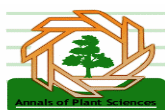

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Original Research Article

Estimation of overall efficiencies of Agrobacterium-mediated transformation system in tomato (*Pusa ruby*) based on the expression of universal marker genes at both RNA and protein levels

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Abstract: Tomato (*Solanum lycopersicum* L.) is a valuable source of nutritional as well as medicinal subtances for human's healthcare. However, as the cultivation of tomato is profoundly affected by both biotic and abiotic stresses, generation of transgenic tomatoes which can tolerate/resist these stresses assumes great importance. In the present study, we have optimized and for the first time estimated overall efficiencies of *Agrobacterium*-mediated transformation system for tomato Pusa ruby cultivar based on the expression of universal marker genes (GUS and GFP) at both RNA and protein levels. Two different formulas of plant growth regulators, 1.5 mg/l of 6-benzylaminopurine (BAP) combined with 0.5 mg/l of indole-3-acetic acid (IAA) and 0.5 mg/l of zeatin combined with 0.1 mg/l of IAA, were used for regeneration of transgenic plants from cotyledonous tissues, and our data revealed significant differences of transient expression of marker genes for these two combinations. The overall efficiencies of the transformation system was calculated as a ratio of the number of the intact transformants (counted as 1 intact plant for 1 explant used for transformation) to the total explants used and were observed to be 4.55 ± 1.02% and 3.11 ± 0.71% for GUS gene and GFP, respectively. Molecular analyses of the GFP transgenic plants indicated various levels of post-transcriptional gene silencing. This system can also be used to efficiently transform gene silencing constructs (RNAi, artificial microRNA expression cassettes) or protein expressing constructs into tomato Pusa ruby cultivar.

Keywords: *Agrobacterium*-mediated transformation, GUS/GFP marker genes, Post-transcriptional gene silencing, Shoot regeneration, Transgenic tomato plants

Introduction

Tomato (*Solanum lycopersicum* L.) belongs to *Solanaceae* or nightshade family. Its fruit contains various bioactive compounds, especially antioxidants like lycopene and essential minerals and vitamins, which constitute a very good source of medicinal subtances for human's healthcare (Beacher, 1998; Renata and Elizabeth, 2009). The plant is native to South America and was introduced to the Old World along with the colonization of Spanish to the America continent (the New World) (Smith, 1994). Nowadays, tomato is being cultivated all over the world and has become an indispensible part of our meals. According to FAO data, the world production of tomato in the year 2010 was about 123 million tones priced at around 45 billion of US dollar (http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor). However, tomato cultivation is potentially hampered by biotic (as well as abiotic) stresses, which include insects, bacteria, fungi, nematodes and viruses. Worldwide, losses due to these pests were estimated to be about 34.4% of attainable tomato yield under current production practices. Furthermore, without crop protection, losses were estimated to increase upto 77.7% of attainable yield (Zalom, 2003; www.ppp.purdue.edu/Pubs/PPP-101.pdf).

As rampant use of chemicals for pest control have led to various problems for environment and human's health, new methods to contain the pests are required. Advanced knowledge in biomolecular engineering has been applied to introduce new, elite and exotic genes into commercially valuable tomato varieties to render them tolerant/resistant against the pests. Traditionally, the introduction of transgenes into plants is carried out via *Agrobacterium tumefaciens* – a soil bacterium that possesses intact apparatus for gene transformation and integration, and transforms the dicotyledonous plants by integrating their genes into the hosts' genome. The apparatus has been modified to transfer genes of interest thereotically into any plant (Gelvin, 2000).

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The *Agrobacterium*-mediated tomato transformation system exhibited various efficiencies for different cultivars. The transformation system for tomato Pusa ruby cultivar was also established using marker gene (Sharma *et al.,* 2009). Efforts have been made to optimize the conditions of transformation stage, however, no quantitative overall transformation efficiency has been reported in these studies (Park *et al.,* 2003; Cortina and Culiáñez-Macià, 2004; Sharma *et al.,* 2009). Here, we report the optimization and estimation of overall efficiencies of *Agrobacterium*-mediated transformation system using tomato Pusa ruby cultivar. We analyzed the transformants by reverse transcription-polymerase chain reaction (RT-PCR) and western blot assays, and estimated the overall transformation efficiencies. This is the first study where the efficiency of the *Agrobacterium*-mediated transformation protocol was systemically analyzed based on the expression (at both RNA and protein levels) of the transformed marker gene in the tomato transformants. We had previously used this method to efficiently transform artificial microRNA into tomato Pusa ruby cultivar, where the stable integration of transgene into the genome was clearly demonstrated at least until T2 generation (Vu *et al.,* 2013), thus emphasizing the utility of the transformation system.

Materials and Methods

Preparation of explants for transformation:

The seeds of the *Pusa ruby* variety (purchased from National Seed Corporation, Pusa, India) were sterilized by treating the seeds with 50% commercial liquid bleach for 20 minutes. Sterilized seeds were grown in Murashige and Skoog (MS) (1962) medium containing 30 g/l of sucrose (pH 5.8) (Table 1) at 25 ± 2ºC under 16 h/8h light/dark conditions. Seven days-old seedlings were collected, and their cotyledons were sliced into 0.2-0.3 cm fragments. The fragments were placed on plates containing pre-culture medium (MS basal salts, Gamborg B5 vitamins containing either 0.5mg/l of zeatin and 0.1 mg/l of IAA or 2.0 mg/l of BAP and 0.5mg/l of IAA, 0.1mM of putrescine and 30 g/l of glucose, pH 5.8; Table 1) for 2 days before transformation.

Preparation of Agrobacteria:

A. tumefaciens LBA4404 cells were separately transformed with pBI121 and pBI121-GFP (constructed by replacing GusA gene by GFP gene at *Xba*I and *Sac*I sites of pBI 121) vectors (Fig. 1). The agrobacteria transformants were cultured in primary medium [yeast extract mannitol (YEM) containing 20mg/l rifampicin, 50mg/l streptomycin, and 50mg/l kanamycin] overnight in a shaking incubator at 28ºC. Secondary culture was prepared by inoculating fresh YEM medium containing the antibiotics with 1/10 volume of the primary culture and incubating in a shaking incubator at 28ºC for 4-5h (OD_{600} = 0.6-0.8). Agrobacterium cells were then collected from the secondary culture by centrifugation at 5000rpm for 10min. The cells were resuspended in liquid MS basal salts (pH 5.2) or fresh YEM containing acetosyringone (AS, 200µM) and were kept in shaking incubator at 28ºC for 1h prior to transformation.

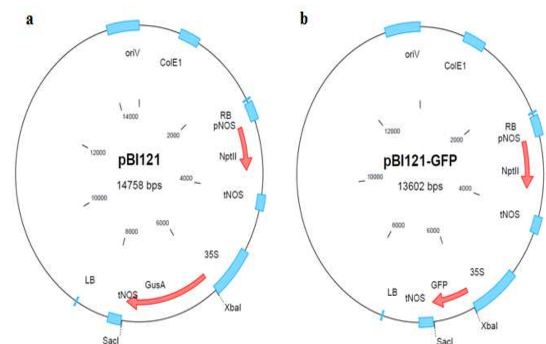

Fig. 1: Maps of the binary vectors used in the present study: (a) pBI121 vector, and (b) pBI121-GFP vector, constructed by replacing GusA gene by GFP gene at *Xba*I and *Sac*I sites of pBI121.

Tomato transformation:

Transformation was carried out by mixing the pre-cultured tomato explants with the agrobacterium cells prepared above followed by incubation for 30 minutes at room temperature. The explants were then transferred to cocultivation medium [containing all of the components in the pre-culture medium plus AS (200µM), pH 5.8; Table 1]. The cocultivation plates were kept in the dark at 25ºC for 2 days, following which the explants were shifted to selection medium containing all of the components in the pre-culture medium plus 250mg/l of cefotaxime and 50mg/l of kanamycin (Table 1). The explants were subcultured at 10-day intervals for 4-5 weeks to achieve the best

regeneration efficiency. Shoot regeneration and organogenesis efficiency was calculated for the explants regenerated for 4 weeks as the ratio of explants exhibiting shoot regeneration or organogenesis to the total number of transformed explants. Regenerated shoots from the selection plates were elongated on elongation medium (MS/2 containing 30g/l of dextrose, 0.5mg/l of BAP, 0.5mM of putrescine, 200mg/l of cefotaxime, 25mg/l of kanamycin, and 8g/l of agar, pH 5.8; Table 1). When the shoots were long enough (1.5-3.0cm), they were transferred to rooting medium (containing all of the components in the elongation medium, except that BAP was replaced with 1mg/l of IBA; Table 1) to generate intact plants. The intact plants from the rooting medium were transferred to vermiculite pots to allow them to harden before shifting to soil pots in a greenhouse at 26 ± 2ºC under a 16h/8h light/dark photoperiod.

Semi-quantitative RT-PCR:

Semi-quantitative RT-PCR was performed as described previously (Vu *et al.,* 2013). Briefly, total RNA was isolated from tomato plant leaves using the TRIZOL reagent following the manufacturer's protocol (Invitrogen). Approximately 2 µg of total RNA was used for first-strand cDNA synthesis, followed by PCR. One microliter of the cDNA mixture was used as a template for PCR amplification with the GFP forward primer (5′-GGCAAGTAAAGGAGAAGAAC-3′) and GFP reverse primer (5′-GAGTTCGTCGTGTTTGTATAG-3′). The RNA samples were also directly used in the PCR reaction as non-RT control to check for the presence of any genomic DNA. The amplified bands (~400 bp) were quantified using ImageJ software, taking total RNA used for the PCR reactions as loading control.

GUS histochemical assay:

Leaves of transgenic plants were stained with X-Gluc to detect the expression of GUS following the protocol of Jefferson *et al.,* (1987). Briefly, the leaf fragments were fixed in 0.3% formaldehyde in 10mM MES, pH 5.6 containing 0.3M mannitol for 45min at room temperature, followed by several washes in 50 mM NaH_2PO_4 (pH 7.0). The fixed explants were stained in a buffer containing 1 mM X-Gluc and 50mM NaH_2PO_4, pH 7.0 at 37°C for varying period ranging from 20 min to several hours. Subsequently, the explants were rinsed in 100% ethanol for 5min, followed by 70% ethanol for 10min and photographed.

Western blot for detection of GFP protein:

Total protein was isolated from both wild type and transformed tomato leaves and was separated on a 10% SDS-PAGE gel. The proteins were then transferred to Hybond-P membrane (GE Healthcare Life Science, UK) using semidry transfer cell (BioRad, USA). The membrane was treated overnight with 3% of BSA at 4 °C before incubation with the primary antibody (1:10,000) raised in rabbit against GFP at room temperature, for 2 h. The membrane was subsequently washed with phosphate buffered saline containing 0.05% tween-20 and incubated with the horseradish peroxidase conjugated secondary anti-rabbit antibody (Sigma, USA, 1:10,000) at room temperature for 1 h. Next, the membrane was washed with the above wash buffer and further incubated with the substrate for horseradish peroxidase (EZ-ECL reagents, Israel Beit Haemek Ltd., Israel). On development, the membrane was photographed by Typhoon Phosphoimager (GE Healthcare Life Science, UK). The intensities of the bands obtained were estimated using ImageJ software.

Results and Discussions

Effect of BAP and zeatin on the somatic regeneration of Pusa ruby cotyledonary explants:

In previous reports, BAP or zeatin have been routinely used as a cytokinin in combination with an auxin [IAA or 1-naphthaleneacetic acid (NAA)] for tomato shoot regeneration, where the regeneration efficiency in each combination was found to be varied depending on the genetic background of each tomato variety (Öktem *et al.,* 1999; Park *et al.,* 2003; Cortina and Culiáñez-Macià, 2004; Sharma *et al.,* 2009; Afroz *et al.,* 2010). In an attempt to optimize the composition of the regeneration medium for plant regeneration from cotyledonary explants, we sought to use BAP or zeatin at various concentrations (Tables 2 and 3) in combination with 0.5mg/l or 0.1mg/l of IAA, respectively. The other components of the medium are described in Table 1. The regeneration experiments were carried out in absence of any antibiotic. Our data showed that when 1.5mg/l BAP and 0.5mg/l IAA (BI3 variant) was used in the regeneration medium, the regeneration efficiency

(calculated as ratio of total number of shoot regenerated explants to the total number of explants used) recorded after 2 weeks was as high as 34.43 ± 4.55%. The capacity for regeneration was reduced at higher concentrations of BAP (Table 2). This result suggests that BAP has beneficial effect (s) (until a concentration of 1.5mg/ml) on the direct shoot regeneration of Pusa ruby cotyledon.

Table.1: Media compositions used in the study

Media	Composition
Germination	MS + 30 g/l of sucrose + 8 g/l of agar, pH 5.8
Pre-culture	PRE1: MS + 30 g/l of dextrose + 0.5 mg/l of zeatin + 0.1mg/l of IAA + 0.5 mM of putrescine + 8 g/l of agar, pH 5.8 PRE2: MS + 30 g/l of dextrose + 1.5 mg/l of BAP + 0.5 mg/l of IAA + 0.5 mM of putrescine + 8 g/l of agar, pH 5.8
Cocultivation	CC1: MS + 30 g/l of dextrose + 0.5 mg/l of zeatin + 0.1 mg/l of IAA + 0.5 mM of putrescine + 200 µM of AS + 8 g/l of agar, pH 5.8 CC2: MS + 30 g/l of dextrose + 1.5 mg/l of BAP + 0.5 mg/l of IAA + 0.5 mM of putrescine + 200 µM of AS + 8 g/l of agar, pH 5.8
Selection	SEL1: MS + 30 g/l of dextrose + 0.5 mg/l of zeatin + 0.1 mg/l of IAA + 0.5 mM of putrescine + 200 mg/l of cefotaxime + 50 mg/l of kanamycin + 8 g/l of agar, pH 5.8 SEL2: MS + 30 g/l of dextrose + 1.5 mg/l of BAP + 0.5 mg/l of IAA + 0.5 mM of putrescine + 200 mg/l of cefotaxime + 50 mg/l of kanamycin + 8 g/l of agar, pH 5.8
Elongation	SE: MS/2 + 30 g/l of dextrose + 0.5 mg/l of BAP + 0.5 mM of putrescine + 200 mg/l of cefotaxime + 25 mg/l of kanamycin + 8 g/l of agar, pH 5.8
Rooting	RE: MS/2 + 30 g/l of dextrose + 1.0 mg/l of IBA + 0.5 mM of putrescine + 200 mg/l of cefotaxime + 50 mg/l of kanamycin + 7 g/l of agar, pH 5.8

MS: Murashige and Skoog medium (1962); IAA: Indole-3-acetic Acid; BAP: 6-Benzylaminopurine; IBA: Indole-3-butyric Acid; MS/2: Half strength of MS.

Table.2: Shoot regeneration efficiencies of Pusa ruby cotyledonary explants after 2 weeks at various BAP concentrations

Variants	Concentration of BAP (mg/l)	Total number of explants used	Total number of shoot regenerated explants	Shoot regeneration efficiencies (%)(±SE)
BI1	0.5	62	6	9.68 ± 2.63
BI2	1.0	61	9	14.75 ± 2.67
BI3	1.5	61	21	34.43 ± 4.55
BI4	2.0	59	11	18.64 ± 3.40
BI5	2.5	62	12	19.35 ± 7.47

SE: standard error

Table.3: Shoot regeneration efficiencies of Pusa ruby cotyledonary explants after 2 weeks at various zeatin concentrations

Variants	Concentration of zeatin (mg/l)	Total number of explants used	Total number of shoot regenerated explants	Shoot regeneration efficiencies (%)(±SE)
Z1	0.1	106	34	32.08 ± 2.90
Z2	0.5	86	61	70.93 ± 6.93
Z3	1.0	88	51	57.95 ± 9.52
Z4	1.5	94	50	53.19 ± 9.66

SE: standard error

Furthermore, when zeatin was used at various concentrations in combination with 0.1 mg/l of IAA in the regeneration medium, much higher shoot regeneration efficiencies (compared to BAP supplement) were observed in all the cases. The highest efficiency (70.93 ± 6.93%), recorded after 2 weeks, was obtained for the medium containing 0.5 mg/l of zeatin (Table 3). The significant difference in the shoot regeneration rates observed when BAP and zeatin was separately used in the medium revealed that zeatin was more effective on direct shoot regeneration (at 2 weeks stage) for cotyledonary explants of tomato Pusa ruby cultivar. The difference in the shoot regeneration rate was more prominent during the period of 2 to 3 weeks. However, shoot regeneration in both the cases (Fig. 2) increased up to 80-90% at later stage (4 weeks, data not shown). The shoot regeneration efficiencies observed in these experiments were significantly higher than that obtained by others using the same tomato variety (Afroz *et al.,* 2010). These suggest that both combinations of the regulators in the medium used in the present study result in improvement in tomato regeneration.

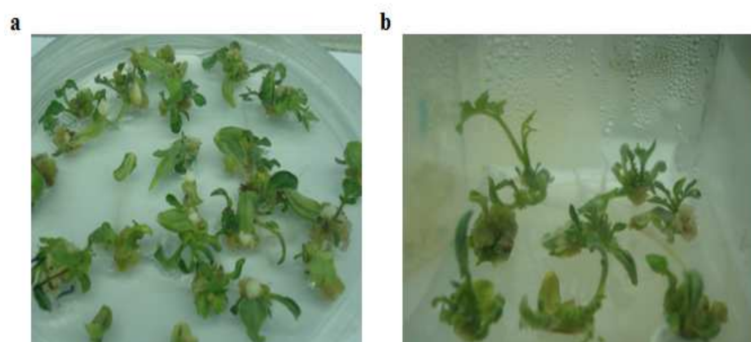

Fig. 2: Direct shoot regeneration of tomato cotyledonary explants. Regenerated shoots after 4 weeks of culture (a), and subsequently on shoot elongation medium (b) are shown.

GUS and GFP transformation:

GUS and GFP are two commonly used marker genes for establishment and optimization of a plant transformation system. The systems using GUS or GFP are endowed with both advantages as well as disadvantages. While with GFP the expression of the gene in transformants can be monitored by direct visualization, the expression of GUS gene in the corresponding transformants can be visualized only by chemically staining the tissue *in planta* [e.g. 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) stained leaf samples]. However, the expression of GUS gene is thought to be a more reliable tool than that of GFP gene due to more background noise generally observed in case of GFP visualization. In the present study, we used both the genes as markers for transformation and compared the *Agrobacterium*-mediated transformation efficiencies with these two genes.

When transformed explants were regenerated, distinct differences in shoot regeneration of the explants were observed at 4 weeks stage with above combinations of the regulators (Table 4). The shoot regeneration rates were found be much lower when BAP was replaced with zeatin in the selection medium. This was presumably due to the reduced growth of the explants under the selection pressure (kanamycin). Our data showed that the number of survival explants (under selection pressure) and organogenesis rate were much higher on the medium containing 1.5mg/l of BAP and 0.5mg/l of IAA than that containing 0.5mg/l of zeatin and 0.1 mg/l of IAA (Table 4). The results also demonstrated that there were no significant variation in the viability of the explants and the organogenesis rate in GUS or GFP transformation events using both the plant regulator combinations. The shoot regeneration and organogenesis rate was as high as 30.41 ± 7.40% in case of GUS gene transformation using 1.5 mg/l of BAP and 0.5mg/l of IAA in the selection medium. The observed rate was higher than that reported by Cortina and Culiáñez-Macià (2004) for different tomato cultivars using similar components in the selection media. This suggests that the shoot regeneration and organogenesis rate might depend on the genetic background of the cultivar as well.

Table.4: Shoot regeneration and organogenesis efficiencies of transformed explants after 4 weeks for either GUS or GFP genes on selection media containing different plant regulator combinations

Gene	Plant regulators in selection media	
	1.5 mg/l of BAP + 0.5 mg/l of IAA (%) (±SE)	**0.5 mg/l of zeatin+ 0.1 mg/l of IAA (%) (±SE)**
GUS	30.41 ± 7.40	12.63 ± 2.16
GFP	22.34 ± 6.16	12.82 ± 2.92

SE: standard error

In the later stages (5-6 weeks), the number of intact shoots derived was not as high as the number of organogenesis explants obtained, perhaps due to the genetic variation leading to the absence of meristem in the regenerated organs. Only intact shoots could grow well on the selection medium (Fig. 3). The shoots were elongated on the elongation medium before shifting to rooting medium whenever the length of the elongated shoots reach at 2-3 cm (Fig. 3). Root growth was observed in almost all the shoots used. Rooted shoots were transferred to greenhouse through acclimization step (Fig. 4) and were subjected to further analyses. The overall efficacies of the transformation system, calculated on the basis of the number of intact transformants (counted as 1 intact plant for 1 explant used for transformation) divided by the total explants used for transformation, were 4.55 ± 1.02% for GUS gene and 3.11 ± 0.71% for GFP. These data indicate towards the possibility of generating normal, healthy and proper transformants under the antibiotic selection and culturing conditions used in the system.

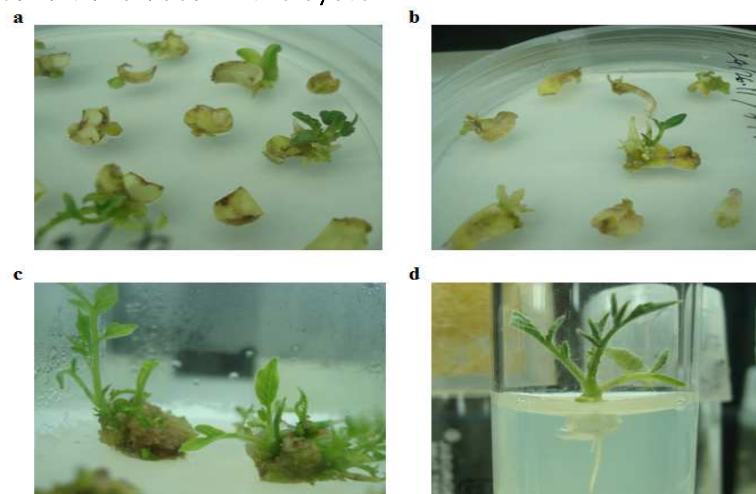

Fig.3: Regenerated shoots at different stages post-transformation. Regenerated shoots on selection media containing 1.5mg/l of BAP and 0.5mg/l of IAA (a), and 0.5mg/l of zeatin and 0.1mg/l of IAA (b) at 4 weeks post-transformation. Regenerated shoots of (a) grown on elongation medium (c) and subsequently on rooting medium (d) are displayed.

Molecular analyses of the transformants:

The tomato transformants in the greenhouse were screened for the presence of transgenes in their cells by polymerase chain reaction (PCR) using genomic DNA isolated from leaf tissue as template. GUS expression in PCR-positive transformants was visualized by staining the GUS-expressing leaf tissues of transformants with X-Gluc (Fig. 5a) following the protocol of Jefferson *et al.,* (1987). GFP expression was visualized under UV light (Fig. 5b). The expression of GUS was detected in 62.5% of total of transformants, while about 44.0% of the transformants exhibited GFP expression in leaf tissues. These indicate that not all the transformants led to the expression of the proteins.

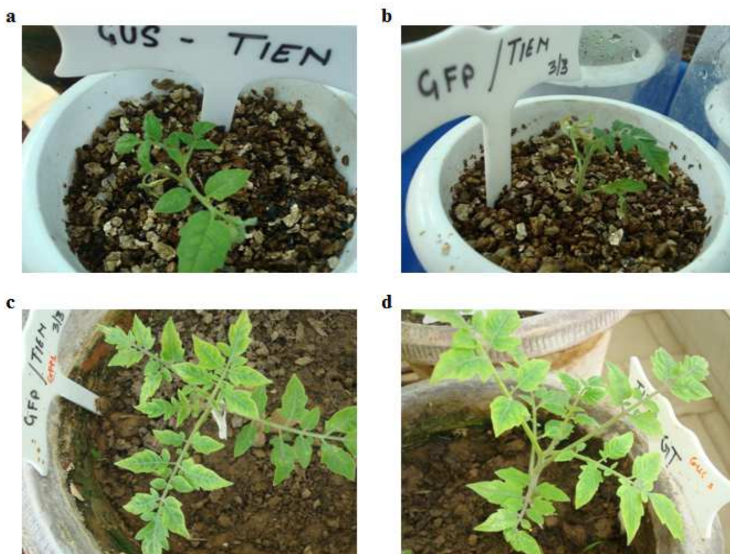

Fig. 4: Tomato transformants grown in greenhouse. Figure shows acclimatization of the transformants on vermiculite (a and b) and subsequently on soil (c and d) for GUS and GFP transformants, respectively.

Further molecular analyses of the expression of the GFP-transgene were carried out by RT-PCR and western blot assays. Total RNA was isolated from 5 GFP PCR positive plants and was used in reverse transcription reaction to synthesize first strand cDNA. Resolution of the products on an agarose gel showed the presence of amplified bands of expected size with different intensities (Fig. 6b). This indicates that the expression of the GFP gene at RNA level was varied in different transgenic lines. The observed difference in RNA levels may be attributed to either RNAi phenomenon or the random intergration of the T-DNA containing the transgene (Vaucheret *et al.,* 2001; Gelvin, 2003; Sanders and Hiatt, 2005). Further work is needed in this direction to resolve the issue.

The expression of GFP protein in tomato transgenic plants is demonstrated in western blot assay using GFP specific antibody (Fig. 6b). Out of the 5 transgenic plants studied, only 2 (lines #1 and #5) showed the expression of GFP. These results further corroborated our earlier notion regarding the correlation of RNA levels and protein level of transgene expression. Furthermore, although the transcript level in the plant line #1 was much higher compared to that of the line #5 (~5.5 fold, Fig. 6a), the amount of GFP protein in the plants showed a reversed correlation (line #1 exhibiting ~7.9 fold reduction in intensity compared to that for line #5, Fig. 6b). The expression of GFP protein in lines #2, #3 and #4 seemed to be suppressed completely, as no signal was observed for these lines on the western blot (Fig. 6b). These findings can be attributed to the phenomenon called "translational repression" which led to the silencing of GFP expression upto 100%, with or without affecting the corresponding mRNA levels. Our western blotting data are in line with that observed for GFP expression using UV light (Fig. 5b). Out of 20 transgenic tomato plants tested, only ~44% showed green fluorescence excitation under UV stimulation.

Fig. 5: Expression of GUS and GFP in the leaves of tomato transformants. GUS expression (a) and GFP expression (b) in the leaves of the respective transformants (collected 4 weeks after transferring to soil) as observed under UV light are displayed. Corresponding healthy plant leaves are also shown on the right side in each panel.

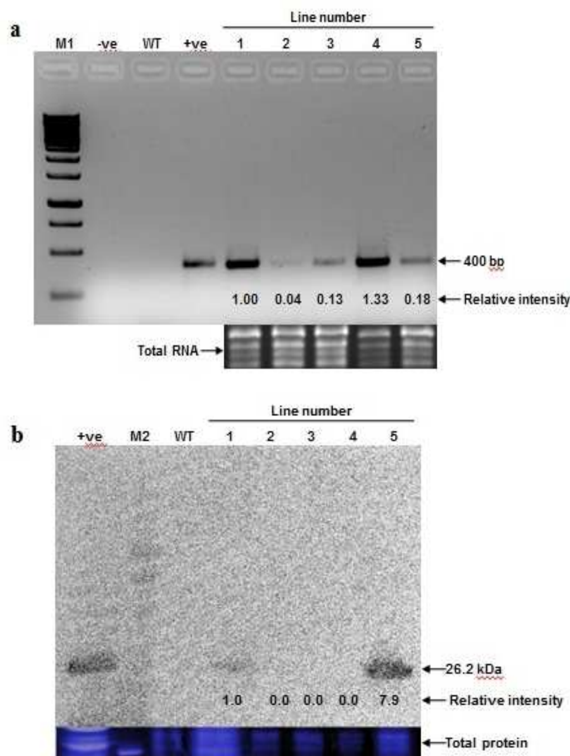

Fig. 6: The expression of GFP in tomato transformants. RNA was isolated from the leaves of the transformants and RT-PCR was performed using GFP-specific primers. RT-PCR products were resolved on a 1.2% agarose gel as shown in panel (a). Total protein from the leaf samples was also isolated and was analyzed through western blotting using GFP-antibody (b). The relative amounts of the transcripts and the translated products formed in different lines were estimated by measuring the intensities of the bands obtained in panels (a) and (b), respectively employing ImageJ software. The respective band intensity of line #1 was arbitrarily assigned a value of 1.0 in each case. Panels (a) and (b) clearly demonstrates the presence of transcripts in the lines #1-5 and of translated products in lines #1 and 5. M1 - 1kb ladder; -ve - negative control; +ve in panel (a) - plasmid pBI121-GFP; +ve in panel (b) - GFP protein expressed in Rosetta *E. coli*; M2 - pre-stained ladder; WT - Healthy plants; lanes 1 to 5 - different GFP transgenic lines.

Taken together, these results demonstrate that the transgenes (GUS and GFP) had been integrated stably into the tomato Pusa ruby genome and successfully expressed at both RNA and protein levels. Our data clearly demonstrates that screening for the most suitable transgenic plants through molecular analyses upto protein levels is required to pick the best transformant to achieve any specific aim.

Conclusions

Regeneration capacity of Pusa ruby tomato cotyledon was optimized in the experiments using BAP or zeatin as cytokinin combined with IAA as auxin. The direct shoot regeneration rates from non-transformed cotyledon fragments were highest in the media containing 1.5mg/l of BAP + 0.5mg/l of IAA and/or 0.5mg/l of zeatin + 0.1mg/l of IAA. Zeatin showed higher shoot regeneration efficiency compared to BAP with these explants at 2 weeks stage. At later stage (4 weeks), however, there was no significant difference in the direct shoot regeneration between the two plant regulator combinations. These combinations were used to regenerate shoot from transformed explants in GUS and GFP transformation events. In these cases, the BAP showed better effect on explants with higher explant survival rates compared to zeatin. The survival rate of transformed explants on the selection medium was as high as 30.41 ± 7.40% with BAP combination. The overall efficiencies of the transformation system, calculated on the basis of the number of intact transformants (counted as 1 intact plant for 1 explant used for transformation) divided by the total explants used, were 4.55 ± 1.02% for GUS gene and 3.11 ± 0.71% for GFP. This is the first report of estimation of overall efficiencies of the transformation system in Pusa ruby tomato based on the expression of genes at both RNA and protein levels.

Notably, our molecular analyses of the GFP expression revealed variations of the transgene expression at RNA and protein levels in the transgenic plants. The analyses indicated towards a gene silencing effect operative in the transgenic plants leading to translational repression. However, further studies are required in this direction to validate the notion.

In summary, we have for the first time estimated overall efficiencies of the *Agro*-mediated transformation system to generate transgenic tomato plants with stable integration of the transgene expressing at protein level. This system is especially suitable for transformation of RNAi- and microRNA-expression cassettes, where the level of protein expression is of lesser

consequence. In addition, in view of the low rate of generation of intact transgenic plants (highest obtained value of 4.55± 1.02%), the number of explants used for transformation or the number of transformation events should be increased to yield better result.

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