Agrobacterium mediated transformation for development of transgenic tomato with ySAMdc gene

INTRODUCTION

Tomato is an ideal model crop plant for genetic improvement (Park et al., 2003). Tomato has a diploid genome (2n=24) with gene size of 950Mbp (Michaelson et al., 1991b) encoding for approximately 35,000 genes that are largely sequestered in flanked euchromatic regions (Van der Hoeven et al., 2002). For any genetic engineering program in a particular system to become successful, the factors that need to be optimized are; receptiveness of the tissue to foreign DNA integration, availability of vectors and screenable markers, regeneration of intact plant from transformed tissues (Hooykaas et al., 1992, Ruppert, 1999). The genetic modification of crop plants with the introduction of desirable traits, such as nutritional enhancement, disease and pest resistance, and enhanced crop productivity, has revolutionized agriculture; this is being increasingly sought as a promising technology to boost food production worldwide.

There are different plant transformation techniques for the introduction of a foreign gene in to a plant system; they include Agrobacterium Ti plasmid vectors, microprojectile bombardment, microinjection, and chemical (poly ethylene glycol) treatment of protoplasts and electroporation of protoplasts (Veluthambi et al., 2003). In planta transformation using floral dip method was also demonstrated (Clough and Bent, 1998). Agroinjection in tomato fruit was also considered as a type of transformation method (Orzaez et al., 2006). Among the above techniques, Agrobacterium based transformation and microprojectile bombardment is currently the most extensive method used in plant transformation (Gelvin, 2009). Agrobacterium is a natural genetic engineer that scientists have used for gene transfer experiments for the past 25 years. In 1986, Mc Cormick et al. reported the transformation in tomato for the first time. Information on the Agrobacterium mediated gene transformation have been reported in different cultivars of tomato grown worldwide for a variety of purposes which includes characterization of gene function, herbicide tolerance, disease and insect resistance, improvement in fruit quality, delay in fruit ripening, improved transformation protocol, and production of
foreign proteins (Metha et al., 2002; Giorio et al., 2007, Guerra et al., 2007, Koc et al., 2007; Youm et al., 2008; Yokotani et al., 2009; Permiakova et al., 2009).

The objective of this study was to standardize Agrobacterium mediated transformation protocol using ySAMdc gene induced with E8 fruit promoter to produce putative tomato transgenic to enhance the lycopene content and improve shelf life of tomato. Also, attempts were made to extract RNA and study the level of expression of transgene in tomato fruit. The morphological characteristics like plant height, no of flowers, fruit number, fruit cluster, fruit weight and biochemical characteristics like lycopene, TSS and titrable acid contents produced in transgenic plants were determined in comparison to control plants.
MATERIAL AND METHODS

1 Selection of the tomato genotype

The genotype Arka vikas, the product of Indian Institute of Horticultural Sciences, Bangalore was selected as the host for the expression of the $\gamma$SAMdc gene. This genotype, a popular variety in India, is a high yielder that produces attractive medium sized firm fruits with uniform ripening quality and suitable for fresh market. It was developed from an American variety Tip top by pure line selection (Lopez et al., 1996). The crop duration of this cultivar was found to be 120-135 days.

2 Selection of transformed Agrobacterium tumefaciens strain

An Agrobacterium tumefaciens strain was transformed in plasmid pFGC5941 plasmid, fused with $\gamma$SAMdc gene and E-8 fruit inducing promoter. The plasmid contained 2 selection markers one for BAR gene resistant to BASTA (Ammonium glufosinate, a powerful herbicide) and another plasmid Km marker resistant to kanamycin. The total plasmid size was 11406 kb and the full length gene size was 1.2kb. While, $\gamma$SAMdc gene region was 488bp in size kindly supplied by Dr. Handa, Purdue University USA was used in the present study. This strain of bacteria was stored in glycerol at -80°C in a deep freezer (Thermoforma freezer model 8517) until use.
Fig. 20. pFGC5941 plasmid construct with ySAMdc gene

Binary vector: Km- kanamycin resistance for selection in Agrobacterium. BAR gene- BASTA resistance for selection of plant cells

Source: Courtesy Dr. Handa’s Lab
3 Preparation of yeast extract peptone media for Agrobacterium culture

The YEP broth was prepared as follows - Yeast (Hi media, Mumbai) - 1.0g, Peptone (Hi media, Mumbai) - 1.0g and NaCl (Hi media, Mumbai) - 0.5g was added to 80 ml distilled water and pH was adjusted to 7.5 and the final volume was made up to 100 ml with SDW in a measuring cylinder.

The YEP agar medium was prepared as follows - Yeast- 1.0g, Peptone- 1.0g and NaCl- 0.5g was added and pH was adjusted to 7.5 to 80 ml distilled water and pH was adjusted to 7.5. Agar (1.5g) (Hi media, Mumbai) was added and stirred. The final volume was made up to 100 ml with SDW in a measuring cylinder. The culture broth and agar media were autoclaved at 121°C, 15lb for 20 min.

4 Preparation of Agrobacterium cell culture

A loop of transformed Agrobacterium suspension was collected from the glycerol stock and streaked on YEP agar medium supplemented with antibiotic kanamycin (50μg ml⁻¹) and incubated in an incubator in darkness, for 12h at 30°C (Plate 12a). This was done 2-3 days prior to its co-cultivation with cotyledonary leaf explants of tomato.

5 Broth culture of transformed Agrobacterium tumefaciens on YEP

A well grown colony that developed on the YEP agar was collected using a sterilized bacterial loop and dispensed into sterilized conical flask containing 25ml of YEP broth appended with 6.25 μl Kanamycin (50μg ml⁻¹). This broth was incubated at 30°C for 12h under constant shaking at 250rpm. The cell concentration of the bacteria in YEP broth was fixed to an OD of 0.5, 0.8 or 1.0 at 600nm (Spectronic Genesys 2). This suspension was used for co-inoculation studies.

6 Preparation of Murashige and Skoog’s (MS) media (Murashige and Skoog, 1952)

The MS medium was prepared by adding inorganic and organic stock solutions, sucrose and agar (Hi-media, India). Initially, the inorganic and organic stock solutions as
well as vitamin solutions were prepared (Table 27) and stored in colored bottles at 4°C. About 750ml of SDW was taken in a beaker and added with the macronutrients (25ml), micronutrients (10ml), Na2Fe EDTA (10 ml), CaCl2 (10 ml), vitamins (10 ml) and stirred to completely dissolve the ingredients. Into this solution, 30g of sucrose was added and the pH was adjusted to 5.8. Eight grams of agar were added and homogenized on a water bath. Following the dissolution of agar, the total volume of the media was made up to one liter using a graduated cylinder and stirred using a glass rod. The molten culture (40°C) media was dispensed into sterilize conical flasks and plugged with cotton plugs and autoclaved at 121°C 15 pounds for 20 min. The media was added with suitable plant growth hormones (PGH) only at the time of pouring to plate/baby food jars at a temperature of 40-55°C under aseptic conditions in a laminar air-flow chamber. Based on the addition of certain ingredients like antibiotics, plant growth hormones (PGH) and selection markers, the MS media were classified into MS-modified A, B, C, D, E and F.

The details of the composition of these modified MS media are as following:

MS modified-A: MS medium + agar (0.8%), pH 5.8.

MS modified-B: MS medium + 100μM acetosyringone (3', 5'-Dimethoxy-4'-hydroxyacetophenone) (Himedia, Mumbai) + agar (0.8%), pH 5.8.

MS modified-C: MS medium + IAA (0.1mg L⁻¹) (Himedia, Mumbai) + BAP (4mg L⁻¹) (Himedia, Mumbai) agar (0.8%) + cefotaxime (250mg ml⁻¹) (Taxim 1g, Alkem Laboratories Ltd, Daman), pH 5.8.

M.S modified-D: MS medium + IAA (0.1mg L⁻¹) + BAP (4mg L⁻¹) agar (0.8%) + cefotaxime (250mg/ml) + BASTA (Ammonium Gufoinate)- (2g L⁻¹) (Himedia, Mumbai) pH 5.8.

M.S modified-E: MS medium + IAA (0.1mg L⁻¹) + BAP (4mg L⁻¹) agar (0.8%) + cefotaxime (250mg/ml) + BASTA (Ammonium Gufoinate)- (4mg L⁻¹) (Himedia, Mumbai) pH 5.8

M.S modified-F: MS medium + IAA (0.5 mg L⁻¹), BAP (0.5 mg L⁻¹) agar (0.8%) + pH 5.8.
Table 27. Composition of Murashige Skoog (1962) media

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Salt concentration in Stock Solution (gL⁻¹)</th>
<th>Final Concentration of salt in 1 liter of medium (mg L⁻¹)</th>
</tr>
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<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>16.50</td>
<td>1650</td>
</tr>
<tr>
<td>KNO₃</td>
<td>19.00</td>
<td>1900</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>KH₂PO₄</td>
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<tr>
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<tr>
<td>FeSO₄·7H₂O</td>
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<tr>
<td><strong>Micronutrients</strong></td>
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</tr>
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<tr>
<td>ZnSO₄·H₂O</td>
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<tr>
<td>KI</td>
<td>0.0083</td>
<td>0.83</td>
</tr>
<tr>
<td>NaMoO₄·2H₂O</td>
<td>0.0025</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.00025</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.00025</td>
<td>0.025</td>
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<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
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<tr>
<td>Myo-inositol</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Thiamine·HCl</td>
<td>0.001</td>
<td>0.1</td>
</tr>
<tr>
<td>Pyridoxine·HCl</td>
<td>0.005</td>
<td>0.5</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.005</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.02</td>
<td>2.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30</td>
<td>30000</td>
</tr>
<tr>
<td>Agar</td>
<td>8</td>
<td>8000</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>5.8</td>
<td></td>
</tr>
</tbody>
</table>
The specific concentrations of hormones used were arrived at after a series of trial and error attempts to obtain the shoot and root primodia from cotyledon explants.

Transformation of tomato (*Solanum lycopersicum*) was done using *Agrobacterium* mediated and the protocol was a modified protocol of (Zhang, 2000; Datsenka and Handa, 2006).

6 Preparation of tomato explants

Tomato seeds (50 seeds) of the genotype Arka vikas were taken and surface disinfected with ethanol (70%, 1 min) and then in Hg Cl₂ (0.01%, 3min) and finally washed in SDW for 3-4 times. The surface disinfected seeds were blotted on autoclaved blotter discs and transferred onto MS modified A medium (Plate 12b). They were incubated in an incubation room (25°C) in darkness for 24 hours and then transferred to the culture room with a light intensity of 30 lux (cool white fluorescent tube) and a temperature of 25°C. The tomato seedlings at the age of 10-12 days were removed carefully using a sterilized forceps and cotyledon explants were prepared and used for further experimentation (Plate 12).

7 Reagents for DNA extraction and PCR

The extractants for DNA and reagents for PCR reaction are similar to that described in chapter-II. The SAM-sense forward primer 5'-TGT CTC TTT CTT GTT CCC ATT TC -3' and SAM-anti reverse primer 5'-TGG TTG TAG TAG CGC ACG TC -3' were kindly supplied by Dr. Handa's laboratory, Purdue University, USA.

8 Preparation of reagents for plasmid DNA

Solution I

a 50mM Glucose

0.45g of glucose (Hi media, Mumbai)

b 25mM Tris (pH 8.0)

0.157g Tris was added to 50ml SDW.
c 10 mM EDTA

The 10 mM EDTA solution was prepared by taking 1ml from 0.5M (stock solution prepared for DNA extraction) to 50ml taken to prepare solution I. This was autoclaved prior to use.

The solution I was made by adding 0.45g of glucose and 0.157g Tris + 1ml 0.5M EDTA and final volume was made up to 50ml.

Solution II

a 10% SDS

1g of SDS was mixed with 10ml of SDW and the solution was autoclaved.

b 0.2N NaOH

400μl of 5N NaOH (stock solution previously prepared). To make solution II, 1% SDS (1000 μl from 10% stock SDS) and 0.2N NaOH (400 μl was added) was added and made to a final volume of 10 ml.

Solution III

a 5M Potassium acetate

49.06g of potassium acetate was added to 100 ml SDW.

b Glacial acetic acid

The solution III was prepared by adding 60 ml of potassium acetate and 11.50ml glacial acetic acid. 28.5ml of SDW was added to make the final volume to 100ml. This was autoclaved prior to plasmid preparation and stored at 4°C.

Reagents like phenol: chloroform isoamyl alcohol, chloroform isoamyl alcohol, 70% alcohol TE buffer that were prepared for DNA isolation was used here also.
10 Reagents for RNA isolation (Sambrook et al., 1989)

10.1 Diethyl pyrocarbonate (0.1%) (DEPC)

This was prepared by adding 0.1ml of diethyl pyrocarbonate to double distilled milliQ water (DDMQ) and volume was made up to 100 ml.

10.2 RNA extraction buffer (pH 7.0)

The extraction buffer consisted of 1.4 M NaCl, 100mM Tris, 20mM EDTA, 2% CTAB (w/v), 1% SDS, 2% β-mercaptoethanol, and 4%- PVP.

10.3 4M Lithium chloride (Hi media, Mumbai)

The solution (4M) was made by dissolving 16.94g of lithium chloride in 80ml double distilled milliQ water and then the volume was adjusted to 100ml and stored at 4°C.

10.4 Saturated phenol (pH 8.0)

The solid phenol crystals were melted at 68°C and 0.1% 8-hydroxyquinoline was added to a final concentration of 0.1%. Equal volume of 0.5M Tris-Cl buffer pH 8.0 was added and stirred on a magnetic stirrer for 15 min at room temperature. After removing the upper aqueous phase, it was equilibrated with Tris and this step was repeated until the pH of the phenolic phase reached 8.0. After equilibration to pH 8.0, the final aqueous phase was removed, and 0.1M Tris-Cl (pH 8.0) containing 0.2% β-mercaptoethanol was added. The saturated phenol solution was stored in this form under 100mM Tris-Cl (pH 8.0) in a DEPC treated amber bottle at 4°C.

10.5 Phenol: chloroform mixture

Equal amounts of phenol with chloroform- isoamyl alcohol mixture (24:1v/v) was mixed and stored at 4°C in a DEPC treated dark glass bottles.
10.6 Chloroform: isoamyl alcohol

This mixture was prepared by adding 96mL of chloroform with 4mL of iso-amyl alcohol and stored in refrigerator (4°C).

10.7 3M Sodium acetate buffer (pH 4.0)

This solution was prepared by dissolving 40.8g of sodium acetate in 50ml DDMW and pH adjusted to 5.2 using newly opened glacial acetic acid. The volume was made up to 100ml and sterilized by autoclaving.

10.8 6x RNA gel loading buffer (pH: 8.0)

The dye was prepared by mixing 50% Glycerol, 1mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF.

10.9 5X Formaldehyde gel running buffer

This buffer was prepared by preparing 0.1M morphalinopropanesulfonic acid (MOPS) buffer pH 7.0 and 40mM sodium acetate with 5mM EDTA pH 8.0 was added. This buffer was prepared just before use and was not stored.

10.10 1% Ethidium bromide

One gram of ethidium bromide was dissolved in 100ml of DDMW. This solution was stirred on a magnetic stirrer for several hours to ensure that the dye dissolved completely. The small vials were DEPC treated and wrapped in an aluminum foil and stored in a refrigerator (4°C).

10.11 Ethanol

100% ethanol was opened just before use and used directly.

10.12 70% Ethanol

Seventy milliliters of absolute alcohol was mixed with 30ml DEPC treated water and stored at 4°C.
11 Co-cultivation of transformed *Agrobacterium* and tomato cotyledon explant

The cotyledon explants were pricked and transferred to the culture broth along with the transformed *Agrobacterium* and swirled for 5 min for promoting infection. The prick-inoculated cotyledons were then removed, and washed in SDW and then with an antibiotic (250 mgL\(^{-1}\)) solution placed on sterile filter paper discs to remove the extra broth and transferred on to plates containing MS modified B medium (appended with 100 μM acetosyringone). The plates were then placed in dark for 24h and then transferred to culture room (25°C, light intensity of 30 lux) and further incubated for 24h.

Two control samples were maintained, a positive control with explants that were not co-cultivated (not infected with transformed *Agrobacterium*) and grown in the absence of the antibiotic and a negative control containing explants that are not co-cultivated and (but infected with transformed *Agrobacterium*) and grown in the presence of the antibiotic.

12 Regeneration of transgenic shoot from *Agrobacterium* infected cotyledon explants

After 48 hours of co-cultivation in the broth, the explants were washed, blotted and transferred with its adaxial side on the surface of the MS modified C medium. All the explants categories were transferred to the fresh medium at an interval of two weeks. (Plate 12d) The calla that were initiated were cut into 10mm square pieces which were transferred to fresh MS modified C medium after treatment with cefotaxim antibiotic solution (250 mgL\(^{-1}\)). After four weeks, the calli was transferred to MS modified D selection medium and cultured for two weeks. Later, they were transferred to M S modified E selection medium (Plate 12f). The calli from cotyledon explants were cultured in selection media for a maximum period of only four weeks.

13 Rooting of transgenic shoot

The shoots (3-4 cm length), that developed from *Agrobacterium* infected calli were excised under sterile conditions and transferred to MS modified F medium (rooting medium) contained in baby food jars (Plate 13a).
14 Transplanting of transgenic plantlets to greenhouse

The plantlets with well developed roots were removed from the baby food jars (Plate 13b) and agar pieces sticking to roots were washed off gently under slow running tap water. The plantlets were dipped in 0.1% carbendazin (50% WP, Meerut Agro Chemical Industries Ltd, Meerut) solution for 15 min. The paper cups (8 cm dia × 12 cm length) containing potting medium (cocopeat) were transplanted with plantlets and were irrigated with sterile water. The plantlets were covered with a transparent plastic bag (size 22 cm × 36 cm) to maintain high humidity (95% RH) and placed under florescent lamps in a growth chamber (Plate 13c). The potting media containing transplants were irrigated with half strength M.S (without hormones and vitamins) once a week. The plastic bags were removed 10 days after transplantation. The plants that grew to 15-20 cm length (2-3 weeks) (Plate 13d) were transplanted into large pots (22 cm × 30 cm) with potting mixture with farm yard manure, red soil and soil (1:2:1) and grown in the green house (Plate 13e and f). The transgenic plants were kept in isolation and carefully monitored (Plate 13f).

Observations were made at vegetative and reproductive phases. The red ripe fruits were harvested at 120 days during maturation (Plate 13g and h).

15 Evaluation of transformants for morphological and biochemical characteristics

The data on the morphological characteristics like plant height, number of flowers and fruits produced, fruit clusters and fruit weight at red ripe stage were collected from each plant. The procedure for collection of data of each parameter is as described in Chapter-I. The data of the number of flowers produced per plant was also collected. Similarly, the data of the biochemical characteristics like total soluble solids, titrable acidity and lycopene content were also collected described in chapter I from each of the plant.

16 Molecular analysis of primary transformants of tomato

The putative primary transformants (transformed or non-transformed with \( y\text{SAMDC} \) gene) of tomato obtained by \textit{Agrobacterium}-mediated transformation method was subjected to PCR.
16.1 Isolation and quantification of genomic DNA from leaf samples

The genomic DNA was isolated from young leaves (at flowering stage) of putative transformed plants and non transformed plants by CTAB method (Porebski et al., 1997) and activated charcoal method (Krizman et al., 2006) with certain modifications as described in Chapter II.

The reagents used for the PCR analysis, preparation of agarose gel electrophoresis and loading of the gel are detailed in Chapter-II.

16.2 Isolation of plasmid DNA

A loop of transformed *Agrobacterium* colony was taken and added to 50ml YEP broth with 6.25 µl Kanamycin (50µg ml⁻¹). This culture was grown overnight to an OD of 0.5. The broth culture 1.5ml was taken into sterile eppendorf tubes and centrifuged at 11269g for 10 min at 4°C. The supernatant was discarded and 100 µl of ice cold solution I was added and vortexed vigorously for 10 min. Freshly prepared solution II was added and incubated for 4 minutes and the tube was inverted twice gently. These tubes were kept on ice. 150 µl of ice cold solution III was added and kept in chilled conditions. These eppendorf tubes were centrifuged to 11269g at 4°C for 15 min. To this supernatant, equal amounts of phenol: chloroform isoamyl alcohol mixture was added and vortexed. The supernatant was collected and equal amounts of chloroform isoamyl alcohol was added and centrifuged at 11269g for 15 min. The supernatant was taken and absolute alcohol + 1/10th potassium acetate was added and kept at 4°C in refrigerated condition for an hour. This supernatant was centrifuged at 4°C to 11269g and the pellet obtained was washed with 70% alcohol and centrifuged at 11269g for 10 min. The pellet was air-dried and 50µl of TE buffer was added. The pure plasmid obtained was stored at -20°C.

16.3 PCR analysis

In order to confirm the integration of ySAMdc gene into cells, the PCR analysis was performed with specific primer pairs like SAM-sense forward and SAM-anti reverse to amplify the above gene having the size of 488bp.
Chapter-III

The amplification was performed in a Thermocycler (PTC-100, MJ research Inc, USA) under the following reaction conditions. The amplified products were resolved on 1.0% agarose gel according to the procedure outlined (Sambrook et al., 1989).

16.4 Total PCR mixture per reaction (20 μL) (Sambrook et al., 1989)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDMW</td>
<td>8.2 μL</td>
</tr>
<tr>
<td>1X Taq assay buffer</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>200μM dNTPs</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>1.5mM MgCl$_2$</td>
<td>1.2 μL</td>
</tr>
<tr>
<td>10 pmol Forward primer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>10 pmol Reverse primer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>10ng ul$^{-1}$Template DNA</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>1.5U (0.3 μL)</td>
</tr>
</tbody>
</table>

16.5 PCR program (Sambrook et al., 1989)

- Hot start /Denaturation 94°C - 5 min
- Denaturation 94°C - 45 sec
- Annealing 55°C - 30 sec
- Extension 72°C - 1 min
- Final extension 72°C - 10 min

The marker used was a 500-5000 bp marker for detection of band size.

17 Extraction of total RNA from transgenic tomato fruits

The red ripe tomato fruits were collected and immediately wrapped in an aluminum foil and freeze-dried in liquid Nitrogen (-190°C). Approximately, 4g of tomato tissue was taken and crushed using a DEPC treated pestle and mortar and taken in a DEPC treated clean oak ridge tube. To this extract, 16ml of extraction buffer (at 65°C) and 13 ml of phenol-chloroform isoamyl alcohol (25:24:1) was added. The mixture was centrifuged at 13,226g using a high speed centrifuge (Kubota 6900, Japan) for 15 min at RT. The aqueous phase was transferred in a clean oak ridge tube and was centrifuged
again at 13,226g with phenol-chloroform isoamyl alcohol. The aqueous phase was mixed with 1/10th volume of 3M sodium acetate (pH 4.0) and 3 volume of ice cold (-20°C) 100% ethanol and was kept for precipitation for an hour. After centrifugation at 15,339g, the supernatant was removed and the pellet was allowed to dry on the laboratory bench for 5 min. The pellet was re-suspended with 2ml DEPC treated DDMW and vortexed. This sample was incubated for 10 min at 50°C. To this, 2ml extraction buffer was added and gently mixed for 2 min. It was further centrifuged at 15,339g at 4°C for 5 minutes. The supernatant was discarded and the pellet was re-suspended with 4ml 2M NaCl and 5ml chilled 100% ethanol. The tubes were incubated at -40°C for 4 hours. After incubation, the tubes were centrifuged again at 15,339g at 4°C for 20 min and the supernatant was removed and the pellet was washed with 70% alcohol. The pellet was allowed to dry for 5 min and added with 2ml 3M LiCl₂ and incubated overnight at -80°C. The suspension was divided into two parts of 500μl each and centrifuged using a micro centrifuge (Eppendorf cold centrifuge 5430 R) at maximum speed. The supernatant was discarded. To the pellet, 2M LiCl₂ was added and centrifuged at maximum speed at 4°C for 15 min. The supernatant was discarded and the pellet was washed with 70% ethanol and air-dried and dissolved with DEPC treated DDMW. The purity of RNA was calculated and stored at -80°C. The RNA concentration was checked at OD_{260nm} / OD_{280nm} concentration. The yield and purity was determined by the using spectrophotometer using the formula:

\[
\text{RNA conc (μg μ⁻¹)} = 40 \times \text{dilution factor} \times \frac{\text{OD}_{260nm}}{1000}.
\]

17.1 Formaldehyde agarose gel electrophoresis

The total RNA from the transgenic tomato fruit was electrophoresed on the denaturing formaldehyde agarose gel as described (Sambrook et al., 1989). The gel was prepared by melting agarose in DEPC treated water, cooled to 60°C followed by the addition of formaldehyde gel-running buffer and formamide to a final concentration of 1X and 2.2M, respectively.
The reaction mixture consisted of

RNA 25µg
5X formaldehyde gel-running buffer 2.0
Formaldehyde (37%) 3.5µl
Formamide 10.0µl

The RNA samples were denatured at 65°C for 15min and snap chilled on ice. The formaldehyde gel loading buffer (2µl) was added and samples were loaded on to the agarose gel and electrophoresed at 60V using a power pack (Biometra, Germany). The gels were stained with ethidium bromide for 30min and visualized under UV light.

17.2 5X formaldehyde gel-running buffer

0.1M MOPS (pH 7.0)
40mM Sodium acetate (pH 4.0)
5mM EDTA (pH 8.0): The above were mixed filter sterilized through 0.2µM and stored at RT away from light.

17.3 Reverse transcription PCR (Sambrook et al., 1989)

The first strand cDNA synthesis was carried out by reverse transcribing the total RNA obtained from ripe tomato fruits using RevertAid™ M-MuLV Reverse transcriptase (Bangalore GeNei®, Bangalore).

The reaction mixture for the above are as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>5ng µl⁻¹</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1mM of each dNTP</td>
</tr>
<tr>
<td>Primer: Oligo(dT)$_{18}$</td>
<td>10 pmols µl⁻¹</td>
</tr>
<tr>
<td>RevertAid™ $M$-$MuLV$-RT buffer</td>
<td>5x</td>
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<tr>
<td>RevertAid™ $M$-$MuLV$-RT</td>
<td>10Uµl⁻¹</td>
</tr>
</tbody>
</table>
The reaction volume for reverse transcription was as follows:

- Total RNA: 5.0 µl
- dNTPs: 2.0 µl
- Primer: Oligo(dT)$_{18}$: 2.0 µl
- RevertAid™ M-MuLV -RT buffer: 2.5 µl
- RevertAid™ M-MuLV –RT: 0.3 µl
- Distilled water: 8.2 µl
- Total reaction mixture: 20.0 µl

The total RNA and the primer (Oligo (dT)$_{18}$) (Bangalore GeNei®, Bangalore) were preheated at 70°C for 5 min in a water bath and immediately chilled on ice for 5 min. Before adding the reverse transcriptase enzyme, all the reaction components were incubated at 37°C for 5 min. After adding the reverse transcriptase, the incubation was carried out at 42°C for 1 hr in a Thermocycler (model PTC-100, MJ Research, USA). The reaction was stopped by heating at 70°C for 10 min and cooling immediately on ice. The reaction product was stored at -20°C.

17.4 Polymerase chain reaction (PCR)

The primers used for running the PCR are as follows.

- a) SAM-sense forward 5’-TGT CTC TTT CTT GTT CCC ATT TC -3’
- b) SAM-anti reverse 5’-TGG TTG TAG TAC CGC ACG TC -3’.

Reagents

- cDNA: From reverse transcription
- dNTPs: 1 mM each dNTP
- *Taq* Polymerase: 3 U/µl
- Assay buffer: 10X
- MgCl$_2$: 25 mM
- Forward primer: 10 pmols µl$^{-1}$
- Reverse Primer: 10 pmols µl$^{-1}$
The amplification of cDNA was done in a total reaction volume of 25μl with the following components:

- cDNA 5.0μl
- dNTPs 5.0μl
- MgCl₂ 0.5 μl
- Taq Pol buffer 2.5μl
- Forward primer 1.0μl
- Reverse primer 1.0μl
- Taq polymerase 0.5μl
- Water 9.5μl
- Total reaction mixture 25.0μl

The amplification was performed in a Thermocycler PTC-100 from MJ research Inc., under the following reaction conditions and PCR program given below. The amplified products were resolved on 1.5% agarose gel according to the procedure outlined by Sambrook et al. (1989).

- Hot start /Denaturation 94°C for 5 min
- Denaturation 94°C for 1 min
- Annealing 36°C for 1 min
- Extension 72°C for 2 min
- Final extension 72°C for 10 min

### Evaluation of T₁ generation tomato lines

The T₁ lines from the seeds collected from T₀ lines were grown in green houses. The data on the morphological characteristics like plant height, number of fruits produced, fruit clusters and fruit weight at red ripe stage were collected from each plant. The data of the number of flowers produced per plant was also collected. Similarly, the data of the biochemical characteristics like total soluble solids, titrable acidity and lycopene content were also collected described in chapter I from each of the plant. The putative primary transformants (transformed or non-transformed with γSAMdc gene) of
tomato obtained by *Agrobacterium*-mediated transformation method was subjected to PCR using gene specific primers. The PCR product was resolved on 1.5% agarose gel and 488bp DNA was confirmed running a 500bp DNA ladder.

**Swab test for confirmation of transgenics in T₁ generation**

The putative transformed as well as non transformed plants were grown in pots (22×30cm size) and healthy young leaves were selected at flowering stage. The healthy young leaves in transformed and non-transformed plants were tagged. The leaves were surface disinfected with SDW. Different concentrations of BASTA (0.5 mg L⁻¹, 1 mg L⁻¹, 2 mg L⁻¹ and 4 mg L⁻¹) were swabbed in the adaxial and abaxial surface of tagged leaves of transgenic and non-transgenic plants. Observations were recorded for 2 weeks.
RESULTS

The seed sample of Arka vikas had 100% germination and produced normal seedlings on MS modified A medium (Plate 12b). The transformed A. tumefaciens produced off-white color smooth shiny round colonies on YEP medium appended with kanamycin as an antibiotic marker for bacterial selection (Plate 12a).

Agrobacterium infection and co-cultivation

When a single Agrobacterium colony was transferred to YEP broth, the bacteria multiplied within 12 hours under constant shaking. The bacteria produced a turbid suspension. The bacterial cell concentration was fixed to 0.8 OD at 600nm. The time required for Agrobacterium infection was determined and the best transformation efficiency was observed when the explants were co-cultivated with Agrobacterium for 3 minutes (confirmed by observation of tiny colonies around the cotyledon explants). Increase in incubation time for co-cultivation resulted in the production of dense bacterial colonies around the cotyledons and the control of Agrobacterium growth was difficult later. The addition of acetosyringone improvised the efficiency of transformation. Different concentration, i.e., 50 μM - 100 μM, of acetosyringone was used in the study. One hundred micro molar of acetosyringone was sufficient for good transformation. Addition of cefotaxime to culture media inhibited the growth of Agrobacterium on medium. Among the different concentrations of cefotaxime used in the study (100, 200, 250 and 500 mg ml\(^{-1}\)), 250mg ml\(^{-1}\) considerably decreased the Agrobacterium growth, thereby increasing the transformation efficiency.

Regeneration and selection

The pin-pricked and Agrobacterium inoculated cotyledon explants started producing pale green to creamish colored nodular calli by 10 days. All the explants (co-cultivated and non co-cultivated with Agrobacterium) produced good callus cultures by three weeks (Plate 12d). Some of the sub-cultures resulted in shoot regeneration in MS modified C medium. When the calli were transferred to MS modified D media containing...
BASTA, certain calli started browning symptoms resulting in senescence. On the other hand, certain other calli showed normal regeneration growth. These normally grown unaffected calli and shoot primordia were further inoculated on MS modified E containing higher concentration of BASTA (4mg L⁻¹). This resulted in senescence of some of the escaped shoot on MS modified D medium (Plate 12e). The calli that survived this stringent condition was excised and re-cultured on MS modified C medium (Plate 12f). The shoots were normal in their appearance with a length of 3-6 cms (Plate 12g and 12h). Only shoots with 3cm length were selected and placed on the MS modified F rooting media that produced 100% rooting (Plate 13b). The shoots obtained from positive control (not co-cultivated and not infected with Agrobacterium or BASTA treated) were healthy and produced good roots. The regenerated plantlets from transformed explants also produced good roots similar to that of positive control. In negative control treatment (not co-cultivated but infected with transformed Agrobacterium), the calli failed to regenerate any shoot and started senescing upon transfer to MS modified D and E. The rooted transgenic plantlets survived 100% on the potting medium (Plate 13c). However, only 80% of them survived when transferred to the greenhouse. In case of positive control, all the rooted plants survived when transferred to green house.

The growth hormones played an important role during transformation. The best combination for regeneration and selection was 0.1mg ml⁻¹ IAA and 4 mg ml⁻¹ BAP. However, zeatin showed poor response to calli formation. It was observed, that a combination of 0.5 mg ml⁻¹ IAA+0.5 mg ml⁻¹ BAP showed good rooting.

Molecular characterization of transgenic tomato plants containing ySAMdc gene

The ySAMdc gene was isolated and cloned in Dr. Handa’s laboratory. The E8 fruit inducing promoter was isolated from pE8mutNR that was kindly obtained from Bob Fischer’s laboratory, USA. The entire gene was cloned to PFGC5941 plasmid and was transferred into Agrobacterium strains. The gene sequence had a SAMdc coding region starting from ATG but the primer attaches prior to this region. The one reverse primer was designed to attach with one region to produce a product size of 488bp and the other
a. Colonies of transformed *Agrobacterium tumefaciens* (with transformed plasmid pFGC5941) in YEP agar medium

b. Arka vikas seeds germinating in MS media A

c. Cotyledons explants placed on MS modified C (MS medium+0.1mgL⁻¹ IAA + 4mg L⁻¹ BAP) medium after co-cultivation

d. Profuse callus growth on regeneration MS modified C (MS medium+0.1mgL⁻¹ + 4mg L⁻¹ BAP) medium

e. Senescing of the non-transformed calli perish during MS modified E (MS medium+0.1mgL⁻¹ IAA + 4mg L⁻¹ BAP+ 4mgL⁻¹ BASTA)

f. The surviving transformed calli growing in MS modified E (MS medium+0.1mgL⁻¹ IAA + 4mg L⁻¹ BAP+ 4mgL⁻¹ BASTA)

g & h. Shoot-lets growing in fresh regeneration MS modified C (MS medium+0.1mgL⁻¹ + 4mg L⁻¹ BAP) medium
PLATE 13

a. Putative transgenic shoot-lets on MS rooting media

b. Profuse rooting of the transgenic shoot-lets on MS modified F (MS medium + 0.5 mg L⁻¹ + 0.5 mg L⁻¹ BAP) medium

c. Putative transgenic lines being hardened in the growth chamber

d. Partially hardened putative transgenic lines ready for transplantation into pots

e. The growth habit of certain putative transgenic lines in the greenhouse conditions

f. Putative transgenic plants producing fruits in the greenhouse

g & h. Close up view of fruits of putative transgenic lines of the genotype-Arka vikas
product reverse primer was designed to contain BamH1 site for a full length PCR product at ~1200bp (Fig. 20).

The genomic DNA was isolated from the leaf and fruit samples of full grown plants using activated charcoal-CTAB method (Plate 14a). Essentially, the same methodology was followed for extraction and yield for high quality DNA as described in Chapter-II. In this experiment, the PCR annealing temperature was at 55°C and extension was at 72°C. However, the final extension was 12 min. The DNA bands due to specific SAM primers produced bands with a band size of 488bp (Plate 14b). No amplification was observed in positive control plants.

Out of the 16 transformed plants produced in 2 events, used for PCR reaction, only seven putative transgenic plant lines (AVT2, AVT3, AVT9, AVT6, AVK11, AVT13 and AVT15) produced conspicuous bands at 488bp region there by confirming the introduction of ySAMdc gene. Some of the transformed tomato lines like AVT2, AVT6 and AVT11 showed intense banding pattern as compared to other lines (Plate 14b). However, the DNA extracted from the plasmid produced highly intense bands as compared to the transgenic plants. The non-transformed control plant did not show any band. Further the RNA extracted from ripened fruits of transgenic plants confirmed the transformation when tested on formaldehyde gel (Plate 15a). The first strand cDNA was synthesized using oligo (dT) primers and M-MuLV-RT enzyme. When this was electrophoresed on 1.0% agarose gel, the banding pattern showed a smearing appearance (Plate 15b). Using this as template, ySAMdc specific primers were used to amplify the specific transcripts. Reverse Transcriptase-PCR analysis produced bands at 488 bp regions indicating the expression of ySAMdc gene that was integrated into the transgenic fruits (Plate 15c).

**Evaluation of putative transgenic tomato plants for morphological and biochemical characteristics**

Seven candidate transgenic plants that were exhibiting normal and healthy growth were selected for determining their morphological and biochemical characteristics. The plants from positive control were also selected for comparison. All the seven plants
a. Genomic DNA on 0.8% agarose gel from putative tomato transgenic leaves

b. PCR analysis of seven transgenic tomato plants (T₀ generation) for the presence of \( ySAMdc \) gene (488bp). The amplified products were resolved in 1.5% agarose gel

c. PCR analysis of four transgenic tomato plants of T₁ generation showing the presence of \( ySAMdc \) gene (488bp). The amplified products were resolved in 1.5% agarose gel
PLATE 15

a. Total RNA extracted from the fruits of seven transgenic lines using LiCl₂ method. The RNA extraction was confirmed in formaldehyde gel. C= control ;1, 2, 3, 4, 5, 6, 7 = transgenic lines

b. cDNA copies of transgenic fruit lines by reverse transcriptase-PCR. PC=water control; C=control; 1, 2, 3, 4, 5, 6, 7 = transgenic lines

c. PCR analysis of cDNA copies using gene specific primers to find out the expression of ySAMdc gene into the fruit
received similar culture conditions within the green house. The data of morphological and biochemical characteristics in transgenic tomato lines are detailed on Table 28. The control plants fared better with morphological traits compared to transgenic lines (Fig 21). Biochemical tests indicated that there was 2-3 fold increase in lycopene as compared to the non-transformed plant suggesting the efficient insertion of ySAMd gene. Also, there was a significant increase in the titrable acidity and TSS (Brix°) value as compared to the control (Table 29) (Fig 22).

**Table 28. Morphological characteristics of transgenic tomato lines (T₀) and control (non-transformed) plants**

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Transgenic Lines</th>
<th>Plant height (cm)</th>
<th>No of flowers</th>
<th>No of fruits</th>
<th>Fruit clusters</th>
<th>Fruit weight(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AVT2</td>
<td>106.7</td>
<td>60.0</td>
<td>22.0</td>
<td>3.00</td>
<td>65.80</td>
</tr>
<tr>
<td>2</td>
<td>AVT3</td>
<td>94.50</td>
<td>63.0</td>
<td>21.0</td>
<td>5.00</td>
<td>66.80</td>
</tr>
<tr>
<td>3</td>
<td>AVT6</td>
<td>100.6</td>
<td>62.0</td>
<td>25.0</td>
<td>4.00</td>
<td>73.50</td>
</tr>
<tr>
<td>4</td>
<td>AVT 9</td>
<td>106.7</td>
<td>61.0</td>
<td>30.0</td>
<td>4.00</td>
<td>72.50</td>
</tr>
<tr>
<td>5</td>
<td>AVK11</td>
<td>97.50</td>
<td>79.0</td>
<td>32.0</td>
<td>2.00</td>
<td>69.70</td>
</tr>
<tr>
<td>6</td>
<td>AVT13</td>
<td>112.8</td>
<td>71.0</td>
<td>27.0</td>
<td>4.00</td>
<td>74.10</td>
</tr>
<tr>
<td>7</td>
<td>AVT15</td>
<td>94.50</td>
<td>69.0</td>
<td>36.0</td>
<td>2.00</td>
<td>60.00</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>118.9</td>
<td>61.0</td>
<td>37.0</td>
<td>6.00</td>
<td>72.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>103.62</td>
<td>65.75</td>
<td>28.75</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LSD₀.₀₁</td>
<td>8.74</td>
<td>6.23</td>
<td>7.61</td>
<td>3.35</td>
</tr>
</tbody>
</table>

**Table 29. Biochemical characteristics of transgenic tomato lines (T₀) and control (non-transformed) plants**

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Tomato transgenic</th>
<th>Lycopene (mg 100g⁻¹)</th>
<th>TSS (%)</th>
<th>TA (mg 100g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AVT2</td>
<td>8.63</td>
<td>5.90</td>
<td>0.544</td>
</tr>
<tr>
<td>2</td>
<td>AVT3</td>
<td>8.26</td>
<td>6.80</td>
<td>0.656</td>
</tr>
<tr>
<td>3</td>
<td>AVT6</td>
<td>8.28</td>
<td>5.40</td>
<td>0.512</td>
</tr>
<tr>
<td>4</td>
<td>AVT9</td>
<td>7.81</td>
<td>5.12</td>
<td>0.540</td>
</tr>
<tr>
<td>5</td>
<td>AVK11</td>
<td>8.46</td>
<td>6.40</td>
<td>0.672</td>
</tr>
<tr>
<td>6</td>
<td>AVT13</td>
<td>9.62</td>
<td>5.60</td>
<td>0.544</td>
</tr>
<tr>
<td>7</td>
<td>AVT15</td>
<td>9.73</td>
<td>7.10</td>
<td>0.576</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>3.53</td>
<td>3.20</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>8.04</td>
<td>5.69</td>
<td>0.547</td>
</tr>
<tr>
<td></td>
<td>LSD₀.₀₁</td>
<td>3.23</td>
<td>3.23</td>
<td>0.948</td>
</tr>
</tbody>
</table>
The T₁ generation transgenic lines were also studied for their characteristics. The seeds procured from the fruits of T₀ plants were germinated and the plantlets were transferred to pots containing potting medium (manure, red soil and sand; 1:2:1). The plants were monitored from the date of sowing until fruit maturation. The fruits were harvested in red ripe stage and tested for biochemical characteristics. The information on morphological and biochemical characteristics of T₁ generation plants are mentioned on table (Table 30 and 31). The PCR analysis of DNA from young healthy leaves showed the presence of bands for 4 transgenic plants (AVT2, AVT6, AVT13, and AVT15). There was a significant difference between control and transgenic lines of T₁ generation. (Fig 23 and 24). However, further work at T₂ generation there on could not be incorporated due to lack of time.

| Sl No. | Transgenics Lines | Plant height (cm) | No of flowers | No of fruits | Fruit clusters | Fruit weight (g) |
|-------|-------------------|------------------|--------------|-------------|---------------|----------------|-----------------|
| 1     | AVT2              | 88.392           | 44.00        | 22.00       | 4.00          | 44.30          |
| 2     | AVT6              | 80.772           | 54.00        | 21.00       | 3.00          | 60.80          |
| 3     | AVT13             | 86.489           | 49.00        | 27.00       | 4.00          | 62.80          |
| 4     | AVT15             | 88.392           | 70.00        | 30.00       | 5.00          | 68.10          |
| 5     | Control           | 106.68           | 58.00        | 30.00       | 6.00          | 68.80          |
| Mean  |                   | 90.14            | 55.00        | 26.00       | 4.40          | 61.96          |
| LSD₀.₀₁ |                  | 13.60            | 13.70        | 9.03        | 4.64          | 14.34          |

**Table 30.** Morphological characteristics of T₁ transgenic tomato lines in comparison to control (non-transformed) plants

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>T₁-Transgenic tomato</th>
<th>Lycopene (mg 100g⁻¹)</th>
<th>TSS (%)</th>
<th>TA (mg 100g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AVT2</td>
<td>8.99</td>
<td>5.2</td>
<td>0.590</td>
</tr>
<tr>
<td>2</td>
<td>AVT6</td>
<td>7.10</td>
<td>5.0</td>
<td>0.540</td>
</tr>
<tr>
<td>3</td>
<td>AVT13</td>
<td>8.67</td>
<td>5.6</td>
<td>0.600</td>
</tr>
<tr>
<td>4</td>
<td>AVT15</td>
<td>7.28</td>
<td>5.8</td>
<td>0.492</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>2.78</td>
<td>4.0</td>
<td>0.279</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>6.96</td>
<td>5.12</td>
<td>0.500</td>
</tr>
<tr>
<td>LSD₀.₀₁</td>
<td></td>
<td>6.86</td>
<td>3.64</td>
<td>1.570</td>
</tr>
</tbody>
</table>

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Fig 21. Morphological characteristics like plant height, fruit weight, no of flowers, no of fruits and no of fruit clusters in T₀ transgenic tomato

Fig 22. Biochemical characteristics like TSS, lycopene and titrable acidity in T₀ transgenic lines and control.

Fig 23. Morphological traits like plant height, fruit weight, no of flowers, no of fruits and no of fruit clusters between T₁ transgenic tomato lines

Fig 24. Biochemical characteristics like TSS, lycopene and titrable acidity in T₁ transgenic tomato lines and control
Swab tests for confirmation in T1 generation

Non-transformed leaves showed necrosis in the localized area of application of BASTA (Plate 16a and b) and (Plate 17a and b). However, concentrations of 0.5mg, 1mg or 2mg L⁻¹ of BASTA showed partial browning, while 4 mg L⁻¹ showed complete necrosis of leaves suggesting the plant to be non-transgenic. However, leaves of transgenic plants remained unaffected by different concentrations of BASTA (Plate 16c and d) and (Plate 17c and d).

Transformation efficiency

Transformation efficiency was computed based on the number of explants infected and number of surviving explants in the selection media. The results indicated an average of 18.4% transformation efficiency (Table 31). These results corroborate the genetic transformation protocol developed in this study using ySAMdc gene (Mehta et al., 2002).

Table 32. Efficiency of Agrobacterium-mediated tomato transformation

<table>
<thead>
<tr>
<th>Events</th>
<th>No. of explants infected</th>
<th>No. of explants survived on BASTA media</th>
<th>No. of explants regenerated</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Event 1</td>
<td>60</td>
<td>19</td>
<td>10</td>
<td>16.7</td>
</tr>
<tr>
<td>Event 2</td>
<td>70</td>
<td>23</td>
<td>13</td>
<td>14.2</td>
</tr>
<tr>
<td>Total</td>
<td>125</td>
<td>42</td>
<td>23</td>
<td>18.4</td>
</tr>
</tbody>
</table>
PLATE 16

a. Swab test of control plant before the application of BASTA to the tagged leaf

b. Swab test of control plant with 2mg L\(^{-1}\) BASTA showing senescence of the leaf

c. Swab test of transgenic line before the application of BASTA to the tagged leaf

d. Transgenic leaf swabbed with 2mg L\(^{-1}\) BASTA remained unaffected after two weeks
a. Swab test of control plant before the application of BASTA to the tagged leaf

b. Swab test of control plant with 4 mg L\(^{-1}\) BASTA showing complete senescence within a week

c. Swab test of transgenic line before the application of BASTA to the tagged leaf

d. Transgenic leaf swabbed with 4mg L\(^{-1}\) BASTA remained unaffected after two weeks
DISCUSSION

Genetic improvement of crops has traditionally been achieved through the process of sexual hybridization, which has resulted in the development of numerous cultivars with high yield and superior agronomic performance. The conventional plant breeding, sometimes combined with classical cytogenetic techniques continues to be the main method of crop improvement. However, more recently, through the use of new tools of biotechnology, crossing barriers have been overcome, and genes from unrelated sources have become available via the introduction of genetic transformation methods in plants (Repellin et al., 2001).

Tomato is highly acquiescent to physiological and cytological studies due to ease of culture and genetic uniformity resulting from autogamy (Devi et al., 2008). The tomato which naturally accumulates lycopene in fruits has attracted the attention of many researchers who have manipulated it to increase or diversify carotenoid accumulation. Also, studies related to fruit ripening in tomato have been focussed on. The genetic manipulation of carotenoid biosynthesis in higher plants has been the objective of a number of biotechnology programs. The study of model plants such as tomato and citrus for pigmentation mutants (Galpaz et al., 2006) and transgenic approaches (Giuliano et al., 2008; Fanciullino et al., 2008; Alque' zar et al., 2009) have provided insights into the regulatory mechanism(s) operating in carotenogenesis. The genetic engineering in fruit ripening has received considerable attention because of vivid changes in a range of metabolic processes that occur before and after ripening. Some imperative changes that occur are the production of aromatic compounds and nutrients, colour, and softening of the flesh to an edible texture, which have evolved to attract animals and promote seed dispersal (Giovannoni, 2004). The molecular pathways that underlie many of these ripening-related phenomena have been sufficiently characterized to allow the modification of fruit nutritional status or organoleptic characteristics through targeted genetic engineering (Davulari et al., 2005 and Rodrigo and Zacarias, 2007). The commercial importance of the crop has hastened for more genetic, molecular and physiological studies (Yokotani et al., 2009).
Factors effecting transformation

Transformation efficiency depended on various factors such as cultivar, type of explant, age of explant, *Agrobacterium* strain and its density, co-cultivation period and time, effect of acetosyringone, and dosage of cefotaxim (Wu *et al.*, 2006; Madhulatha *et al.*, 2007; Jabeen *et al.*, 2009). The present study corroborated with these findings.

Selection of tomato genotype

Arka vikas seeds were procured from IIHR, Hessarghatta, Bangalore. This variety is a high yielding crop and commonly grown in Karnataka. Also, from the studies deduced from Chapter-1, Arka vikas was found to exhibit an average response to different parameters of the study. However, from the point of view of organoleptic characteristics, this genotype was highly acceptable to the consumers. A study was conducted on the transgenic tomato in Arka vikas (Shivakumar *et al.*, 2007), however, the fruit quality of this transgenic genotype Was not evaluated. For this reason, this genotype was identified for introducing lycopene gene with good shelf life via *Agrobacterium* mediated transformation.

Type and age of explants

Previous studies have suggested that high shoot forming ability was found in apical meristem region followed by hypocotyl and cotyledon (Yasmeen, 2009). However, Schutze and Weiczorrek (1987) suggested that hypocotyl was not as efficient as cotyledon in generating transgenic shoots and it is not a safe bet to use the latter in the transformation experiment. Although, tomato genotypes have high totipotency, the right choice of explant depended upon the genotype. In this study, cotyledon was used as the explant for transformation.

There was an observation by Hu and Phillips (2001) that the increase in the age of seedlings resulted in the decrease of regeneration capacity. Hu and Phillips (2001) used 8 day-old-plants for callogenesis and regeneration and reported that younger explants are more responsive to callus formation and shoot regeneration. Hamza and Chupeau (1993)
also used 8-10 days-old-explants for regeneration and transformation. However, in the present study, the regeneration capacity was found to be high when 10-12 days-old-explants were used, instead. In contrast to these observants, Dai et al. (1988) reported that the regenerative capacity increased with an increase in the age of explants.

**Agrobacterium cell concentration/density**

In the present study, different concentrations of *Agrobacterium* were tested. Among the optical density, at 600nm, (of 0.5, 0.8, 1.0 concentrations of *Agrobacterium* was used) the OD at 0.8 produced optimum results upon co-cultivation of cotyledon explants with bacterial cells at 48 hours of incubation. An increase in the incubation period caused the over-growth of *Agrobacterium* on explant surface in spite of giving several washes and addition to cefotaxime. The blotting of surface disinfected explants on sterile blotting discs helped in minimizing the growth of bacteria over the explants. The blotting did not affect the callusing ability of the explants.

**Co-cultivation period and time**

Cotyledons were carefully put into the conical flasks containing cell suspension of *Agrobacterium* and swirled for 5 min. The explants did not survive on the selection media when they were co-cultivated for 24 hours whereas the survival ability increased when it was co-cultivated for 2 days. The above findings corroborated with those of the other researchers (Jabeen et al., 2009; Yasmeen, 2009).

Optimization of co-cultivation time was done by keeping the co-cultivation period steady. The transformation efficiency varied with respect to time of co-cultivation. Explants co-cultivated for 1 minute died on selection media as there was hardly any bacterial infection in them, while explants co-cultivated for more than 5 minutes caused excessive growth of *Agrobacterium* which resulted in reduced transformation efficiency. In this study, the cotyledon explants showed good transformation efficiency when immersed in *Agrobacterium* medium for 3 min which is in accordance to studies conducted by Jabeen et al. (2009). However, Wu et al. (2006) and Sumithra et al. (2010) reported that 10 minutes of the inoculation duration in tomato evoked good response.
Effect of acetosyringone

Acetosyringone is an external signal molecule that induces *vir* genes and is also the key factor in *Agrobacterium*-mediated transformation (Guo *et al.*, 1998). Knowledge is available to introduce tDNA by *A. tumefaciens* into recalcitrant host plant (Jones *et al.*, 2005). In the present study, 100 μM acetosyringone treatment gave optimum transient gene expression. Several studies indicated that a concentration of 50-200 μM acetosyringone in the co-cultivation medium is ideal to improve the efficiency of transformation in tomato. Wu *et al.* (2006) and Jabeen *et al.* (2009) have reported that 50 μM acetosyringone was found to be effective for the optimum efficiency of transformation in tomato cv. Lichaň. However, concentration higher than the above was also found to be less effective (Raj *et al.* (2005); Paramesh *et al.*, (2010)). In contrast to the above, Sharma *et al.* (2009) optimized a simple efficient transformation procedure which did not require acetosyringone or suspension feeder plate.

Dosage of cefotaxime

The most commonly used antibiotic chemicals in genetic transformation experiments are cefotaxime, carbenicillin, timentin and vancomycin. An addition of cefotaxime into the medium increased the efficiency of transformation and inhibited bacterial growth. Cefotaxime significantly increased the number of regenerated shoots of apple and strawberry (James *et al.*, 1990). An addition of cefotaxime can improve the frequency of shoot multiplication and transformation (Kaur *et al.*, 2006). When cefotaxime was not added into the medium, high bacterial growth resulted in the death of explants. Among the different concentration of cefotaxime (100, 200, 250 and 500mg L⁻¹) used in the study, the concentration of 250 mg L⁻¹ inhibited the bacterial growth thereby increasing the transformation efficiency. However, at the concentration of 500 mg L⁻¹ cefotaxime, the efficiency of transformation decreased. Studies have shown that concentration above this was detrimental to the explant.

Usage of kanamycin

Kanamycin, an antibiotic, was used as a selective marker for the expression of pFGC5941 plasmid DNA. In the present study, 50μg ml⁻¹ of kanamycin was used. A
study by Sarker et al. (2009) also showed that 50mgL\(^{-1}\) of kanamycin could be used as the selection marker to transform GUS and \textit{npt II} genes via \textit{Agrobacterium} mediated transformation.

\textbf{Usage of BASTA (Ammonium glufosinate)}

BASTA/ Rely/ Finale or Challenge, are the trade names for the ammonium salt, glufosinate-ammonium, which is the product now registered for use in more than 40 countries. It is a broad-spectrum contact herbicide and is used to control a wide range of weeds after the crop emergence or for total vegetation control on land not used for cultivation. In \textit{Agrobacterium} mediated transformation, BASTA was used as a selection marker for BAR gene present in the plasmid. BASTA was used for the selection of transformants at very low concentration of 2 mg L\(^{-1}\) or 4mg L\(^{-1}\). It was used as \textit{bar} gene resistant plant marker. Jones \textit{et al.} (2005) reported the usage of bar gene which confers tolerance to glufosinate ammonium in transformation of wheat. It is reported that under conditions of non-recommended usage of glufosinate ammonium, a detrimental effect on the health of both users and consumers is extremely unlikely (Jewell, 1998).

\textbf{Effects of growth regulated hormones}

MS medium (Murashige and Skoog, 1962) is the most commonly used commercial tissue culture medium and the same was used for the study after optimization of hormone strength and supplementation with the required chemical substances.

The plant growth hormones (PGH) are small organic molecules which elicit defined responses either directly at the site of production in specific organs or tissues where they are synthesized or are transported to other organs and tissues. Each class of PGH affect a large number of developmental processes, and therefore, most developmental processes are controlled by more than one class of hormone (Bhatia \textit{et al.}, 2004). It is accepted that without plant growth regulators, the \textit{in vitro} culture of plants is almost impossible. For tomato regeneration, a wide variety of plant growth regulators have been used at different concentrations and its effect of a particular cytokinin or auxin that is being used affected the regeneration medium.
The standardization of tissue culture medium was taken up for cotyledon explant culture. The transformation efficiency of 18.4% was obtained due to a combination of IAA+BAP. The BAP alone showed extensive growth of callus and shoot regeneration but supported poor transformation. Generally, IAA is used for tomato transformation but cytokinin choice is unresolved (Hamza and Chupcau, 1993). Among the different concentration(s) and combination(s) of IAA+BAP used in the study, 0.1mgL⁻¹ IAA and 4mgL⁻¹ BAP showed high regeneration and transformation efficiency (18.4%). This observation is in accordance to that of Karthikeyan (2009) who reported maximum shoot regeneration of calli derived from hypocotyls (32%) of tomato cv. Nagina in MS supplemented with 4mgL⁻¹ BAP and 0.5mgL⁻¹ IAA. High regeneration efficiency in cotyledons and hypocotyl explants was reported by Velcheva et al. (2005) when 2.5 mg L⁻¹ BAP and 0.1mg L⁻¹ IAA was used. Devi et al. (2008) studied the effect of growth regulators on morphogenesis response of tomato and found that BAP (3.0mgL⁻¹) and IAA (2.5mgL⁻¹) caused better callus induction and shoot generation. Any increase and decrease in the concentration of IAA or BAP showed a significant decline in the plant regeneration and transformation efficiency.

**Optimum condition for PCR**

The DNA extraction by modified CTAB method with activated charcoal was used. The extraction protocol yielded pure DNA. The CTAB is the most popular method used in molecular biology experiments for DNA extraction.

For good banding pattern, 20ng of DNA was recommended (Sambrook, et al., 1989) and the same amount was used in the study. The MgCl₂ content was essential for Taq polymerase activity and 1.5mM (Anon., 2011z) was found to be ideal in the present study. The dNTPs provide nucleotide for the synthesis of new complimentary strand during PCR. 50µM of each dNTP was ideally used for PCR reactions as recommended by Ranjan et al. (2010), and similarly, 1.5U Taq DNA polymerase produced good amplification in the present study. The primer concentration also plays a very important role in PCR. The over usage of primer concentration was shown to result in smearing of
the PCR product (Sambrook et al., 1989). Thus 2.5µL of 10pmol each of forward primer and of reverse primers was ideal for identifying putative transgenics.

The first cycle was designed for denaturation of template DNA at 94°C for 5 min. The cycle consisted of denaturing at 94°C for 45 sec, followed by annealing at 55°C and extension at 72°C for 1 min and primer annealing for 40 cycles. The final extension time was reduced to 10 minutes followed by storage at 8°C. The insertion of the transgenic DNA was confirmed by observing the PCR products separated on 1.5% agarose gel containing ethidium bromide. The gene size was estimated at 488bp by using a DNA ladder (0.1-1Kb and/or 0.5-5Kb).

**Extraction of RNA from fruits**

The RNA preparation is an important step in the functional genomics. There are different methods for extracting RNA from the tissues. Some of the common methods used in extraction of total RNA are TRIzol® reagents (Rosas-Cárdenas et al., 2011), lithium chloride method, guanidine thiocyanate method (Chomczynski and Sacchi, 2006) or CTAB method (Carra et al., 2007). Rosas-Cárdenas et al. (2011) reported that lithium chloride method produced high yield and quality of RNA. The RNA precipitate with lithium chloride increased the stability of RNA and improved the cDNA synthesis. Hence, lithium chloride method was the most preferred method (Sambrook et al., 1989; Manickavelu et al., 2007). Certain critical conditions have to be considered for maintaining the quality of RNA. The disruption of cellular material lead to the release of RNases, that rapidly degrade RNA. Hence, inhibition of RNases was very important. In order to achieve this, the RNase-free consumables such as tubes and pipette tips, reagent bottles, beakers, pestle and mortars were used throughout the purification procedure. These equipments were cleaned with 0.1% DEPC treated water and autoclaved several times. The cleanliness was of paramount importance: the working surface, pipettes, and stands were wiped with diluted bleach followed by 70% (DEPC treated) alcohol. Handling of all the equipments and reagents was done using sterilized hand gloves. The most common method that was used to assess the integrity of total RNA was by running an aliquot of the RNA sample on a denaturing formaldehyde agarose gel stained with
The polymerase chain reaction depends on a DNA polymerase to amplify the starting template of DNA. Therefore, to quantify the gene expression, cell or tissue RNA was first converted to a DNA template before amplification could occur. For this, reverse transcriptase was used to translate information in mRNA to DNA. The primer used for cDNA synthesis was Oligo (dT). It consisted of short strands (usually ~18 bases long) of thymine deoxynucleotides. These oligo-T primers were used to prime reverse transcription giving rise to 3'-end. The reverse transcriptase-PCR reaction produced complementary DNA (cDNA) copies of the different RNA molecules in the sample. The reverse-transcriptase procedure consisted of RNA denaturation step (heated at 70°C) followed by quick cooling on ice and addition of the reverse transcriptase enzyme. This enzyme extends the target site of poly A tail of mRNA at 3'-end producing RNA-DNA hybrid molecule; cDNA of the mRNA strand. Since the 3'-end of the cDNA single strand is used with a primer in the reaction, a short hairpin loop is generated at this end. This hairpin loop gets cleaved by a single-strand-specific nuclease to yield a regular DNA duplex (Singh, 2010a). The reverse transcriptase enzyme buffer and dNTPs was added for synthesis the cDNA strands. Once the reaction mixture was ready, it was subjected to incubation at 42°C in a thermocycler for one hour and the reverse transcriptase enzyme was deactivated by heating to 70°C and snap chilling on ice. The cDNA resulting from the reverse transcriptase-PCR reaction was used as template. The gene specific primers used were SAM-sense forward primer 5'-TGT CTC TTT CTT GTT CCC ATT TC -3' and SAM-anti reverse primer 5'-TGG TTG TAG TAG CGC ACG TC -3'. The thermocycling conditions were hot start denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min. The PCR product obtained from cDNA was run on 1.5% agarose gel containing ethidium bromide (0.5μg ml⁻¹). The expression of ySAMdc gene was confirmed by observing the band having 488bp using a DNA ladder/marker (0.1-1Kb and/or 0.5-5Kb). The gene expression is measured by the quantification of cDNA converted from a mRNA corresponding to this
gene. The conventional method using normal PCR condition to the cDNA is undisputable (Sambrook et al., 1989). However, recent methods using SYBR green PCR master mix (real time-PCR), reverse transcriptase -PCR kits is less time consuming which gives good clear bands, amplifying even the small amount for expression studies of RNA. The northern blot technique could be incorporated using radioactive probes, however, in the present study, this technique could not be employed.

Many attempts have been made to study the fruit quality and the increase in carotenoid level in tomato. Apel and Bock (2009) demonstrated the potentials of plastid engineering to enhance the lycopene and β-carotene levels that in turn influences the accumulation of total carotenoid accumulation in tomato fruits. The genes involved in the fruit quality and in defining the molecular basis of texture, flavor, color and aroma have been identified (White, 2002). The expression of an antisense RNA gene in genetically engineered fruit reduced the enzymatic activity of pectin methylestrase by 95% (Janick, 1993). To increase the shelf life of tomato (Flavr Savr) fruit antisense RNA technology was introduced in 1994. The key enzymes involved in fruit softening and the genetic regulatory factors that influence the fruit texture and shelf life in tomato are being characterized (Seymour et al., 2002). Meli et al. (2010) studied the suppression of N-glycan processing enzymes, α-mannosidase and β-D-N-acetylhexosaminidase members of glycosyl hydrolase families 38 and 20, which were isolated and the functional characterization to produce enhanced fruit shelf life in tomato was determined. An antisense gene for pectin methylestrase in tomato was introduced to create high solid fruits.

*Agrobacterium* mediated plant transformation of tomato with ySAMdc gene resulted in the accumulation of higher polyamines, spermidine, and spermine during tomato fruit ripening. These polyamines in turn increased the shelf life of tomato by 2-3 folds due to increase in lycopene content that enhanced the juice and nutritional quality. In addition, the high spermidine-spermine tomatoes have high fructose/glucose and sugar/acid ratio (Srivastava et al., 2007; Mattoo et al., 2010). Lycopene is considered to be the most effective singlet oxygen quencher (Cantrell et al., 2003). The protective effects of lycopene have been shown on oxidative stress, cardiovascular disease, hypertension, atherosclerosis, cancers, diabetes and others (Kong et al., 2010).
Effect of ySAMdc gene

S-adenosyl methionine is a precursor for the biosynthesis of both ethylene and polyamines, and its utilization into both pathways is not reversible. A competition that exists between polyamine and ethylene biosynthesis for utilization of SAM has been postulated to explain some of their antagonistic relationships in plant growth and development (Quan et al., 2002). During the ripening process, the levels of spermidine and spermine decline in fruits. Thus the enzyme SAMdc catalyzes decarboxylation of SAM, with the product (SAMdc) donating the aminopropyl moieties needed for the biosynthesis of spermidine and spermine at the cost of putrescine (Liu et al., 2006; Srivatsava et al., 2007). SAMdc belongs to a small class of decarboxylases which uses a covalently bound pyruvate as prosthetic group. Polyamines, especially spermidine and spermine, that compete with ethylene for the common substrate, SAM, makes it plausible to modulate post harvest fruit development. Although the inverse relationship between polyamine and ethylene is present in many species, it is supposed that such a relationship is only true when the polyamine levels are high enough to affect ethylene production.

The result of the present study indicated that molecular changes also influenced the agronomic and biochemical traits. The transgenic lines showed the decrease in the plant height, fruit weight and number of fruits than in non-transgenic lines (Mattoo et al. 2007; Neelam et al. 2008) The slight reduction in fruit weight was attributed probably due to high levels of metabolism and decreased the sugar content (Mattoo et al. 2007; Neelam et al. 2008). Pedro et al. (1999) observed accumulation of SAMdc gene that produced small tubers in potato. However, an over-expression of ySAMdc gene in tomato resulted in the enhancement of polyamine content. The simultaneous two to three fold increase in lycopene and high titrable acidity and TSS values as compared to non-transgenic lines in T0 and T1 generation (Table: 30, 31, 32, 33). These results are in accordance to the work done by Mehta et al. 2002; Srivastava et al. 2007 and Neelam et al. 2008).

In the present study, the ySAMdc gene of 488bp was successfully incorporated into the tomato explants using Agrobacterium mediated transformation. There was a significant increase in lycopene levels, high TSS and titrable acidity as compared to control. The expression study using conventional method of RNA extraction and reverse transcriptase PCR further confirmed the expression of transgenic lines.
CONCLUSION

Agricultural innovation can have a huge potential to address some of the requirements for mankind in medicine, agriculture and food. It has an impending impact for developing crops of improved nutritional quality and crops that will grow under previously inhospitable conditions thereby contributing to alleviating hunger and malnutrition. This could help to prevent the otherwise inevitable future pressure to encroach on natural resources.

Taking necessary precautionary measures, the present study on *Agrobacterium* mediated transformation of ySAMdc gene was performed. A transformation efficiency of 16.7 and 14.2% was achieved in two batches, respectively. The gene of interest was 488bp and was confirmed by running a DNA ladder during gel electrophoresis. Further test for confirmation was done by foliar application of BASTA, a powerful herbicide, to the leaves of transgenic and control plants. Putative transgenics showed significant difference at 99% probability levels with 2-3 fold increase in lycopene, high TSS and titrable acidity levels when compared to control. The RNA work was initiated with reverse transcriptase- PCR only. This suggests that the gene action of ySAMdc using E8 fruit inducing promoter is only limited to the tomato fruit. Recent techniques such as real time PCR using SYBR green dye or northern blot techniques could throw light on gene action. Apart from confirming the integration of ySAMdc gene in T1 generation, expression studies of the gene could not be taken up. However, morphological and biochemical observations were satisfactory with no drastic changes in T1 generation as compared to T0 generation.

Further work on gene sequencing, analysis of gene expression, variation, protein work and observation of the physiological changes over the generations could be taken up for a greater understanding. The validation, in T-2, T-3 generations of transgenics can be looked into for segregation patterns, stability and expression of the desired gene of interest.