TRANSFORMATION OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) WITH *ARABIDOPSIS* EARLY FLOWERING GENE *APETALAI* (*API*) THROUGH *AGROBACTERIUM* INFILTRATION OF RIPENED FRUITS

M. HASAN[1], A. J. KHAN[2], S. KHAN[3], A.H.SHAH[4], A. R. KHAN[4] AND B. MIRZA[5]

[1]*G.P.G.C. Bannu*
[2]*Gomal Centre of Biotechnology and Biochemistry, Gomal University, D.I. Khan, Pakistan*
[3]*Kohat University of Science and Technology, Kohat.*
[4]*Department of Biological Sciences, Gomal University, D. I. Khan,*
[5]*Faculty of Biological Sciences, Quaid-e-Azam University Islamabad, Pakistan.*

Abstract

In order to develop an easy, rapid and efficient protocol for the genetic transformation of tomato (*Lycopersicon esculentum* Mill.), without involving tissue culturing, ripened fruits of tomato were infiltrated with *Agrobacterium* harboring plasmid pROKII *GUSin*t *AP1*. The plasmid contains *Arabidopsis* early flowering gene *AP1, GUS* reporter gene and *NPTII* as a selectable marker gene. Both transient and stable *GUS* expressions were studied with different parameters such as various incubation periods (48, 72 and 96 hours) and tissue type effect.

The effects of infiltration on germination of seeds both on selection and non-selection media were studied. The percentage of germination of seeds on non-selection medium was 14% lesser than control, while the germination percentage on selection medium was significantly higher for treated seeds (65-74%) as compared to control (5%), with 48 hours incubation period having maximum (74%). Among kanamycin resistant plants 94.9% to 87.9% showed stable *GUS* expression, while 5.1% to 12.07% were escapes. Transient *GUS* expression exceeded stable *GUS* expression. Different tissues of the same plant showed variable stable *GUS* expression (data not shown here). The effect of incubation period was highly significant (p<0.05), with 48 hours incubation period having maximum efficiency (68%). The effect of tissue type on both transient and stable *GUS* expression was also prominent. Overall, high transformation efficiency 68% to 54% was obtained in seedlings raised from seeds collected from infiltrated fruits. Transformation was confirmed by analyzing the PCR amplified product of *AP1*, *GUS* and *NPTII* genes. This depicted a novel method of transformation in plants in general and tomato in particular.

Introduction

Tomato (*Lycopersicon esculentum* Mill) is a member of the Solanaceae family. Its entire genome consists of 12 chromosomes. Genetic transformation of plants occurs naturally. However, for any genetic engineering program to become successful, the factors need to be optimized for the particular system are; receptiveness of tissue to foreign DNA integration, availability of vectors and screen-able markers, regeneration of intact plant from transformed tissues (Ruppert, 1999, Hooykaas *et al*., 1992).

Transformation is basically of two types; 1) protoplast based transformation, in which the desirable gene is transferred into the protoplast in the presence of calf thymus carrier DNA and polyethylene glycol. These have successfully been used by many workers initially, and transgenic plants have been produced (Shimamoto *et al*., 1989, Datta, *et al*., 1990); 2) non-protoplast based transformation, which, unlike the use of *Agrobacterium*, is plant genotype-independent and relies upon the bombardment of accelerated DNA coated on any inert material (micro-projectile) into target cells. The

most commonly used instruments for accelerating DNA coated particles are those powered by burst of helium generated by a rupture membrane mechanism (Kikkert, 1993), or by a shock wave generated by high voltage discharge through a watered droplet (McCabe & Christou, (1993).

Genetic transformation can be transient or stable, and transformed cells may or may not give rise to gametes that pass genetic material on to subsequent generations. Transformation of protoplasts, callus culture cells, or other isolated plant cells is usually straightforward and can be used for short-term studies of gene function (Gelvin & Schilperoort, 1998). The manipulation of tomato fruit through genetic engineering is reasonably advanced. The genes involved in fruit quality and in defining the molecular basis of texture, flavor, color and aroma have been identified (White, 2002). Key enzymes involved in fruit softening and the genetic regulatory factors that influence fruit texture and shelf life in tomato are being characterized (Seymour *et al*., 2002). To increase the shelf life of tomato (Flavr Savr) fruit antisense RNA technology was introduced. An antisense gene for pectin methylestrase, in tomato has been introduced to create high solid fruits. Expression of an antisense RNA gene in genetically engineered fruit reduced the enzymatic activity of Pectin methylestrase to up to 95% (Janick, 1993).

Strategies for the manipulation of tomato fruit through genetic engineering to increase the levels of the carotenoids ß- carotene and the lycopene have been described (Bramley, 2002). Recently, an *Arabidopsis CBF1* gene was used to generate tomato plants with increased chilling tolerance and increased disease susceptibility. Salt resistant tomato which can grow up to 200mM NaCl has been developed by the modification of a trait that encodes a protein that exports excess salt to vacuole before it damages the tissues (Hsieh *et al.,* 2002, Zhang & Blumwald, 2001).

The current protocols used for tomato transformation are based on shoot regeneration from leaf disk/cotyledon tissue co-cultivated with disarmed *Agrobacterium tumefaciens* harboring binary vector (Fillatti *et al*., 1987). The efficiency of such procedures is generally low because most of the transformed leaf cotyledon cells could not develop into shoots (Hamza & Chupeau, 1993). *Agrobacterium* mediated transformation in tomato has been studied on different media and conditions have been optimized (Park *et al*., 2003).

Arabidopsis transformation without tissue culture was carried out in which *Agrobacterium* was applied, plants were grown to maturity in the absence of any selection and the progeny seeds were collected and germinated on antibiotic containing media to identify transformed plants (Bechtold *et al*., 1993).

Ovule transformation was convincingly demonstrated when constructs containing a *GUS* marker gene were used to document sites of delivery of T-DNA and the additional promoters used for *GUS* fusions were *Arabidopsis* ACT11 (Ye *et al.,* 1999, Desfeux *et al*., 2000).

Agrobacterium-mediated transient assays for gene function are increasingly being used as alternatives to genetic complementation and stable transformation. Transient expression provides a rapid method for assaying the functions of some types of genes. The range of genetic and physiological factors affecting transient expression following *Agrobacterium* infiltration has been analyzed and a protocol for efficient and routine transient assays in several plant species has been developed (Wroblewski *et al.,* 2005). The durability of expression could be enhanced by the co-expression of viral silencing suppressors. Future enhancement of assays for transient gene expression will probably involve optimization of plant's physiological condition, manipulation of factors

promoting T-DNA transfer, optimization of transcription and the application of silencing suppressors. For the screening of transformed cells, various bacterial indicator genes have been developed, which allow selection or screening of transformed cell (Voinnet *et al*., 2003, Klee *et al*., 1997).

The aims of this project were; To develop an easy, rapid and efficient protocol for transformation, without involving tissue culturing, of tomato with *Agrobacterium tumefaciens* strain EHA105 containing pROK AP1 *GUSint* NPTII plasmid.

1. To study the effect of various factors on transformation efficiency.
2. Production of transgenic tomato plants with *Arabidopsis* early flowering gene (*AP1*).
3. Molecular analysis of transgenic plants.

Material and Methods

Fresh, healthy and mature fruits were rinsed thoroughly with tape water and dried with blotting paper. The construct pROKII *AP1 GUSint* was generated by cloning cDNA sequences (1.2 kb fragment) of the *Arabidopsis APETALA (AP1)* gene into the Sma1 restriction site of vector pROKII under the control of Cauliflower Mosaic Virus 35S promoter (CaMV 35S). The construct was verified by DNA sequencing and its functionality has been tested on *Arabidopsis* plant. This vector was incorporated into *Agrobacterium tumefaciens* EHA 105 strain for further use in genetic transformation. The plasmid pROKII *AP1 GUSint* contains *GUS* gene along with *NPTII* gene as a selection marker. The *Agrobacterium tumefaciens* strain EHA105 containing plasmid pROKII *AP1 GUSint NPTII* was used for the transformation**.**

Agrobacterium was grown for three days (72 hours) in Luria Broth Base (LB) medium (tryptone 1% (w/v), yeast extract 0.5 % (w/v) and Sodium chloride 1% (w/v)) (Appendix-II). pH of the medium was adjusted to 5.7. Medium was supplemented with rifampicin 40 mg/l and kanamycin 50 mg/l. Antibiotics were added to cold medium after autoclaving under laminar flow hood. Inoculum was taken from glycerol preserved cultures stored at –20. After inoculation the culture was maintained at 28°C and 130 rev/min in shaking incubator. Surface sterilization was done by washing with 0.1% aqueous Mercuric chloride solution for 1 minute. Fruits were then rinsed thrice with autoclaved distilled water and dried with sterilized blotting paper. For inoculation of fruits with *Agrobacterium* culture 1 ml sterile hypodermic syringes were used to evenly inject the culture throughout the fruit. 1 ml of the above mentioned culture was injected in each fruit. Fruits were incubated at 28°C for 48, 72 and 96 hours incubation periods. For each incubation period 15 fruits, three replications were selected and placed in already cleaned and autoclaved 500 ml beaker inside the laminar flow hood.

Transient *GUS* assay was performed to observe the efficiency of transformation in various tissues of fruit and to observe transient expression of *GUS* gene under the control of constitutive promoter (CaMV 35S Promoter). Histochemical *GUS* assay was carried out to study the effect of incubation time on transformation efficiency in various fruit tissues. For histochemical GUS assay, fruit samples of mesocarp, base and tip of fruit, center of columella and seeds were dipped individually in GUS solution and incubated at 37°C for 24 hours. The samples were studied for 48 hours, 72 hours and 96 hours incubation.

Transverse sections of fruit were observed to analyze relative transient *GUS* expression in different tissues of the fruits. Germination medium was sterilized at 121°C, 15 psi and sealed with aluminium foil and for selection medium, kanamycin was added at

the rate of 100mg/L to cold medium before solidification, autoclaved and sealed with aluminium foil. To observe the effect of *Agrobacterium* treatment on the germination of seeds, as compared to control (untreated), seeds were inoculated on non-selection medium. Seeds were collected from *Agrobacterium* injected fruits and air-dried on blotting paper and sterilized before inoculation on germination and selection medium. For germination of seeds on selection medium, seeds were inoculated and kept in dark for three days at 25±2°C. Germinating seeds were then transferred in growth chamber. After 28 days the plants were transferred individually to small pots containing soil. Acclimatization was done for 15 days.

For stable *GUS* assay, stem, leaf, cotyledon and root tissues from 45 days old kanamycin resistant plants were assayed. They were incubated for 24 hours at 37°C and observed in stereo microscope (400 X magnification). The plant showing stable *GUS* expression in all the three tissue types i.e. stem, root and leaf was considered transformed. Genomic DNA from plant leaves was extracted by non-organic method and stored at –20°C

For the confirmation of the transformation and integration of the *NPTII*, *GUS* and *AP1* gene molecular analysis was carried out through PCR. For PCR analysis genomic DNA was isolated from transformed seedlings, and plasmid DNA was also isolated from the strain of *Agrobacterium tumefaciens* EHA 105 harboring *pROKII AP1 GUSint NPTII* plasmid by chloroform-isopropanol method and stored at –20⁰C**.** Minipreparations of plasmid DNA were obtained by alkaline lysis method.

Polymerase chain reactions for the detection of *NPTII, AP1* and *GUSint* genes were performed following the standard method (Hsieh *et al*., 2003). PCR products were electrophresed on 2% agarose gel, containing ethidium bromide and visualized with UV transilluminator (Life Technology, U.S.A.)

NPTII gene was detected using forward primer5$^/$AAGATGGATTGCAC GCAGGTTC-3$^/$ and reverse primer, 5$^/$- GAAGAACTCGTCAAGAAGGCGA-3$^/$. PCR reaction was performed using 0.3µg DNA, 0.25µg of each of the forward and reverse primer, 0.2mM of each of dNTPs, 2 mM MgCl$_2$ and 2 units of Taq polymerse and 5µl of 10X PCR buffer. The reaction was carried out through 37 cycles that consisted of 30 seconds denaturation at 95°C, 30 seconds annealing at 54°C and 30 seconds extension at 72°C, while in the last cycle, extension was done at 72°C for 10 minutes and in the 1st cycle, denaturation was done at 95°C for 5 minutes. The amplified product of 781bp was obtained.

AP1 gene was detected using the forward primer 5$^/$-TAGACAAGTGA CATTCTCGA-3$^/$ and the reverse primer 5$^/$-GAAGCAGCCAAGGTTGCAGT-3$^/$. The reaction was carried through 37 cycles that consisted of 30 seconds denaturation at 95°C, 30 seconds annealing at 47°C and 30 seconds extension at 72°C, while in the last cycle, extension was done at 72°C for 10 minutes and in the 1st cycle, denaturation was done at 95°C for 5 minutes. The amplified product of 712 bp was obtained.

GUS gene was detected using the forward primer 5$^/$-AACTGGACAAGGC ACTAGCGG-3$^/$ and reverse primer 5$^/$- TGCGACCTGACCGTACTTGAA-3$^/$. The reaction was carried through the same 37 cycles with the same denaturation, annealing and extension periods as stated for above NPII gene, but the amplified product of 1100 bp was obtained.

PCR products were electrophoresed on 2% agarose gel containing Ethidium bromide and visualized with UV transilluminator (Life-Tech, USA).
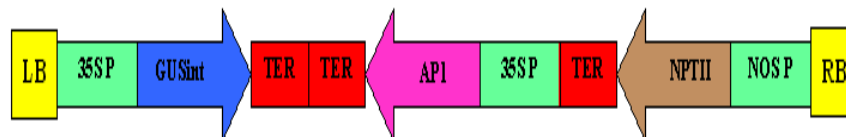
Fig. 1. Schematic diagram of the T-DNA region of transformation vector pROKII *AP1 GUSint*.
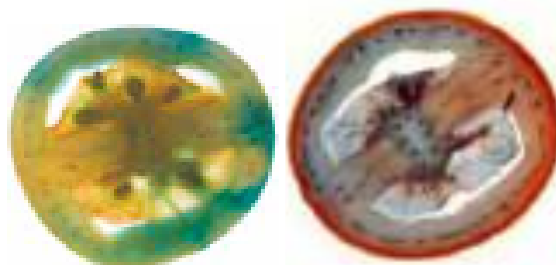

Fig. 2. T.S of fruits showing transient *GUS* expression.

Results

In order to develop rapid, easy and efficient protocol for transformation of tomato, without involving tissue culturing, ripened fruits of tomato were infiltrated with *Agrobacterium tumefaciens* harboring plasmid pROKII (Fig. 1) carrying *Arabidopsis* early flowering gene *APETALA1* (*API*). Seeds collected were germinated on kanamycin selection medium and the plants raised were analyzed for stable transformation.

Mature fruits injected with *Agrobacterium* culture were sampled for the transformation efficiency in various tissues after different incubation periods. Transient *GUS* expression was observed in transverse section of fruits, which were previously injected and incubated with *Agrobacterium* culture (Fig. 2).

Co-cultivation has highly significant effect on the percentage of transient *GUS* expression in different tissues of tomato fruits (Tables 1 & 2). The highest value was observed for 48 hours incubation period (81.107%) followed by 72 hours (68%) and 96 hours (61.333%) respectively (Fig 3), however, the effect of tissue type on percentage of transient *GUS* expression was non-significant. Highest percentage of transient *GUS* expression was observed in tip of the fruits (75.556%) followed by fleshy epicarp (73.333%), loculus of the fruit (68.889), center of the columella (68.511%) and base of the fruit (64.444%) respectively.

The interaction effect of incubation period and tissue type on percentages of transient *GUS* expression was non-significant (p>0.05) (Table 3). However a range of values of percentages of transient *GUS* expression was observed for different incubation period and tissue type combinations. Highest value of 86.667% transient *GUS* expression was observed for the tip of the fruit incubated for 48 hours while lowest value of 53.333% was observed for the base of the fruit incubated for 96 hours. Seeds collected from *Agrobacterium* injected fruits showed that highest *GUS* expression was observed for 48 hours (88%) followed by 72 hours (78%) and 96 hours (76%) incubation periods. There was no marked difference in the later two incubation periods (Table 2).

Table 1. ANOVA table showing effect of *Agrobacterium* incubation period, tissue types and their interaction on transient *GUS* expression.

Source of variation	df	SS	MS	F-value	P-value
Incubation period (A)	2	3036.069	1518.035	21.2705	0.0000
Tissue type (B)	4	685.65	171.412	2.4018	0.0719
AB	8	179.833	22.479	0.315	
Error	30	2141.04	71.368		
Total	**44**	**6042.592**			

Table 2. Transient *GUS* expression in seeds from *Agrobacterium* injected fruits.

Incubation period	No. of seeds assayed	*GUS* + ve	*GUS* %
48 Hours	50	44	88
72 Hours	50	39	78
96 Hours	50	38	76

Table 3. Germination of seeds from *Agrobacterium* injected fruits.

Treatment	No. of seeds inoculated	Germination on non-selection medium	Germination on selection medium	Survival rate on kanamycin*	Transformation %$^\psi$
Control	100	96	5	0	0
48 Hours	100	82	74	72	68
72 Hours	100	83	65	63	54
96 Hours	100	82	66	64	56

Table 4. ANOVA table for stable *GUS* assay in kanamycin resistant seedlings.

Source of variation	df	SS	MS	F-value	P-value
Incubation Period	2	446.263	223.131	13.642	0.0000
Error	33	539.737	16.356		
Total	**35**	**986.000**			

Efforts were made to get stably transformed plants from the seeds of *Agrobacterium* injected fruits. Seeds collected from *Agrobacterium* infiltrated fruits and non-infiltrated fruits were inoculated on ½ MS medium without Kanamycin. The germination percentage was 96% in untreated seeds (control) while it was 82% in seeds incubated for 48 hours and 96 hours and 83% with 72 hours incubation period (Table 3). Control showed higher germination percentage than treatments, which shows prominent effect of *Agrobacterium* treatment on germination. The germination percentage on selection media with kanamycine was high for 48 hours incubation period and lowest for 72 hours while 96 hours incubation showed similar effect to 72 hours incubation period. Survival rate of seedling on selection medium was noted after 28 days. The survival rate ranged from 72 to 63% for seeds from injected fruits. It was highest for 48 hours incubation period followed by 96 and 72 hours incubation periods. The survival rate was 0 % for control seedlings (Table 3).

Among the 100 seeds collected and germinated for producing stably transformed plants 48 hours incubation resulted in maximum transformed plants (68%), followed by 96 hours (56%) and 72 hours (54%). The later two incubation periods had no marked different impact on stable transformation efficiency (Table 3).

Stable *GUS* assay was made after 45 days of inoculation of seeds. The various parts assayed for stable *GUS* expression were cotyledons, leaves, stem and roots. Different incubation periods showed highly significant (p>0.01) impact on the stable transformation in germinating seeds. Maximum transformation was recorded for 48 hours (Table 4) followed by 72 hours and 96 hours, which were non-significantly different from each other and significantly different from 48 hours incubation period. In the same plant some tissues showed *GUS* positive while other gave no expression. Stable *GUS* expression efficiency in kanamycin resistant plants ranged from 95 to 88%. It was highest for 48 hours incubation period while there was no marked difference for 72 and 96 hours incubation periods (Fig. 4). Cotyledons and leaves showed highest *GUS* assay followed by root and stem.

For molecular analysis of transformed plants the PCR products, after running through gel electrophoresis, were analyzed under U.V. illumination and the amplified product of *NPT11* (781 bp)*, AP1* (712 bp) and *GUSint* (1100 bp) genes were observed in DNA collected from transformed plants. No amplification in PCR product was observed in genomic DNA obtained from plants raised from untreated seeds. Amplified product was also obtained in the plasmid DNA.

Discussion

The current protocols used for tomato transformation are based on shoot regeneration from leaf disk/cotyledon tissue co-cultivated with disarmed *Agrobacterium tumefaciens* harboring binary vector (Dominguez *et al*., 2000). The efficiency of such procedures is generally low (Frary & Earle**,** 1996) because most of the transformed leaf cotyledon cells do not develop into shoots.

Germ-line transformation is the common feature that allows avoidance of tissue culture and regeneration in the vacuum infiltration, seed transformation, in plant transformation, and floral-dip methods. Previous studies have shown that T1 transformants are typically hemizygous, carrying T-DNA at only one of two alleles of a given locus (Clough & Bent, 1998). The results indicate that female tissues such as developing ovules within the gynoecium of young flowers are the primary targets of *Agrobacterium*-mediated floral-dip transformation of *Arabidopsis* (Spolaore *et al*., 2001). This study has been carried out to develop an easy, efficient and rapid protocol for the genetic transformation of plants in general and tomato in particular, without involving or at least minimizing tissue culturing, with *Agrobacterium tumefaciens* harboring pROKII *AP1 GUSint* plasmid through infiltration of fruits. The effect of different incubation periods and tissue types on transformation efficiency was also studied and 56-70% transformation efficiency was obtained. These results are very encouraging and depict a novel method of transformation (Fig. 5).

Analysis of variance showed a highly significant effect (p<0.01) of the length of co-cultivation period on the percentage of transient *GUS* expression in different tissues of tomato fruits (Table1). Maximum transient *GUS* activity was observed in tomato fruits incubated for 48 hours. These results are in agreement with that reported earlier, where maximum *GUS* activity was observed in commercially ripened fruits two days after fruit injection with *Agrobacterium* culture, as compared to 3 and 5 days incubation period. Transient assays are usually performed 48 hours after *Agrobacterium* inoculation or particle bombardment (Spolaore *et al*., 2001, Onde, *et al*., 2001), which, this study gave the best results.
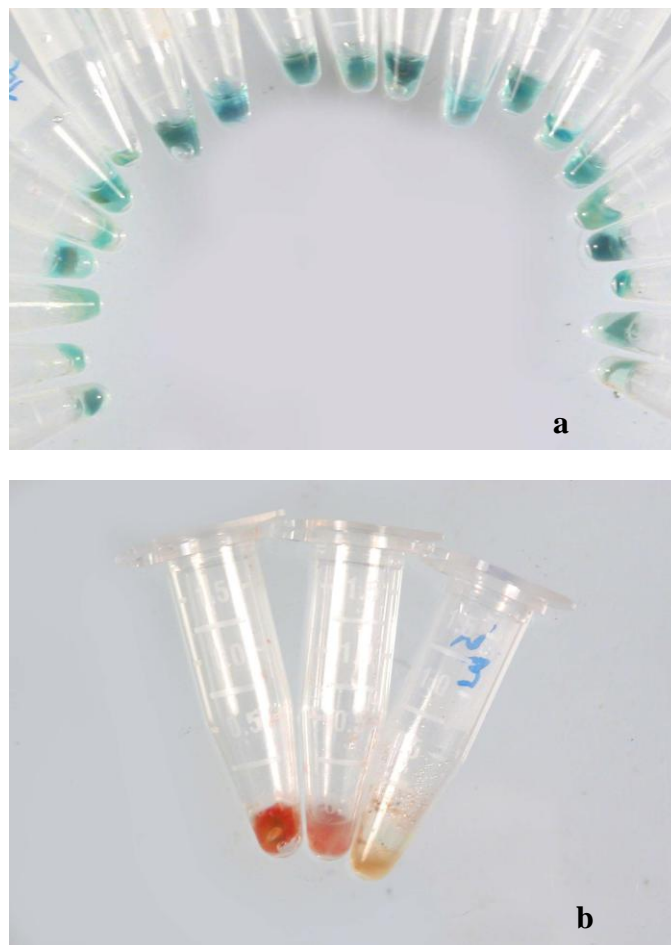
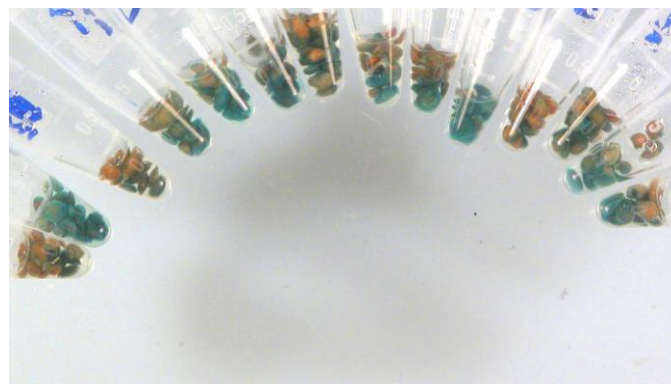Fig. 3. (a) Transient *GUS* expression in different tissues from *Agrobacterium* injected fruits, (b) Control.

Fig. 4. Transient *GUS* expression in seeds from *Agrobacterium* injected fruits.
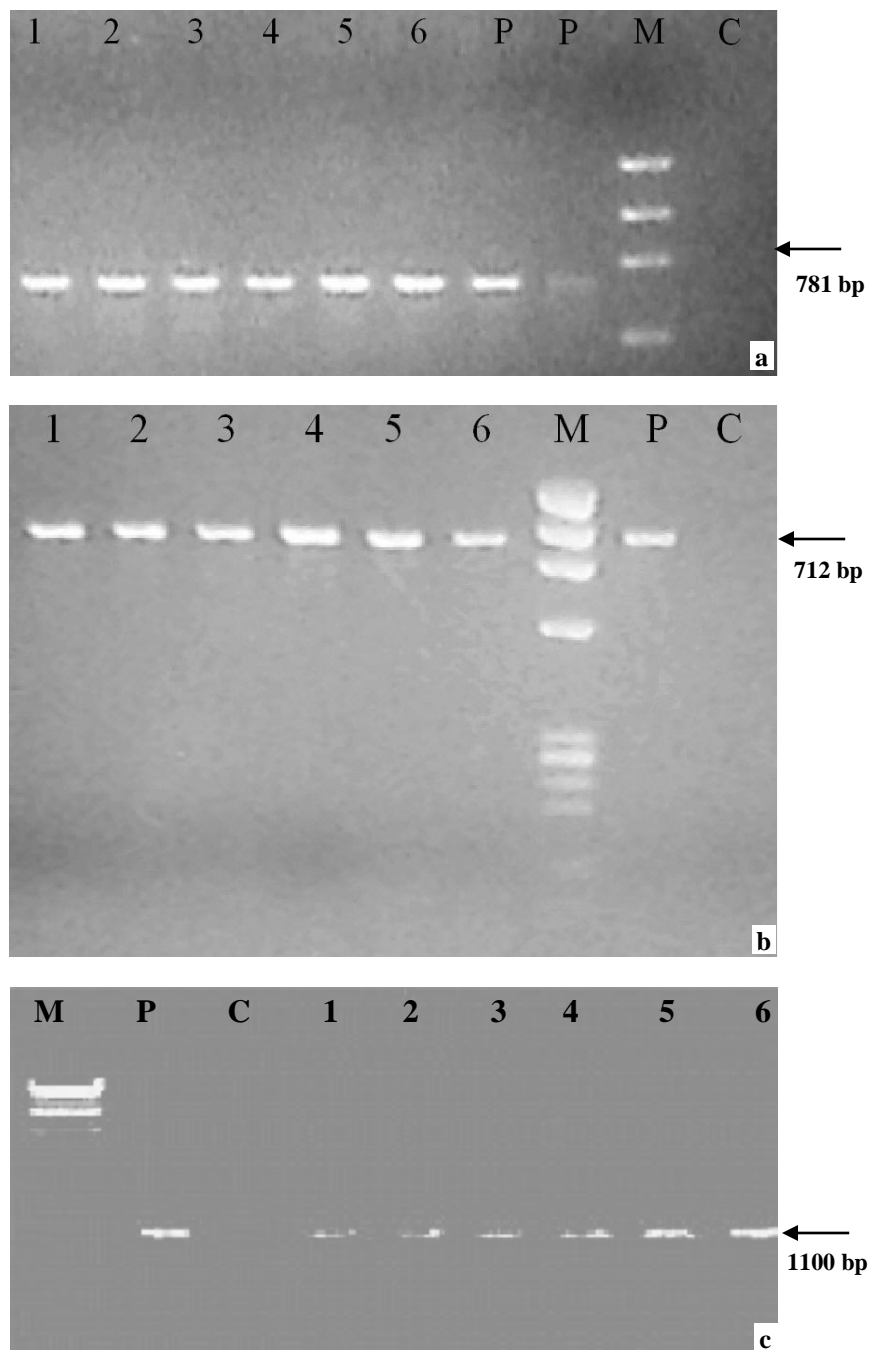
Fig. 5.Amplified product of *NPT11* gene (a); *AP1* gene (b); *GUSint* gene(c), control (C) showed no amplification of transgene. Numbers 1-6 represent transgenic lines; P – plasmid; M – marker.

One possible explanation is that in tomato β-glactosidase II is produced during fruit ripening and that its concentration is maximum at the breaker stage of ripening and progressively decreases in later stages. As the enzyme also acts on X-gluc it is probable that it may have some supplementary effect on transient *GUS* expression and in our experiment the relative efficiency for different incubation periods may have some positive correlation with this enzyme (Smith *et al*., 1998).

Another possible explanation for the significant ($p<0.01$) decrease in the percentage of transient *GUS* expression with the length of time might be attributed to the detrimental effects of low pH inside the fruit on the activity of *Agrobacterium.* The activation of some other genes expression inside the host or bacteria cells that might prevent stable integration of our target DNA or *GUS* expression or both could not be excluded completely (Spolaore *et al*., 2001, Smith *et al*., 1998).

The effect of tissue type on percentage of transient *GUS* expression was non-significant ($p>0.05$) as evident from analysis of variance (Table 3). However it was significant at 92% confidence level ($p=0.0719$). Highest percentage of transient *GUS* expression was observed in tip of the fruits (75.556%) followed by fleshy epicarp (73.333%), loculus of the fruit (68.889), center of the columella (68.511%) and base of the fruit (64.444%) respectively. These variations may be due to the differences in micro-environment inside different tissues of the fruits which would directly or indirectly effect transient *GUS* expression. As reported earlier that the relative efficiency of transient gene expression is usually consistent from ecotype to ecotype, tissue to tissue and many other physiological and environmental conditions. The high value of transient expression in the tip and fleshy epicarp of the fruits may be due to their looseness of the tissues, which make them easier for *Agrobacterium* penetration and positively affect transient *GUS* expression. This study is in agreement with the previous reports (Clough & Bent**,** 1998, Spolaore *et al*., 2001).

After *Agrobacterium* infiltration and isolation of seeds from fruits 72 % to 63% seedlings were obtained geminating and surviving on selection medium. Among these survivors 87.9-94.9 were stably transformed (as observed from stable *GUS* assay), with 48 hours incubation period having maximum followed by 96 and 72 hours incubation periods. Overall, 70-56% transformation was observed through fruit injection. The difference in transformation values between 48 and 72, 96 hours incubation period was marked (14%) but the difference in 72 and 96 hours incubation periods was less prominent (2%). These results show that 48 hours is optimum incubation period, among the three periods studied, and that transformation efficiency decreases with increasing incubation period.

The effect of incubation period on transformation efficiency was found significant. This finding can be explained by the reduction in activity of *Agrobacterium* with the length of post inoculation period (Onde *et al*., 2001) while working on in-planta transformation of *Arabidopsis* suggested that *Agrobacterium* persists for a limited period at levels high enough to achieve reasonable rates of transformation in newly forming flower buds. The presence or absence *GUS* expression in different tissues within the same kanamycin resistant seedling may be due to the several reasons such as:

1. Shielding effect of transformed to untransformed tissue (Cervera *et al*., 1998).

2. Gene silencing or reduced expression which may be due to the presence of multiple copies of the transgene and methylation of foreign DNA and insertion of incomplete *GUS* gene (Linn, *et al*., 1990).

Higher transformation frequency (86.667%) has been obtained on the basis of transient expression of *GUS* gene than stable transformation (70%) during this study. The same have been reported by (Mahmoudian *et al*., 2002). The transient activity is supposed to be the consequence of expression of T-DNA carrying *GUS* gene, which was transferred into the plant nuclei but not integrated into plant chromosomes. Thus the progressive decrease in transient *GUS* expression with increasing co-cultivation period may be due to stable gene expression.

During these studies, it has been observed that some of the kanamycin resistant plants failed to give *GUS* positive assay in some of their tissues (of the same plant) and escaped. The same is true of the previous results (Hamza & Chupeau, 1993). In attempt to suppress the possible chimeric effect of transformation and different physiological conditions in plant tissues, 4 parts of each plant were used for sample preparation and the percentage of stable *GUS* expression was calculated on the average of their mean values. Transformation efficiency obtained for stable *GUS* expression was very promising and ranged from 56% (72 hours) - 70% (for 48 hours incubation period). These results are in accordance with the results (2.9-70%) already obtained by Trieu *et al*., (2000) by inoculating intact flowers and seedlings of *Medicago truncatula* with *Agrobacterium*, then collecting and testing the seeds for transformation.

Conclusion

Agro-infiltration of ripened fruits of tomato presented an excellent protocol for transformation. Transient *GUS* assay of whole fruit, its various tissues, and seeds were not only positive but showed very high efficiencies of transformation. The growth of seedlings on kanamycin selection medium was another encouraging clue in this direction. Stable positive *GUS* assay and PCR amplification of *NPT11, GUSint*, and *AP1* genes finally confirmed the transformation. The length of co-cultivation period showed significant effect on transformation.

The ease and high efficiency of transformation obtained leads us to conclude that the method should be further refined and tested for tomato as well as other plants and if we succeed then this would be a novel method in the field of *Agrobacterium* mediated transformation.

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