Heitz 1993).

\( I \) = Amount of insert DNA in ng needed for ligation reaction.

\( V \) = Amount of vector DNA in ng needed for ligation reaction.

\( T \) = Amount of total DNA required for ligation reaction.

\( S_i \) = Size of insert DNA

\( S_v \) = Size of vector DNA

\( R_{IV} \) = Insert to vector molar ratio.

### 3.2.2.6 Preparation of CaCl Competent cells

One ml of overnight grown *E. coli* culture (DH5a) was added to 99 ml of fresh LB medium and the culture was allowed to grow to an \( O.D_{600} \) of 0.4-0.6 on an orbital shaker maintained at 37°C and 220 rpm. The log phase culture obtained was chilled on ice for 30 min and centrifuged at 5000 rpm for 5 min at 4°C. The pellet was suspended in equal volume of ice cold sterile water and centrifuged at 5000 rpm for 5 min at 4°C. The bacterial pellet was resuspended in 100 ml of ice cold 0.1 M CaCl\(_2\) and incubated on ice for 1 h prior to centrifugation at 5000 rpm for 5 min at 4°C. The bacterial pellet was resuspended in half volume of 0.1 M CaCl\(_2\) and centrifuged at 5000 rpm for 5 min at 4°C. Finally the pellet was resuspended in 2 ml of ice cold 0.1 M CaCl\(_2\) and 200 ul
3.2.2.7 Bacterial transformation

The 20 μl ligated sample was added to a 200 μl aliquot of CaCl₂ competent cells and incubated on ice for 30 min. The competent cells were given a heat shock for 90 sec. at 42°C and incubated on ice for 5 min. To the heat shock treated competent cells, 800 μl of fresh LB medium was added and the culture was incubated at 37°C for 1 h on an orbital shaker maintained at 220 rpm. A 200 μl of the transformed bacterial culture was plated on LB semi-solid medium containing kanamycin at 50 μg/ml and plates were incubated overnight at 37°C.

3.2.2.8 Colony hybridization

About 133 transformed colonies were picked and plated in two replicates on the selection medium and the colonies were allowed to grow for exactly 12 h. A rectangular nylon membrane, cut to the size of the plate was placed over colonies and pressed gently and evenly. The membrane was transferred onto the whatman #1 filter disc soaked in 10 ml of Solution A for exactly one minute and then subsequently transferred to whatman #1 filter discs containing Solutions B, C, D' and E in sequential order. At each step the nylon membrane was placed on filter paper for exactly 1 min. Finally the membrane was air-dried and baked at 80°C for 1 h.
Reagents:

Solution A: 25% Sucrose solution and 50 mM Tris (pH-8.0). Lysozyme at 1.5 mg/ml was added after autoclaving.

Solution B: 0.5 M NaOH and 0.2% SDS.

Solution C: 0.5 M NaOH.

Solution D: 1 M Tris (pH-7.5).

Solution E: 0.15 M NaCl in 0.1 M Tris (pH-7.5).

3.2.2.9 Preparation of electrocompetent cells

A starter culture of Agrobacterium tumefaciens strain EHA105 was raised in 5 ml liquid LB medium having tetracycline at 15 mg/L. Agrobacterium was cultured for 48 h at 28°C on an orbital shaker maintained at 220 rpm. Starter culture of 1 ml was added to 100 ml of fresh LB medium containing tetracycline 15 mg/L and the culture was allowed to grow to log phase OD₆₀₀ 0.4 - 0.6. The log phase culture was chilled on ice for 30 min and centrifuged at 5000 rpm for 15 min at 4°C. The pellet was suspended in equal volumes of ice cold sterile double distilled water and centrifuged at 5000 rpm for 5 min at 4°C. The bacterial pellet was resuspended in equal volume of 0.1 mM HEPES (pH-7.0) and incubated on ice for 1 h before centrifuging at 5000 rpm for 5 min at 4°C. The obtained bacterial pellet was suspended in equal volume of 10% ice-cold glycerol and centrifuged at 7000 rpm for 10 min at 4°C. Finally the pellet was suspended in 300 µl of 10% glycerol and aliquots of 40 µl were made and stored at -70°C till further use.
3.2.2.10 Agrobacterium transformation by electroporation

To a 40 μl of electrocompetent cells about 10 ng of plasmid DNA was added and an electric pulse of Resistance-201 Ω, Capacitance-25 μF and Voltage-2500 V was given in an electroporator. For all the successful electroporations, pulse time of 5.25 milli sec was recorded. Immediately after the pulse treatment, 1 ml of fresh LB medium was added to the electro-transformed competent cells and incubated for 1 h in an orbital shaker maintained at 28°C and 220 rpm. After 1 h of expression, 50 μl of the transformed cells were plated on LB semi-solid plates with corresponding antibiotics for selection of transformed clones, and the plates were incubated at 28°C for 2 d.

3.2.3. Analysis of transgenic plants

3.2.3.1 GUS assay

Transient expression of the uidA gene in calli immediately after cocultivation and stable expression in calli that were proliferating on selective regeneration medium were analyzed. Also expression in transgenic leaf tissue was examined following the protocols stated in IRRI reports (Datta et al., 1997) with slight modifications. Explants were immersed in 5 ml of GUS staining solution containing 10 mg X-Gluc (5-bromo-4-chloro-3-indolyl-α-D-glucuronide), 100 mM Tris (pH-7.0), 50 mM NaCl, 2 mM Potassium ferricyanide, 0.1% Triton X-100, 0.2% Sodium azide and sterile water. Explants were incubated for 16 h in dark at 37°C and decolorized with 2-3 rinses of absolute ethanol 30 min each before detecting the blue spots under a low magnification laboratory compound microscope. Photographs were taken using a high sensitive
photographic film (Kodak 400 ASA) using the attached camera facility.

3.2.3.2 In vitro HPT assay

In vitro HPT assay using rice leaf tissues was performed as described by Wang et al., (1997). A fully juvenile leaf grown to about 10-12 cms was cut into pieces of about 1 cm in length. The leaf pieces were placed over HPT assay medium (Table VII) consisting of MS basal medium fortified with 1 mg/L BA and 100 mg/L hygromycin. The leaf tissues were incubated at 25°C for one week with a 16/8 light/dark regime.

3.2.3.3 Isolation of genomic DNA “Mini-prep”

Genomic DNA was isolated following a genomic DNA mini prep protocol stated at website http://biologi.uio.no/molbiol/protocol/miniprep2.htm. About 50 mg of leaf tissue was ground to a fine powder in a 1.5 ml micro centrifuge tube using liquid nitrogen and micro pestle. To the powdered tissue 700 µl of prewarmed extraction buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0. 100 mM NaCl. 1% SDS and 10 mM á-mercaptoethanol) was added and incubated at 65°C for 15 min. To this, 220 µl of Acetate buffer (3 M Potassium + 5 M Acetate) was added and incubated on ice for 30 min. The cell lysate was centrifuged at 10000 rpm for 5 min at 4°C and to the supernatant 550 µl of isopropanol was added, mixed well by inversion and incubated at room temperature for 1 h. The precipitated DNA pellet was obtained by centrifuging at 11000 rpm for 30 min at 4°C. The DNA pellet was washed in 70% ethanol and dissolved in 100 µl of TE after air drying.
3.2.3.4 Genomic DNA isolation from rice leaves using modified CTAB method

Genomic DNA was isolated from young leaves following modified CTAB stated in CIMMYT protocols (Saghai-Maroof et al., 1984). Leaf tissue of 300 mg was frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. The powdered tissue was transferred to 50 ml conical flasks and 9 ml of pre warmed CTAB extraction buffer (500 mM NaCl. 50 mM Tris-HCl (pH 8.0). 50 mM EDTA. 1% (v/v) β-mercaptoethanol) was added, followed by addition of 6% polyvinylpyrrolidone (PVP. 25kDa) and 2% SDS before incubating the mixture for 1 h at 65°C. The sample was centrifuged at 12000 rpm for 10 min at room temperature. The top aqueous phase was collected and to it 30 ul of 10 mg/ml RNaseA was added and incubated at room temperature for 1 h. To the aqueous phase 5 M Potassium acetate was added to a final concentration of 0.5 M and the DNA was precipitated with the addition of 0.6 volume isopropanol. The DNA pellet was dissolved in TE and purified by Phenol-Chloroform-Isoamyl alcohol extraction. The genomic DNA was precipitated by adding 50 al of 5 M NaCl and 2.5 ml of ethanol and washed once with 76% ethanol containing 10 mM ammonium acetate, air dried and dissolved in 250 µl of TE. The DNA concentration was measured by spectrophotometer, and its quality was checked on 0.8% agarose gel.

3.2.3.5 PCR analysis of putative transgenic rice plants of cv “Manasarovar”

The gene specific primer sequences were designed using online Primer3 software at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi and the following primer sequences were synthesized from MWG. Germany.
Primer sequences for *uidA* gene

\[ \text{gusF} \ 5' - \text{GCCATTTGAAGCCGATGTCACGCC} - 3' \ (Tm\ 62^\circ C) \]

\[ \text{gusR} \ 5' - \text{GTTCTGCGACGCTCACACCGATAC} - 3' \ (Tm\ 60^\circ C) \]

Primer sequences for *hpt* gene

\[ \text{hptF} \ 5' - \text{GAAGATCTTTACCATGAAAAAGCCTGAACCTCACCG} - 3' \ (Tm\ 65^\circ C) \]

\[ \text{hptR} \ 5' - \text{ATTCGAGCTCTATTTTTTTGGCCCTCGGACGA} - 3' \ (Tm\ 68^\circ C) \]

Plants regenerated from the transformed calli on selective regeneration medium were initially analyzed by PCR. Gene specific primers that are homologous to the *uidA* gene and *hpt* gene were used. PCR amplifications using 40 ng of genomic DNA were performed in 20 μl volumes containing 100 mM Tris-HCl (pH 9.0), 500 mM KCl, 25 mM MgCl$_2$, 1% Triton X-100, 2.5 mM of each dNTP, 5 μM of primers, 0.8U of *Taq* DNA polymerase and a primer concentration of 0.1 mM each.

Amplifications were performed in a DNA engine (PTC 200, M J Research, Inc.). The amplification temperature cycle for the *uidA* gene was as follows. Preheating at 94°C for 5 min and 1 cycle of 1 min each at 94°C, 59°C and 72°C and 34 cycles of 1 min each at 94°C, 55°C and 72°C. The amplification temperature cycle for the *hpt* gene was as follows - pre-heating at 94°C for 5 min and 1 cycle of 1 min each at 94°C, 55°C and 72°C and 34 cycles of 1 min each at 94°C, 61°C and 72°C. The programs ended at 4°C for cooling. Products were electrophoresed on 1.0% agarose gel and visualized under UV light by ethidium bromide staining.
3.2.3.6 PCR analysis of apxl rice transgenics cv *Pus a basmati*

Genomic DNA was isolated from 8 *apxl* sense expressing and 7 *apxl* anti-sense expressing independent putative transgenic rice lines, following modified CTAB method. About 40 ng of genomic DNA isolated from rice transgenics was used to amplify a 512 bp region corresponding to maize *apxl* coding, using gene specific primer sequences at an annealing temperature of 59°C.

\[
\text{apxlF} \ 5' - \ CGGTCGACAAGGCCAAGCGTAAG - 3' \ (Tm \ 60^\circ C) \\
\text{apxlR} \ 5' - \ TGGGCGGAAGGATGGATCAGAGA - 3' \ (Tm \ 60^\circ C) \\
\]

Similarly, a 1.029 kb region corresponding to *hpt* gene was amplified using the same reaction conditions as stated in section 3.2.3.5. The amplified samples were fractionated on 1% agarose gel stained with ethidium bromide.

3.2.3.7 RT-PCR amplification of maize *apxl* gene in rice transgenics

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) following manufacturer's protocols. RNA was isolated from leaf tissues of *apxl* sense and anti-sense expressing transgenic rice lines that have shown *hpt* and *apxl* gene amplifications using genomic DNA.

By using the above RNA samples, RT-PCR was performed using “SUPERSCRiPT™ One-Step RT-PCR” kit obtained from Invitrogen. RT-PCR reaction was carried out in a 50 ul reaction volume containing 1 µg of total RNA, 1.2 mM MgSO₄, 0.2 mM dNTPs, 0.5 µM forward and reverse primers corresponding to maize *apxl* cDNA. 1 ul of RT/Platinum *Taq* mix and 2 X reaction buffer. The reaction mix
was initially incubated at 50°C for 30 min and the PCR amplification was performed at an annealing temperature of 49°C for 40 cycles, as recommended in the manufacturers guide lines. The amplified samples were resolved on 1% agarose gel stained in ethidium bromide.

3.2.3.8 Restriction digestion and agarose gel electrophoresis

About 30 μg of genomic DNA isolated from apx1 sense and anti-sense expressing independent transgenic lines was digested with 30U of Eco RI restriction enzyme (NEB) in a 50 μl reaction volume, incubated at 37°C for 16 h. The genomic DNA isolated from untransformed plant (negative control) was also digested with Eco RI. The digested samples were fractionated on 1% agarose gel (13 cm gel length) casted in TAE buffer. Electrophoresis was carried out for 12 h in a 25 cm X 13 cm gel tank (Broviga) with an applied voltage of 0.5 V/cm.

3.2.3.9 Southern blotting

After electrophoresis, the agarose gel resolving the restricted DNA was depunnated in 250 ml of 250 mM HCl solution for 30 min followed by washing with sterile double distilled water. The depurinated gel was denatured for 15 min in 250 ml of denaturation solution (1 M NaCl and 0.5 M NaOH) on a rocker maintained at 15 cycles per minute. The depurinated gel was thoroughly washed using double distilled water and neutralized for 15 min in 250 ml of neutralization solution [1.5 M NaCl and 0.5 M Tris (pH-7.0)] and the gel was kept overnight for capillary transfer.
Capillary blotting

Restricted DNA from the above pretreated gel was transferred onto N+ Nylon membrane (Amersham Pharmacia) by capillary blotting. In a 25 cm x 25 cm glass trough, the gel casting tray (13 cm x 13 cm) was kept in an inverted position with the bottom side up and whatman # 1 filter paper wick was placed on top with its free ends dipped in 500 ml of 20X SSC (175.3 gms/L NaCl and 88.2 gms/L Sodium citrate. pH-7.0) solution present in the glass trough. Three whatman # 1 sheets, cut to the size of the gel, were placed and above this the gel was placed in an inverted position so that the bottom even surface faces up. A nylon membrane cut to the size of the gel was placed over the gel ensuring no air bubbles trapped in between nylon membrane and the agarose gel. Above the nylon membrane, three whatman # 1 papers, cut to the size of the gel, were placed and a pile of rough filter papers cut to the size of the gel were placed over the whatman # 1 filter papers. On top of all, a weight of about 200 gms was placed and the setup was left undisturbed overnight. After capillary transfer the blot was air dried and baked at 80°C for 1 h in vacuum.

Random labeling of DNA fragments

To 200 ng of purified DNA fragment corresponding to maize apxl coding sequence, 5 µl of random primer was added and the DNA was denatured by placing the tube in boiling water for 5 min and immediately transferred to ice. To this denatured DNA, 4 µl of each dATP, dGTP, dTTP, 5 µl of α-32P-dCTP, 5 µl of 10 X reaction buffer and 1 µl of klenow (10U) was added and the total volume was made up to 50 µl using sterile water. The reaction mix was incubated at 37 C for 1 h and the synthesized probe
was purified using Sephadex G-50 spin column.

Southern Hybridization:

a) Pre-hybridization.

The nylon membrane after baking was placed inside a hybridization bottle containing 150 ul/cm² pre-hybridization solution (0.5 M Na₂HPO₄, 7% SDS and 1 mM EDTA pH 7.0) and incubated for 30 min in a hybridization oven maintained at 65°C.

b) Hybridization

The synthesized probe was denatured in boiling water for 5 min and immediately transferred to ice for 10 min. The Prehybridization solution was removed and equal volume of fresh Prehybridization solution was added, to this the denatured probe was added and kept for overnight hybridization at 65°C in the hybridization oven.

The hybridization solution was decanted and the membrane was washed in solution containing 2X SSC and 1% SDS, for 30 min at 65°C. The blot was then washed twice with solution containing 1% SDS and 0.5X SSC for every 10 min. Finally, the membrane was rinsed in 2 X SSC solution at room temperature, air-dried and covered with Saran wrap for autoradiography.

3.2.3.10 Autoradiography and X-ray film development

An X-ray (Kodak) film was placed over the dried Southern blot wrapped in saran wrap, in the dark room under red light. The film was allowed to expose for sufficient length of time in the X-ray cassette depending on the intensity of counts recorded using a GM counter. The X-ray cassette was stored at -70°C by wrapping it in a black cloth. The autoradiogram was developed in the dark room first by rinsing it in
the developer solution for 2 min followed by a brief wash using distilled water for 1 min. Finally the film was immersed in fixer solution for another 2 min and washed thoroughly using tap water and later air-dried.