Materials & Method
3.1 Somatic embryogenesis

3.1.1 Explant collection and preparation

Male flower buds of banana cultivars Robusta (AAA), Elakki Bale (AB), Grand Naine (AAA) and Giant Cavendish (AAA) were collected from the field-grown plants, 1 to 10 weeks after flowering. The male bud was shortened to 6-8 cm in height by removing the enveloping bracts outside the laminar hood and these explants were surface sterilized in 70 % ethanol for about 5 minutes and rinsed three to five times in sterile water.

3.1.2 Preparation of media

The culture media for the establishment of embryogenic cultures were prepared as per the basal composition described by Murashige and Skoog (MS; 1962) and Schenk and Hildebrandt (SH; 1972). The medium was supplemented with / without the adjuvants. Analytical reagents and distilled water were used to prepare the culture medium. Stock solutions of macro (20 X), micro nutrients (200 X), iron salt (200 X), Vitamins and Myo-inositol (200 X) were prepared separately and stored at 4°C. The individual media components (major and minor salts, vitamins, Fe EDTA, growth regulators and sucrose) were mixed and the final volume was adjusted using glass-distilled water.

3.1.3 Culture vessels

Erlenmeyer flasks (150 ml), test tubes (25 mm x 150 mm) made up of Borosil glass and commercial bottles (250 ml) were used as culture vessels. All the culture vessels and glassware used in preparation of the media were cleaned with Tween-
20 and followed by prolonged washing in tap water. The glasswares were finally dried in an oven at 70 °C and then autoclaved for 1 hr.

### 3.1.4 Sterilization of media

All the media was adjusted to pH 5.3 - 5.8 using NaOH (0.1 N) or HCl (0.1N). The gelling agent (0.2% Gelrite, Sigma, USA) was added as per the desired concentrations in case of semi solid media and was heated to melt the gelling agent. Subsequently, media were dispensed into autoclaved test tubes, petri plates and conical flasks. The mouth of the culture vessels was plugged with non-absorbent cotton wrapped in gauze cloth. The vessels were then autoclaved at a pressure of 1.21 kg/cm² for 20 min at approximately 121°C. After sterilization the culture vessels were immediately transferred to an aseptic air-conditioned culture room.

### 3.1.5 Aseptic techniques

All the inoculations and manipulations involving sterile cultures or media were carried out under aseptic conditions in a laminar air flow cabinet. The working surface was cleaned with 70 % alcohol. The tools such as forceps and scalpel were sterilized by dipping them in 70 % alcohol followed by flaming and cooling. This was done at the start of an inoculation and also several times during the operation. During inoculation, first the cotton plug of the culture vessel was removed and the neck of the vessel was flamed over a spirit lamp, kept in the cabinet. The sterile and trimmed explants were quickly transferred to the culture vessels containing suitable culture medium, using sterilized forceps. The neck of the culture vessels was once again flamed and quickly closed by cotton plug.

### 3.1.6 Culture conditions
After inoculations, all the cultures were incubated in the culture room at 26 ± 1°C and were exposed to florescent light (1000 lux) for 10-12 hrs/day, with relative humidity of 70-80%. The cell suspension cultures were inoculated in to 250 ml Erlenmeyer flasks and were grown on an illuminated two tier gyratory shaker at the speed of 70 rpm.

3.1.7 Culture media

a) Induction

The male buds were reduced to 1.5 cm in length for culture and immature male flower clusters from position 0-15 were removed and cultured on Murashige and Skoog medium (MS) supplemented with 18.10 µM 2,4-dichlorophenoxy acetic acid (2,4-D), 5.71 µM indole-3-acetic acid (IAA), 5.37 µM α-naphthalene acetic acid (NAA), d-biotin (1 mg l⁻¹), sucrose (3%) and Gelrite (0.2%) as a gelling agent. Hereafter, this medium was designated as Banana Callusing Medium (BCM).

b) Maintenance

For the proliferation and maintenance of the callus, MS medium supplemented with 2,4-D (4.52 µM), biotin (1 mg l⁻¹), malt extract (100mg l⁻¹), glutamine (100 mg l⁻¹), sucrose (4.5 %) and gelrite (0.2 %) was used. Hereafter, this medium was designated as M2 medium.

c) Somatic embryo induction

For the development of the somatic embryos, SH medium supplemented with MS vitamins, glutamine (100 mg l⁻¹), malt extract (100 mg l⁻¹), picloram (1mg l⁻¹), 4.5 % sucrose and 0.2 % Gelrite. Hereafter, this medium was designated as Banana embryogenesis medium (BEM).
Table 4. Composition of Murashige and Skoog (MS)(1962) and Schenk and Hildebrandt (SH)(1972) nutrient media

<table>
<thead>
<tr>
<th>Constituent's</th>
<th>Final concentration (mg/l)</th>
<th>MS</th>
<th>SH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INORGANIC NUTRIENTS</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Major Salts</strong></td>
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</tr>
<tr>
<td>Ammonium nitrate NH₄NO₃</td>
<td>1650</td>
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<td>-----</td>
</tr>
<tr>
<td>Ammonium dihydrogen orthophosphate</td>
<td>300</td>
<td>-----</td>
<td>300</td>
</tr>
<tr>
<td>Calcium chloride CaCl₂.2H₂O</td>
<td>440</td>
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<td>200</td>
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<tr>
<td>Magnesium sulphate MgSO₄.7H₂O</td>
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<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
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<td>-----</td>
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<tr>
<td>Potassium nitrate KNO₃</td>
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<tr>
<td><strong>Minor Salts</strong></td>
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<tr>
<td>Boric acid H₃BO₃</td>
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<td>5.0</td>
</tr>
<tr>
<td>Cobalt chloride CoCl₂.6H₂O</td>
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<td>0.1</td>
</tr>
<tr>
<td>Copper sulphate CuSO₄.5H₂O</td>
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<td>0.2</td>
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<td><strong>Iron Source</strong></td>
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<tr>
<td>Ethylene diamine C₁₀H₁₄N₂Na₂O₈</td>
<td>37.3</td>
<td>20.0</td>
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<tr>
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</tr>
<tr>
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<td><strong>ORGANIC NUTRIENTS</strong></td>
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</tr>
<tr>
<td><strong>Vitamins</strong></td>
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<td></td>
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<tr>
<td>Nicotinic acid</td>
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<td>5.0</td>
<td>5.0</td>
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<td>Pyridoxine HCl</td>
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</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Sugar alcohol</td>
<td></td>
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</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>
d) **Somatic embryo conversion and regeneration**

For somatic embryo to plant conversion, MS medium supplemented with benzyl aminopurine (BAP, 2.22 µM), IAA (1.14µM), sucrose (3%) and gelrite (0.2%) was used hereby designated as M4), while for further plant regeneration, MS medium supplemented with BAP (8.9 µM), sucrose (3%) and gelrite (0.2%) was used (hereby designated as BAD).

e) **Rooting**

For the root induction of plants with good shoot system, MS medium was supplemented with NAA (5.37 µM), sucrose (3 %) and gelrite (0.2 %).

f) **Hardening**

The well-developed tender rooted plantlets were washed under running tap water and treated with 0.1 % systemic fungicide. The rooted plantlets were separated and transferred into poly bag containing fine sterilized sand, sterilized soil and FYM (1: 1: 1) mixture. These plantlets were maintained at a temperature of 28 °C and RH greater than 90 % for the first 15 days. After 15 days, plantlets were kept under diffused normal light. The plants were maintained for nearly 50-60 days for further hardening and growth.

3.1.8 **Initiation and growth studies of embryogenic cell suspension cultures**

Whitish embryogenic tissue from 6-month-old callus cultures were placed in a 25 ml Erlenmeyer flask with 8 ml M2 medium (MS) supplemented with 2,4-D (4.5 µm), d-biotin (1 mg l⁻¹), L-glutamine (100 mg l⁻¹), malt extract (100 mg l⁻¹), and sucrose (247 mM) and adjusted to pH 5.3. Culture flasks with cotton plug and aluminum foil were maintained on a gyratory shaker at 70 rpm under a photoperiod of 16 hr at 26 °C ± 1 °C. These suspensions were subcultured every seven to ten days and were used for the growth and regeneration experiments.

3.2 **Agrobacterium tumefaciens mediated genetic transformation of banana embryogenic cells**

3.2.1 **Agrobacterium strain, binary vector and preparation of Agrobacterium suspension**

For transformation of banana cultivar Robusta ECS, the disarmed *A. tumefaciens* strain EHA105 (Hood et al. 1993), harboring the binary vector pCAMBIA 1301 (CAMBIA, Canberra, Australia) was used. The T-DNA of pCAMBIA 1301 contains a
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gusA gene interrupted by a modified castor bean catalase intron and an hpt II gene conferring resistance to hygromycin. The former is driven by single CaMV 35S promoter and the latter by double enhanced 35S promoter.

A single Agrobacterium colony was grown overnight in liquid YENB (0.75% yeast extract, 0.8% nutrient broth) medium supplemented with 50 mg/l kanamycin at 26°C with an orbital shaking of 150 rpm. Next morning the culture was resuspended in the same medium such that OD_{600nm} is approximately 0.1. It was then grown for 4-5 hrs under same conditions until an OD_{600nm} of 0.8-1.0 was reached. The suspension was pelleted down at 6000g for 10 minutes. The pellet was subsequently resuspended in M2 medium supplemented with 100μM acetosyringone (ACS) at a final OD_{600nm} of 0.1 or 0.2, to be used for infection.

3.2.2 Transformation of ECS

Seven days post sub cultured cells were sieved through an 85-μm sieve to remove larger cell clumps. Approximately 0.05 g SCV of sieved ECS was cocultivated with Agrobacterium in liquid M2 medium supplemented with 100μM acetosyringone. For semisolid cocultivation, ECS were aspirated onto glass fiber discs with a Buchner apparatus and transferred to semisolid M2 medium containing 100μM ACS and maintained in dark for 3-7 days. Post-coculture ECS along with filters were transferred to solidified M2 medium containing carbenicillin (200mg/l) and cefotaxime (200mg/l). Three days further ECS and filters were transferred separately to BEM containing carbenicillin (200mg/l), cefotaxime (200mg/l) and hygromycin (5mg/l). Regeneration was carried out in selective regeneration medium as described earlier. For liquid cocultivation, 10 ml M2 medium supplemented with 100μM ACS was used. The cultures were incubated in dark under two conditions- at 100 rpm for 3-7 days (condition A) and initial 12 hours at 40 rpm and thereafter at 100 rpm for 3-7 days. The remaining steps are similar to semisolid cocultivation. At all conditions, one set was centrifuged at 1000 rpm for 5 min prior to cocultivation. Three replicates were used for each experiment.

3.2.3 GUS histochemical assay

Transformed tissues (cells from suspension, developed somatic embryos, leaves and roots from transgenic plants) were analyzed for β-glucuronidase expression by using X-GlcA (5-bromo-4-chloro-3-indolyl- β-D-glucuronide cyclohexylammonium salt) as the
substrate (Jefferson 1987). The histochemical reaction was allowed to proceed at 37°C for 4 h to overnight. Subsequently, the tissues were cleared in 70% (v/v) ethanol. To serve as control against any background GUS staining, un-transformed tissues were included at all staining occasions.

3.2.4 Fluorometric GUS activity assay

β-glucuronidase specific activity was measured in transformed ECS and leaves using the fluorometric assay (Jefferson 1987). ECS were harvested six days post-cocultivation, from all cocultivation conditions and three independent transformation events. Leaves from plants showing positive GUS histochemical staining was also excised. Total protein extracts obtained using GUS extraction buffer (50mM NaHPO4 pH 7.0, 10mM β-mercaptoethanol, 10mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (w/v) Triton X-100) were used for GUS specific activity using 2mM MUG assay buffer. Fluoroscence was measured using a fluorimeter with emission and excitation filters set at 465nm and 360 nm respectively. Total protein concentration in samples was determined as described previously (Bradford 1976).

3.2.5 Polymerase chain reaction (PCR)

Reagents stock used:

10X assay buffer (100 mM Tris, pH 9.0, 500 mM KCl, 15 mM MgCl2, and 0.1 % gelatin)

Taq DNA polymerase 3U/µl

dNTP mix (2.5mM each)

Sterile Milli Q water

Primers

Genomic DNA was isolated from leaves of putatively transformed plants using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). This DNA was fractionated on 0.8% agarose gel along with pCAMBIA 1301 plasmid loaded in one of the wells. High molecular weight genomic DNA, considerably larger than pCAMBIA 1301 plasmid was eluted from the gel and used for PCR. Following primers were synthesized based on the sequence of pCAMBIA 1301 Ti-plasmid.

Primer A 5’ AACAGGTATGGAATTTCGCCGATTT 3’

Primer B 5’ TTTATCCTAGTTTGCAGCTATATT 3’
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Primer C 5’ CCTAGATCTCTTCGGTAATGAAAAA 3’

Primers A and B were used to amplify a 450 bp β-glucuronidase gene fragment which lies within the T-DNA borders. Primers A and C were used to amplify a 1,585 bp fragment, out of which 550 bp lies within the T-DNA borders and the remaining 1,035 bp is outside T-DNA. Eluted genomic DNA from one of the transgenic plants mixed with 10 pg pCAMBIA 1301 plasmid was also used as a template for both primer combinations. Genomic DNA isolated from untransformed Robusta leaves was treated as negative control whereas 1301 plasmid was taken as positive control. A 50 µl PCR mix contained 100 ng of each primer, 1 unit of Taq polymerase, 200 µM of each dNTP, 1X PCR buffer and 500 ng of eluted plant genomic DNA as template. PCR conditions used were 94°C for 5 min for initial melting followed by 35 cycles of amplification with each cycle consisting of the following steps: 94°C for 1 min, 54°C for 1 1/2 min and 72°C for 2 1/2 min with a final extension at 72°C for 15 min.

3.2.6 Southern analysis of transgenic plants

Southern analysis was carried out as previously described (Sunil Kumar et al. 2005). Genomic DNA was isolated by standard CTAB protocol as described by Sambrook et al. (1989).

The chemical reagents, tips (0.1-10 µl, 20-200 µl and 1000 µl), eppendorf tubes (1.5 ml and 0.5 ml), PCR tubes, DNA extraction buffer, mortar and pestle and all other related equipments were autoclaved at 121°C for 15 minutes under 1.06 Kg/cm² pressure. The glasswares were also autoclaved after washing and rinsing with distilled water.

Isolation of DNA

Reagents:
A) 10% CTAB
B) 4M NaCl
C) 1M Tris (pH8)
D) 0.5 EDTA (pH8)
E) 2% CTAB Extraction Buffer (pH8) contained :
10% CTAB buffer (20ml), 4M NaCl (35ml), 1 M Tris-Cl (pH 8.0) (10ml), 0.5 M EDTA (pH 8.0) (4ml) and double distilled water (31ml).
F) 0.2% β mercaptoethanol (added freshly in extraction buffer before use)
G) Chloroform (CHCl₃): Isoamyl alcohol (24:1)
H) TE buffer (10mM Tris, 1mM EDTA, pH- 8.0) (For 100 ml buffer 1M Tris-Cl (1.0ml)
and 0.5 M EDTA (0.2ml) was added 98.8 sterile Milli Q
I) 3M Sodium acetate (pH-5.2)
J) Phenol: Chloroform (1:1) mixture

**Protocol**

The different steps in the method included the following:

- Around 1.00 gm of the leaf tissue was ground to fine powder in liquid nitrogen.
- To the powdered tissue, 20ml of DNA extraction buffer (with 0.2 % β-mercaptoethanol) was added and mixed well by gentle inversion and incubated at 65°C for 1 hr in a water bath.
- To the sample CHCl₃: Isoamyl alcohol mixture was added, mixed slowly and centrifuged at 8000 rpm for 10 min at room temperature and then supernatant was collected into a fresh tube.
- To the collected supernatant, equal volume of chilled isopropanol was added, mixed well and the DNA was allowed to precipitate at –20°C for 30 min.
- The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. The collected pellet was washed with 70 % alcohol by adding 500 µl of 70 % ethanol, briefly centrifuging it in the centrifuge and then the supernatant was discarded. The washing was repeated twice and re-pelleted by centrifugation at 10,000 rpm.
- The collected pellet was air dried in the laminar airflow and dissolved in required quantity of TE buffer.
- DNAse free RNAse-A was added at a final concentration of 20 µg⁻¹ml and incubated at 37°C for 1 hr in hot water bath.
- To the incubated samples, equal volumes of phenol: chloroform (1:1) mixture was added and centrifuged at 10,000 rpm for 10 min.
- The aqueous phase was collected in fresh tube and the above step was repeated till no white interface was seen.
• The aqueous phase 0.1 volume of sodium acetate and 2 volumes of ice-cold absolute ethanol was added, mixed well and kept at -20°C for 1 hr.
• The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. The collected pellet was washed with 70 % alcohol by adding 100 µl of 70 % ethanol, briefly centrifuged and then the supernatant was discarded. The washing procedure was repeated twice and re-pelleted by centrifugation at 10,000 rpm.
• The collected pellet was air dried in laminar flow and dissolved in required quantity of TE buffer and stored at -20°C.

**DNA quality testing and quantification**

DNA quality was checked by loading DNA sample on 0.7 % agarose gel. The DNA sample was diluted with MilliQ water and the Optical Densities (O.D) of different diluted samples was taken at 260 nm. The amount of DNA present in the original sample was calculated using the formula:

$$\text{O.D} \times 50 \times \text{Dilution Factor}$$

**Agarose gel electrophoresis**

**Reagents:**

A) 10X Tris acetate (TAE): Tris-base (48.44g), Glacial acetic acid (11.42ml) and 0.5 M EDTA (20ml). Final volume was adjusted to 1000 ml. Working stock used was 1X TAE

B) Loading dye: 0.25 % bromophenol blue, 0.25 % xylene cynol and 30 % glycerol in water.

Running on 0.7 % agarose gel ensured the checking of the DNA samples qualitatively. For the preparation of agarose gel, agarose @ 0.7 gm⁻¹100 ml was melted in 1X TAE buffer by heating to boiling. After cooling the solution to about 45°C, ethidium bromide @ 3-5 µg⁻¹ ml was added. To the DNA samples, 6X gel loading dye was added so that the final concentration was 1X before loading. The gel was run at 60 V for 1 hr.

For Southern analysis, 10-20 micrograms of genomic DNA isolated from four independently transformed plants was digested with NcoI and Eco721 to release ~2 kb uidA gene from the T-DNA region of pCAMBIA1301. The digested DNA was electrophoresed on 1% agarose gel and transferred to Hybond-N+ membrane (Amersham
Pharamacia Biotech, UK) using standard protocols (Sambrook et al. 1989). These DNAs were hybridized against a random primed radiolabelled probe of 947 bp fragment obtained by PCR amplification of pCAMBIA1301 with uidA specific primers mentioned below.

Forward primer 5' AAGCCAGACAGAGTCTGATATCTACC 3'
Reverse primer 5' GAGTGAAGATCCCTTTCTTGTTACC 3'

Results were visualized by autoradiography.

3.2.7 Primer sequences used for PCR validation of Rasthali and Robusta plants transformed with ripening related genes.

ACO F: CTCATTTTCCTCCTCGACGTCAAG
NOST: GGACTCTAATCATAAAAACCC
MADS XbaI : GGGCGGGTCTAGATCGCGATGGGGAGGGGGAGGGTG
EXP R1: GACTGGTAAAGCTTCCACCGATGC
EXP R2: GCAATGCCCGGAGGGAGGAGATTG
SEQ 1: GAATCCGCACTAGTTCACAGCC
GUS R: ATTCCACAGTTTTCGCGATCCAGAC
101F: TCGTATGTTGTGTGGAATTGTGAG
IFR1.2 SEQ 2: TCACAAATGGGTCGACATTGGAATCC
IFR1.4 SEQ 1: AAAGAGTAGAGGTCGACCATTAT

3.3 Promoter related studies

3.3.1 Cloning of chitinase, expansin and glucanase promoter in place of CaMV 35S promoter in pCAMBIA-1301 binary vector

Three banana fruit-specific promoters namely chitinase (BAC) promoter (Genbank accession no. AY525367), expansin (EXP) promoter (Genbank accession no. AF539640) and glucanase (GLU) promoter (US patent application 20030226175A1) were amplified from banana cv. Rasthali derived genomic DNA using the following primers:

**BAC:** Fw 5’ ATTACTGCAGTCAAAGTTAGAAAAATCTTTACCAAGACG 3’
Rv 5’ TGATAGATCTACCATAGTGAATGGAGGAGGCTATCG 3’

**EXP:** Fw 5’ AGCTAAGCGTTAAAATCCACCGATGAGCCTCTTC 3’
Rv5’ AGCTAGATCTACCATTTAGGAAATTATCTCCACTAAAAGAGATTGGA 3’
GLU: Fw 5’ ATTACTGCAAGGTATTTAGCCTAACCATTTCCGGACTC 3’
Rv 5’ AGCTAGATCTACCATGACAACAACACTCTGCTCAAAGG 3’

Subsequently, the CaMV 35S promoter driving β-glucuronidase in pCAMBIA 1301 binary vector (CAMBIA, Canberra, Australia) was replaced by these promoters using appropriate restriction enzymes (underlined in primer sequences) to obtain three binary vectors called pBAC-1301, pEXP-1301 and pGLU-1301 respectively. These binary vectors were mobilized into the *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation.

3.3.2 *Agrobacterium strain, binary vector and preparation of Agrobacterium suspension*

For transformation of banana cultivar Robusta ECS, the *A. tumefaciens* strain EHA105 harboring pCAMBIA-1301, pBAC-1301, pEXP-1301 or a pGLU-1301 binary vector was used. A single *Agrobacterium* colony was grown overnight in liquid YENB (0.75% w/v yeast extract, 0.8% w/v nutrient broth) medium supplemented with 50 mgL$^{-1}$ kanamycin at 27$^\circ$C with an orbital shaking of 150 rpm. Subsequently, the culture was resuspended in the same medium at an OD$_{600nm}$ of 0.1 and grown under same conditions until an OD$_{600nm}$ of 0.8-1.0 was reached. The suspension was centrifuged at 6000g for 10 minutes and the pellet was subsequently resuspended at a final OD$_{600nm}$ of 0.1 in M2 medium (Cote *et al.*, 1996) supplemented with 100µM acetosyringone.

3.3.3 Transformation of ECS and optimization of transient expression of β-glucuronidase

The initiation and maintenance of ECS was carried out as mentioned in above section. Seven days post sub-cultured cells were sieved through an 85-µm sieve to remove large cell clumps. Approximately 0.05 g settled cell volume of sieved ECS was co-cultivated with *Agrobacterium* (prepared as above) in liquid M2 medium supplemented with 100µM acetosyringone. ECS were aspirated onto glass fiber discs with a Buchner apparatus and transferred to semi-solid M2 medium containing 100µM acetosyringone and maintained in dark for 6 days. Post-coculture ECS were scraped from the glass fiber filters and induced with the respective agent.
Further, 200 mg fresh weight of banana ECS transformed with pCAMBIA-1301 were put for GUS histochemical staining on the 4th, 5th and 6th day post infection to ascertain the day at which the transient expression is maximum.

### 3.3.4 Induction of co-cultivated ECS

Five days post co-cultivation, banana ECS transformed with different constructs were induced with ethephon at different concentrations (0.25mM, 0.5mM, 1mM and 2mM). ECS were scraped from the glass fiber disc and suspended in liquid M2 medium to which ethephon was added. The cells were kept on a gyratory shaker at 100 rpm in dark and at a temperature of 25 ± 2°C for 15 hours. Subsequently, cells were harvested and β-glucuronidase specific activity was measured using the MUG-based fluorometric assay (Jefferson, 1987). Total protein extracts obtained using GUS extraction buffer (50mM NaHPO4 pH 7.0, 10mM β-mercaptoethanol, 10mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (w/v) Triton X-100) were used for GUS specific activity using 2mM MUG assay buffer. Fluorescence was measured using a fluorimeter with emission and excitation filters set at 465nm and 360 nm respectively. Total protein concentration in samples was determined as described previously (Bradford, 1976).

After ascertaining the ethephon concentration at which maximum β-glucuronidase expression was obtained for different constructs, effect of other elicitors on β-glucuronidase expression was determined. Elicitors used for above experiments were MJ, MS, ABA and IAA. For ABA treatment, five days post transformed ECS were cultured in liquid M2 medium supplemented with 5mM, 10 mM and 15mM ABA under the conditions mentioned above. For studying the synergistic action of ethephon and ABA on promoter activity, ABA treatment was followed by ethephon induction as described above. Similarly for studying the combinatorial effect of ethephon with IAA, ethephon with MJ and ethephon with MS, transformed ECS were cultured in liquid M2 medium supplemented with 5mM, 10 mM and 15mM IAA, MJ or MS respectively under the conditions mentioned above. This was followed by overnight ethephon induction after which fluorometric assay was performed.

### 3.4 Fruit ripening related studies

#### 3.4.1 Plant material and treatment
Mature green transgenic and control bananas (variety Rasthali, genome AAB type) were harvested from field and treated with 100 µL/L ethylene for 24 h in an air-tight container as described earlier (Pathak et al., 2003). Fruit pulp from three bananas (pooled together) was first harvested at 12 h and thereafter every 24 h for a period of 6 days after ethylene treatment, frozen in liquid nitrogen and stored at minus 70 °C until further use.

For 1-MCP treatment, mature green bananas were preexposed to 1 µL/L 1-methyl cyclopropene (Ethyl Bloc from Biotechnologies for Horticulture Inc, Walterboro, SC, USA) for 12 h followed by ethylene treatment as described above. Samples were collected from the same stages as described above.

**3.4.2 Determination of fruit firmness**

Three fruits from each treatment were used to measure fruit firmness. To measure the firmness, peel from one side of the banana finger was removed and measurement was carried out at three different places using Penetrometer (Model FT327 and FT011, McIormick Fruit Tech, USA) and recorded as force in Newton (N). Average of three readings was taken as measure of firmness for individual fruit. Data are expressed as mean ± standard deviation (S.D.) of all replicates.

**3.4.3 RNA isolation from fruit pulp**

RNA was isolated from pulp tissue as described by Asif et al, (2005). The chemical reagents, tips (0.1-10 µl, 20-200 µl and 1000 µl), eppendorf tubes (1.5 ml and 0.5 ml), PCR tubes, DNA extraction buffer, mortar and pestle and all other related equipments were autoclaved at 121°C for 15 minutes under 1.06 Kg/cm² pressure. The glasswares were also autoclaved after washing and rinsing with distilled water.

**Solutions and reagents**

Extraction buffer: 100 mM Tris-Cl pH 8.2, 1.4 M NaCl, 20 mM EDTA (pH 8),
2% CTAB
10 M LiCl
3 M Na Acetate pH 5.2
2-mercaptoethanol
DEPC treated and autoclaved MilliQ grade water
Absolute EtOH
70% EtOH
Water saturated phenol
Chloroform : isoamyl alcohol (24:1)

**RNA extraction protocol**

- A ratio of 10 mL extraction buffer / gram of tissue was used. 1 µl of 2-mercaptoethanol per mL of buffer was added just before use.
- The frozen tissues were homogenized in mortar pestle in the presence of liquid nitrogen to which preheated (65°C) extraction buffer was added.
- The homogenate was transferred to a clean 30 mL centrifuge tube and incubated at 65°C for 1 h, with gentle vortexing every 15 min.
- The tube was cooled to room temperature and an equal volume of chloroform isoamyl alcohol mixture was added. The tube was shaken vigorously until the two phases formed an emulsion.
- Tube was centrifuged at 12000 g for 15 min at room temperature.
- The aqueous phase was collected and re-extracted with an equal volume of chloroform isoamyl alcohol mixture and centrifuged as above.
- The aqueous phase was collected and 10 M LiCl to a final concentration of 3 M was added and RNA was allowed to precipitate at 4°C overnight.
- RNA was recovered by centrifugation at 17000 g at 4°C for 20 min.

The pellet was dissolved in DEPC treated water and extracted once with phenol, phenol : chloroform (1:1), and chloroform, sequentially.
- The aqueous phase was collected to which 1/30 volume of 3 M Na acetate pH 5.2 and 0.1 volume of 100% EtOH was added, mixed well and kept on ice for 30 min. It was then centrifuged in a microfuge at 4°C for 25 min. A white jelly-like pellet consisting mostly of polysaccharides was obtained and discarded.
- To the clear supernatant 3 M Na acetate pH 5.2 to a final concentration of 0.3 M and 3 volumes of 100% ethanol were added.
- The RNA was allowed to precipitate at -70°C for 3 h to overnight. RNA was recovered by centrifugation in a microfuge at 4°C for 20 min. Pellet was washed with
equal volume of 70% EtOH, vacuum dried and re-suspended in an appropriate volume of DEPC treated water (100 µl/g starting material).

- RNA was quantitated spectrophotometrically at wavelengths of 240, 260, 280, 300 and 320 nm.

### 3.4.4 RT-PCR analysis

RT-PCR was carried out using the Promega Access RT-PCR System in a single tube reaction protocol. The reverse transcriptase reaction was carried out at 48°C for 1 h, followed by heat inactivating the AMVRT at 94°C for 2 min. The PCR reaction was carried out for 40 cycles; conditions for each cycle being denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 68°C for 2 min 30 s. The final extension was carried out at 68°C for 7 min. An amplicon of the expected size was obtained for the desired gene.

### 3.4.5 Semiquantitative PCR

To study the expression pattern of MADS and expansin, semi-quantitative RT–PCR was carried out using cDNA prepared from DNA-free RNA of different ripening stages. RT–PCR analyses of different genes were carried out using GeneAmp PCR system 9700 (Applied Biosystems, CA). RT–PCR analysis was performed for

**MADS**

ACO F: CTCATTTCGTCCCTCGACGTCAAG  
MADS XbaI : GGGCGGGTCTAGATCGCGATGGGGAGGGGGAGGGAGGGTG

**Expansin**

EXP R1: GACTGGTAAAGCTTCCACCGATGC  
EXP R2: GCAATGCCCGGGAGGGAGGAGATTG

**Actin**

(ACTF: 5’ GAGAGTTTTTGATGTCCCTGCCATG3’;  
ACTR: 5’ CAACGTCGCATTTCATGATGGAGT3’)

Actin was used as internal control. Reactions for RT–PCR were subjected to 35 cycles of amplification, each consisting of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min. The amplified products were run on agarose gel along with the marker.