Plant Cell Rep (2013) 32:239–247
DOI 10.1007/s00299-012-1358-1

ORIGINAL PAPER

Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom

V. J. Chetty · N. Ceballos · D. Garcia · J. Narváez-Vásquez · W. Lopez · M. L. Orozco-Cárdenas

Received: 19 August 2012 / Revised: 7 October 2012 / Accepted: 9 October 2012 / Published online: 26 October 2012

Abstract
Key message ***Agrobacterium tumefaciens* strains differ not only in their ability to transform tomato Micro-Tom, but also in the number of transgene copies that the strains integrate in the genome.**
Abstract The transformation efficiency of tomato (*Solanum lycopersicum* L.) cv. Micro-Tom with *Agrobacterium tumefaciens* strains AGL1, EHA105, GV3101, and MP90, harboring the plasmid pBI121 was compared. The presence of the *nptII and/or uidA* transgenes in regenerated T_0 plants was determined by PCR, Southern blotting, and/or GUS histochemical analyses. In addition, a rapid and reliable duplex, qPCR TaqMan assay was standardized to estimate transgene copy number. The highest transformation rate (65 %) was obtained with the *Agrobacterium* strain GV3101, followed by EHA105 (40 %), AGL1 (35 %), and MP90 (15 %). The mortality rate of cotyledons due to *Agrobacterium* overgrowth was the lowest with the strain GV3101. The *Agrobacterium* strain EHA105 was more efficient than GV3101 in the transfer of single T-DNA insertions of *nptII* and *uidA* transgenes into the tomato genome. Even though *Agrobacterium* strain MP90 had the lowest transformation rate of 15 %, the qPCR analysis showed that the strain MP90 was the most efficient in the transfer of single transgene insertions, and none of the transgenic plants produced with this strain had more than two insertion events in their genome. The combination of higher transformation efficiency and fewer transgene insertions in plants transformed using EHA105 makes this *Agrobacterium* strain optimal for functional genomics and biotechnological applications in tomato.

Keywords Tomato · Micro-Tom · Cotyledon explants · *Agrobacterium*-mediated transformation

Abbreviations

MS	Murashige and Skoog medium
YEP	Yeast Extract and Peptone medium
Z	Zeatin
WT	Wild type
GUS	*β*-Glucuronidase
*npt*II	Neomycin phosphotransferase
*uid*A	*β*-Glucuronidase
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
bp	Base pair

Introduction

The global market value for biotech/genetically modified crops exceeded $13.3 billion in 2011 and its forecast for the year 2012 is $14 billion (James 2011). Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops throughout the world, with an annual production of 153 million metric tons in 2009 (Krylod 2012). The successful completion of the tomato genome project (Sato et al. 2012) would enable the development of high-yielding and nutritionally improved varieties by both traditional and molecular breeding, or by genetic transformation. Efficient plant transformation systems are

Communicated by B. Li.

V. J. Chetty · D. Garcia · J. Narváez-Vásquez · W. Lopez · M. L. Orozco-Cárdenas (✉)
Plant Transformation Research Center,
University of California Riverside, CA, Riverside, USA
e-mail: mlorozco@ucr.edu

N. Ceballos
Universidad de Caldas, Manizales, Caldas, Colombia

essential for the agro-biotech industry, and the functional analysis of genes involved in different physiological, biochemical, and molecular mechanisms of metabolic pathways (Pereira 2000; Tyagi and Mohanty 2000; Ostergaard and Yanofsky 2004; Lee et al. 2004; Dan et al. 2006; Sun et al. 2006).

Micro-Tom is a miniature dwarf determinate tomato cultivar, originally bred for home gardening (Scott and Harbaugh 1989). Micro-Tom differs from standard tomato cultivars by harboring two recessive genes, which confer the dwarf phenotype (Meissner et al. 1997). This miniature tomato cultivar is preferred as a model system for functional genomics, because it shares several unique features with *Arabidopsis*, such as its small size, which enables it to grow at a high density (1,357 plants/m^2), short life cycle (70–90 days to collect mature fruits), seed setting under fluorescent light, and small genome (350 Mbp). Micro-Tom has been used in tomato molecular genetic studies through mutagenesis, gene tagging, and promoter trapping using *Agrobacterium*-mediated transformation (Meissner et al. 1997; Mathews et al. 2003; Dan et al. 2006; Sun et al. 2006).

Much progress has been made recently in the development of high throughput and efficient *Agrobacterium*-mediated transformation protocols for Micro-Tom (Sun et al. 2006; Dan et al. 2006; Cruz-Mendivil et al. 2011). However, different *Agrobacterium tumefaciens* strains have been used for tomato transformation with variable efficiencies (McCormick et al. 1986; Lazo et al. 1991; Hood et al. 1993; Sun et al. 2006; Dan et al. 2006; Cruz-Mendivil et al. 2011). To our knowledge, no studies have been conducted comparing the transformation efficiencies of different *Agrobacterium* strains and their ability to incorporate specific copy numbers of the transgene in plants. Transgene copy number may influence levels of expression of integrated genes, gene silencing, and stability. Gene copy number is also an important parameter for plant biotechnology, since transgenic plants with single gene insertions are preferentially approved by regulatory agencies.

In the present study, the ability of four commonly used *Agrobacterium tumefaciens* strains, GV3101, EHA105, AGL1, and MP90, to transform tomato Micro-Tom, and incorporate specific copy number in the host plant-cells was compared using a duplexed TaqMan qPCR analysis (Ingham et al. 2001). *Agrobacterium* strain GV3101 demonstrated the highest transformation rates and lowest percentage of plants with single transgene insertions, whereas strain MP90 had the lowest transformation capacity but the highest frequency of plants with single transgene copies. *Agrobacterium* strain EHA105 had the best combination of high transformation efficiency and single insertional events of the transgenes. These results may be of significant value for the large-scale genetic manipulation and screening of transgenic Micro-Tom plants for tomato research and biotechnological applications.

Materials and methods

Seed sterilization and germination

Micro-Tom seeds (Ball^{TM} Seeds Co., Chicago, IL, USA) were sterilized with 95 % ethanol for 2 min, 25 % commercial bleach with 0.1 % Tween 20 for 20 min, rinsed six times with sterile distilled water, and planted on to seed germination (SG) medium [0.5× Murashige and Skoog (MS) salts (Sigma-Aldrich Corp, St. Louis, USA), 20 g/l sucrose, Gamborg's B5 vitamins (Gamborg et al. 1968), pH 5.8 and 0.8 % agar]. The seeds were incubated at 25 °C under 16-h light/8-h dark cycle with fluorescent lights (60 µmol m^{-2} s^{-1}) for 1 week. Cotyledon explants of 7-day-old seedlings were used for *Agrobacterium* transformation.

Agrobacterium strains and transformation vector

The *Agrobacterium tumefaciens* strains AGL1, EHA105, GV3101, and MP90 transformed with the binary vector pBI121 (CloneTech, Palo alto, CA, USA; Jefferson et al. 1987) were used in this study. Table 1 describes some characteristics of the different *Agrobacterium* strains (Hellen and Mullineaux 2000). The plasmid pBI121 contains β-glucuronidase (*uidA*) as a reporter gene, and neomycin phosphotransferase II (*nptII*) as a selective marker gene, both genes are driven by the *CaMV* 35S promoter. *Agrobacterium* cultures were initiated from glycerol stocks, colonies were grown on plates with solid YEP medium supplemented with 100 mg/l kanamycin, and incubated for 48 h at 28 °C in the dark. Selected single colonies were analyzed by colony PCR using gene-specific primers to amplify the *npt*II gene (Table 2). PCR positive colonies were inoculated into 3 ml of YEP medium supplemented with 100 mg/l kanamycin, and incubated at 28 °C for 48 h with shaking at 200 rpm. The bacterial cultures were centrifuged and the pellets were washed twice with 10 ml of liquid MS medium (Murashige and Skoog 1962), and re-suspended at an $OD_{600} = 0.6$ with MS liquid medium containing 72.5 mg/l acetosyringone (Sigma-Aldrich) for transformation of cotyledons.

Plant tissue transformation

Cotyledons were sectioned into two halves across the midvein region, and incubated (adaxial side down) for 2 days on pre culture/co-cultivation (PC/CC) medium [4.3 g/l MS

Table 1 Details of *Agrobacterium tumefaciens* strains used in this study (Hellens and Mullineaux 2000)

Agro strain	Ti plasmid	Opine	Marker gene
AGL1	p*TiBo542ΔT-DNA*	Succinamopine	Rifampicin, carbenicillin
EHA105	p*EHA105* (p*TiBo542ΔT-DNA*)	Succinamopine	Rifampicin
GV3101	Cured	Nopaline	Rifampicin
MP90	p*MP90* (p*TiBo542ΔT-DNA*)	Nopaline	Rifampicin

Table 2 Description of primers and probes for end-PCR and TaqMan qPCR

Gene primer/probe	Sequence (5′–3′)	Length (bp)	Annealing (°C)	Product size (bp)
End-PCR				
*npt*II F1	GGATTGCACGCAGGTTCTCC	20	55	773
*npt*II R1	AACTCGTCAAGAAGGCGATA	20		
TaqMan qPCR				
*npt*II F2	ATCCATCATGGCTGATGCAATGCG	24	60	81
*npt*II R2	CGATGTTTCGCTTGGTGGTCGAAT	24		
*npt*II Probe–HEX	TGCATACGCTTGATCCGGCTACCT	24		
*uid*A F2	TCCATCGCAGCGTAATGCTCTACA	24	60	100
*uid*A R2	TCAACAGACGCGTGGTTACAGTCT	24		
*uid*A Probe–HEX	ACGATATCACCGTGGTGACGCATGT	25		
Prosys F1	GTGACGTGAAAGCAATATCAAGAGCCC	27	60	117
Prosys R1	CGCGCATTATGTTGAGATGTGTGC	24		
Prosys Probe–FAM	TCTTTCTTCTCGTGAAGTATAGGAGCGCT	29		

salts (Sigma-Aldrich Corp, St. Louis, USA), 30 g/l sucrose, Gamborg's B5 vitamins, 1 mg/l zeatin, pH 5.8, and 0.8 % agar]. The cotyledons were gently poked using sharp forceps, transferred to 50-ml conical tubes with the *Agrobacterium* suspension, placed horizontally in a shaker at 50 rpm, and incubated at room temperature for 20 min. The inoculated explants were blotted to dry on a sterile paper towel, and then placed onto PC/CC medium and incubated for 2 days. After 2 days of co-culture, the cotyledons were transferred onto shoot induction (SI) medium [4.3 g/l MS salts, 30 g/l sucrose, 100 mg/l inositol, Nitsch vitamins (Nitsch and Nitsch 1969), 0.05 mg/l folic acid, 2.0 mg/l zeatin, pH 5.8, 0.8 % agar, 100 mg/l kanamycin, 250 mg/l cefotaxime, and 500 mg/l carbenicillin]. Carbenicillin was not added to the tissue culture medium used for AGL1 strain. The explants were sub-cultured twice on to SI medium, every 2 weeks for shoot regeneration. At this stage, the mortality of cotyledon explants was determined. When shoots were about 1-cm long, they were detached from the cotyledons and transferred to shoot elongation (SE) medium [4.3 g/l MS salts, 30 g/l sucrose, 100 mg/l inositol, Nitsch vitamins (Nitsch and Nitsch 1969), 0.05 mg/l folic acid, 1 mg/l zeatin, pH 5.8, 0.8 % agar, 100 mg/l kanamycin, 250 mg/l cefotaxime, and 500 mg/l carbenicillin] to induce shoot elongation. When shoots developed to a height of about 5 cm, they were transferred to rooting (RT) medium (4.3 g/l MS salts, 30 g/l sucrose, 100 mg/l inositol, Nitsch vitamins, 0.05 mg/l folic acid, pH 5.8, 0.8 % agar, 50 mg/l kanamycin, 125 mg/l cefotaxime, and 250 mg/l carbenicillin). Rooted plants were transferred to soil and grown to maturity.

One hundred explants were inoculated with each *Agrobacterium* strain in triplicates. The percentage of explants with shoots and the number of shoots per explant were recorded, and the Shoot Forming Capacity (SFC) index was calculated according to Cruz-Mendivil et al. (2011) as follows: SFC index = (% explants with shoots) × (mean number of shoots per explants)/100. Transformation efficiency was determined by dividing the total number of transgenic plants (identified by end-PCR), by the number of explants inoculated, and then multiplied by 100. Only one plant regenerated per cotyledon explant was considered to calculate transformation efficiency. The calculated transformation frequencies included the cotyledon mortality rates. The percentage of escapes was defined as the number of transgenic plants analyzed minus the number of PCR-positive plants, divided by the number of plants analyzed for each individual strain, and then multiplied by 100.

Histochemical β-glucuronidase (GUS) assay

Tissues from 20 selected transgenic plants obtained with each *Agrobacterium* strain were assayed for histochemical GUS expression according to the procedure of Jefferson et al. (1987). In brief, leaf tissues from randomly selected kanamycin-resistant plantlets were incubated in GUS histochemical buffer (50 mM sodium phosphate buffer, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM $K_4Fe(CN)_6$; 0.1 % Triton X-100; 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) at 37 °C overnight. Tissues were cleared by incubating with 70 % ethanol at 37 °C. This process was repeated 3–4 times to fully clear the tissues.

PCR analysis of regenerated plants

End-PCR

Approximately 100 mg of leaves from putative transgenic (i.e., kanamycin resistant) tomato plants were used to extract genomic DNA for PCR analyses. The leaf material was grounded in an Eppendorf tube with 450 µl of extraction buffer (200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8.0), 0.5 % SDS), followed by chloroform extraction, and isopropanol precipitation of nucleic acids. DNA pellet was washed with 70 % ethanol two times, and re-suspended in 50 µl of TE buffer. An aliquot of each of the DNA samples was diluted to have a final concentration of 20 ng/µl using nuclease free water. For regular end-PCR analysis, 100 ng of DNA was added to a 20-µl PCR mix containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 1 U Ex-Taq DNA polymerase (Life Technologies®, NY, USA), and 0.5 µM of each primer pair for the amplification of the *nptII* gene. Primer sequences, probes and expected PCR products are presented in Table 2. The PCR was performed using a C1000™ Thermal Cycler (Bio-Rad Laboratories, Hayward CA, USA), and the conditions of the reactions for end-PCR were 94 °C for 5 min, followed by 29 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were visualized after electrophoresis on 0.8 % agarose gels. The gel was scored for the presence or absence of the *npt*II product (773 bp).

qPCR assay to estimate copy number

The comparative qPCR ($\Delta\Delta C_t$) technique for duplex TaqMan reactions was used for copy number estimation (Ingham et al. 2001; Mason et al. 2002; Weng et al. 2004). Forty-two plants regenerated from independent cotyledon explants with each *Agrobacterium* strain were selected for the Taqman assay. PCR assays to detect the *npt*II or the *uidA* genes were duplexed with the tomato prosystemin gene (McGurl et al. 1992), which is an endogenous, single-copy reference gene. TaqMan qPCR assays were executed on a MyiQ2 Two Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hayward CA, USA). All primers and probe sequences, and their corresponding annealing temperatures and amplicon sizes for each gene are shown in Table 2. Duplex qPCRs were performed in triplicate 25 µl volumes using SensiMix II™ kit (Bioline, Taunton, MA, USA), 400 nM of each gene specific primer, 200 nM of specific TaqMan probe, and 25 ng of sample DNA. The qPCR protocol included 10 min activation step at 95 °C, followed by 40 cycles featuring a 10 s denaturation step at 95 °C, and a 1 min annealing and extension step at 60 °C. Data acquisitions were done at 72 °C as the machine ramped from annealing/extension to denaturation.

TaqMan assay efficiencies for *npt*II/*Prosystemin* and *uidA/Prosystemin* primers and probe sets were determined to be optimal from serial dilutions of template DNA ($R^2 > 0.99$). The advanced relative quantification option in the MyiQ2 software was used to determine sample C_t values and calculate target/reference ratios (ΔC_t). The resulting target/reference ratios were normalized against a known positive control containing one copy of the *npt*II and *uidA* transgenes, determined by Southern blot analysis (Ingham et al. 2001). Normalized copy number data clustered around values of 1, 2, 3, and more copies.

Southern blot analysis

Genomic DNA from selected PCR-positive plants transformed with each *Agrobacterium* strain was used for Southern blot analysis. DNA from wild-type (non-transformed) Micro-Tom plants was used as a negative control. Five microgram of DNA from each plant was digested with restriction endonucleases (New England Biolabs® Inc, Ipswich, MA, USA), fractionated on 0.8 % agarose gels, transferred to nylon membranes and hybridized with P^{32}-radioactive probes to detect either the *nptII* or the *uidA* genes by autoradiography. Probes were labeled using the DECA prime II Labeling Kit (Life Technologies®, NY, USA). The membranes were prehybridized in 20 ml of prehybridization buffer (2× SSC, 5× Denhardt's solution, 1 % SDS, 10 % dextran sulfate sodium salt, 100 µg/ml salmon sperm DNA), for 2 h at 65 °C, and hybridized for 12–16 h at 65 °C with P^{32}-radioactive probes. Membranes were washed 20 min twice at 62 °C with 2× SSC, and exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 2 weeks at −70 °C.

Statistical analysis

A randomized design was applied to the experiments. Three replicates per treatment were used in each experiment.

A replicate consisted of 100 cotyledon explants for each *Agrobacterium* strain. Data were subjected to analysis of variance, and treatment means were compared using the PROC GLM software SAS (SAS, Institute, NC, USA, Version 9.0).

Results and discussion

The ability of *A. tumefaciens* strains AGL1, EHA105, GV3101 and MP90 harboring the plasmid pBI121 to transform tomato Micro-Tom was evaluated in this study. A total of 458 putative transgenic plants were regenerated from 1,200 cotyledon explants treated with the *Agrobacterium* strains, and selected on medium with 100 mg/l kanamycin. Figure 1 illustrates different stages in the plant regeneration process from cotyledon explants. Mortality rate of cotyledon explants was determined 4 weeks after inoculation of explants. As observed in Fig. 2, the lowest percentage of cotyledon mortality occurred with the strain GV3101 (10 %), whereas the remaining strains showed similar mortality rates of ~30 %. Because identical bacterial densities were used to inoculate the explants with each *Agrobacterium* strain, the difference in mortality of cotyledons must be related with the strain virulence and the specific interaction with the tomato host plant (Hansen 2000; Khanna et al. 2007). In fact, plant cell death (PCD) following *Agrobacterium* infection still remains a significant limitation for plant transformation. Because *Agrobacterium* triggers a defense response, expression of many genes in the host cell may lead to PCD (Khanna et al. 2007). In several plant species, such as maize and bananas, calli infected with *Agrobacterium* undergoes a rapid, hypersensitive PCD response, resulting in limited T-DNA transfer and transformation efficiencies (Hansen 2000; Khanna et al. 2007). Therefore, cotyledon mortality caused by *Agrobacterium* strains is an important parameter to improve the conditions for tomato Micro-Tom transformation.

Remaining cotyledons were able to develop green compact calli and shoots after culture in SI selection medium for 4 weeks, followed by another 2 weeks in SE medium for shoot elongation (Fig. 1). To evaluate and compare the effect of each *Agrobacterium* strain on the regeneration capacity of tomato cotyledons, the percentage of explants with shoots and the number of shoots per explant were measured to calculate the shoot formation capacity (SFC index). Significant differences ($P \leq 0.05$) in the shoot regeneration were observed among the cotyledons treated with the *Agrobacterium* strains (Table 3). The percentage of explants with shoots ranged from 26 to 78 % and the number of shoots per cotyledon ranged from 1.98 to 2.72, and the SFC index varied from 0.52 to 1.90 (Table 3). The higher values of shoot formation were obtained with the cotyledons inoculated with the *Agrobacterium* strain GV3101, followed by EHA105. The SFC index has been used for the optimization of regeneration protocols in tomato and other plant species (García-Saucedo et al. 2005; Cruz-Mendivil et al. 2011), but it has not been used to compare the effect of *Agrobacterium* strains in plant transformation.

Rooting of shoots was obtained in RT medium after 3–4 weeks, and significant differences ($P \leq 0.05$) were observed among *Agrobacterium* strains (Table 3). The percentage of rooted shoots ranged from 12 to 73 %. As shown in Table 4, the highest percentage of rooted shoots was observed in cotyledon explants inoculated with strain GV3101, whereas the lowest root formation percentage was observed for strain MP90 (Table 3).

The most important parameter in *Agrobacterium*-mediated transformation is the transformation efficiency determined as the percent of inoculated explants producing PCR positive, independent transformation events (transgenic plants). *Agrobacterium* strain GV3101 presented the highest transformation rates of 65 %, followed by EHA105 and AGL1 with 40 and 35 %, respectively. The strain MP90 demonstrated the lowest transformation efficiency

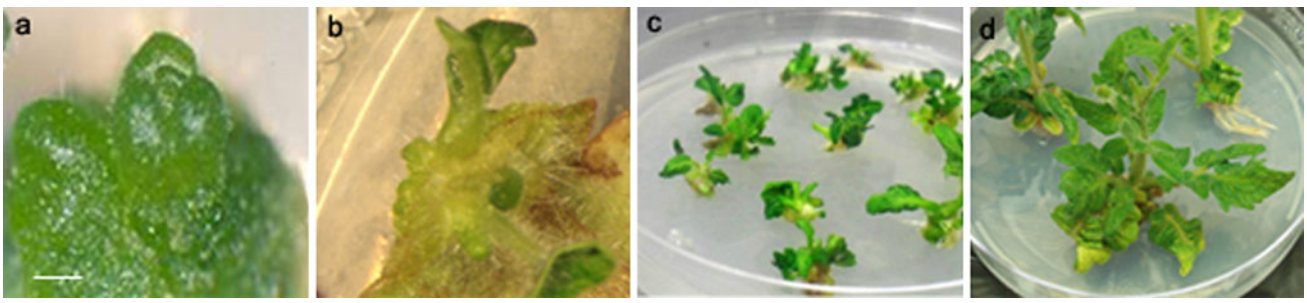

Fig. 1 *In vitro* plant regeneration of Micro-Tom tomato plants from cotyledons inoculated with *Agrobacterium tumefaciens* harboring the binary plasmid pBI121. **a**, **b** Shoot induction on SI medium, **c** shoot elongation in SE medium, and **d** rooting and plant development in RT medium. *Scale bar* 1 mm

with only 15 % (Table 3). The transformation efficiencies of all *Agrobacterium* strains were significantly different ($P < 0.05$), except between EHA105 and AGL1. Different *Agrobacterium* strains have been successfully used to transform tomato; however, transformation frequencies have varied greatly between experiments (McCormick et al. 1986; Lazo et al. 1991; Hood et al. 1993; Park et al. 2003; Dan et al. 2006; Sun et al. 2006; Qiu et al. 2007; Cruz-Mendivil et al. 2011). Using the EHA105 strain, Cruz-Mendivil et al. (2011) reported transformation efficiencies of up to 19 %, whereas Sun et al. (2006) have reported transformation rates ranging from 4–36 % with EHA105. *Agrobacterium tumefaciens* strain EHA105 is routinely used for the transformation of other agronomically important plant species including bananas (Escuola et al. 2011), *Vitis vinifera* L. (Torregrosa et al. 2002), Ginger (Suma et al. 2008), white spruce (Le et al. 2001), and rape seed (van Roekel et al. 1993; Radchuk et al. 2000). Studies comparing the relative transformation efficiencies of *A. tumefaciens* strains have demonstrated that strain AGL1 is superior to C58, GV3101, and EHA105 in its ability to deliver T-DNA into Switch grass seedlings (Chen et al. 2010).

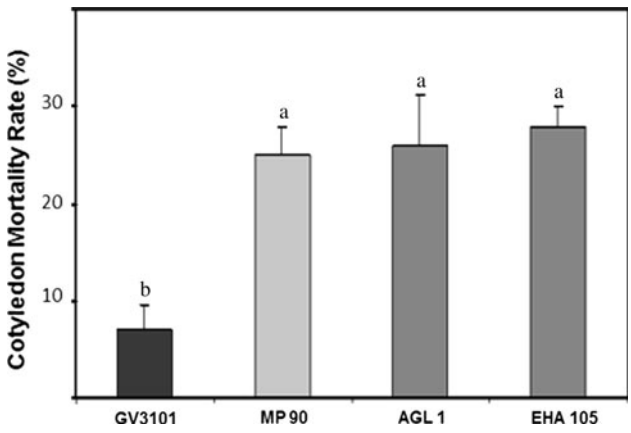

Fig. 2 Mortality rate of Micro-Tom cotyledons infected with different *Agrobacterium* strains. Means with different letters indicate significant differences ($P \leq 0.05$)

The percentage of non-transgenic (PCR negative) plants regenerated on kanamycin selective medium (% escapes) ranged from 7 to 11 % for all *Agrobacterium* strains. Although the differences were small among all strains, they were statistically significant ($P \leq 0.05$) (Table 3). The relative low number of escapes suggests that kanamycin-resistance conferred by the *nptII* gene constitutes a good selection system for regeneration of transgenic tomato plants. Escapes have been reported in leaf disk transformation experiments of 15 cultivated tomato lines using *Agrobacterium* and kanamycin as a selection system (McCormick et al. 1986).

DNA samples isolated from 42 plants obtained with each *Agrobacterium* strain were analyzed to estimate the copy number of *nptII* and *uidA* gene transgenes using the duplexed TaqMan assay. Normalized ΔC_t values were compared against a control DNA sample having a known single insertion of each transgene, using the relative quantification option in the MyiQ2 software (Ingham et al. 2001). Cut-off values for the normalized relative quantification were 0.5–1.5 for one copy, 1.5–2.5 for two copies, and >2.5 for over two copies of the transgenes. The results of the TaqMan assay are presented in Table 4. Interestingly, the four different *Agrobacterium* strains used in this study had an impact upon the extent of transgene transfer into Micro-Tom plants. The qPCR results showed that the strain MP90 had the highest percentage of plants with a single insertion of both *nptII* (76 %) and *uidA* (67 %) transgenes, followed by the strain EHA105 with 67 and 62 %, respectively, for both transgenes (Table 4). Noticeably, none of the 42 transgenic plants evaluated by qPCR that were produced by MP90 had more than two copies of the transgenes. However, the strain MP90 had the lowest transformation efficiency (15 %, Table 3). Strain GV3101 had the highest transformation rate (65 %, Table 3), it also had the lowest percentage of single insertions of both transgenes (52 and 55 %), and the highest percentage of double insertions (33 and 31 %) of all strains (Table 4).

Table 3 Effect of different *Agrobacterium* strains on plant regeneration and transformation of Micro-Tom cotyledons

Agrobacterium strains	Explants with shoots (%)	Number of shoots/explant	SFC index	Rooted shoots (%)	Transformation efficiency (%)	Escapes (%)
AGL1	46 c	2.72 a	1.25 a	32 c	35 b	7 b
EHA105	69 b	2.48 ab	1.71 a	48 b	40 b	9 ab
GV3101	78 a	2.44 ab	1.90 b	73 a	65 a	11 a
MP90	26 d	1.98 b	0.52 c	12 d	15 c	8 ab

Means with different letters within a column indicate significant differences (least significant difference, $P \leq 0.05$)

SFC shoot-forming capacity index = (% explants with shoots) × (mean number of shoots per explants)/100

Transformation efficiency was determined by dividing the total number of transgenic plants (identified by end-PCR), by the number of explants inoculated, and then multiplied by 100. Only one plant regenerated per cotyledon explant was considered to calculate transformation efficiency

Table 4 Percentage of transgenic plants with 1, 2 or >2 copies of *nptII* or *uid*A transgenes in their genomes, based on a duplexed TaqMan qPCR assay (see "Materials and methods")

Agrobacterium Strain	Transgene copy number (%)					
	nptII			*uid*A		
	1	2	>2	1	2	>2
AGL1	52	24	24	52	29	19
EHA105	67	21	12	62	25	13
GV3101	52	33	15	55	31	14
MP90	76	24	0	67	33	0

A total of 42 plants obtained with each *Agrobacterium* strain were used for the TaqMan assay

The strain EHA105 had the second highest both in transformation efficiency (Table 3) and in proportion of plants with single copy events for both transgenes (Table 4).

In general, there was a close correlation in the number of insertional events of both transgenes in the same plants found with the TaqMan assay (Table 4). To reconfirm the results of the qPCR analysis of gene copy number, histochemical analysis of GUS expression and Southern blot analyses of transgenic plants were performed (Figs. 3, 4). Wild-type plants used as negative controls did not show any GUS expression (Fig. 3). All the transgenic plants analyzed that were positive for the presence of the *uid*A gene in the TaqMan assay, also showed constitutive GUS expression in leaves, indicating the presence of a full-functional transgene (Fig. 3). In addition, there was a good correlation (over 90 %) between the number of inserted copies of *nptII* and *uidA* genes found with the TaqMan assay and the Southern blot analyses, including the escapes with no copies of the transgenes (Fig. 4). The PmeI restriction enzyme that do not cut inside the T-DNA, reconfirmed whether the two reference genes were integrated (Fig. 4). These results were reconfirmed with the use of other restriction enzymes, including EcoRI, and HindIII (not shown). Differences in copy number obtained with either assay may have resulted from partial restriction digests of genomic DNA, insertion of tandem repeats, re-arrangements or truncations of the inserted T-DNA (Ingham et al. 2001; Mason et al. 2002; Weng et al. 2004; Yang et al. 2005). On the other hand, transgene insertion sites (i.e., positional effects), and loss of primer binding sites due to truncational/rearrangement events may also lead to variable C_t values in the TaqMan assay (Mason et al. 2002; Yang et al. 2005).

Although much progress has been made in understanding the infection process, T-DNA transfer, and protein delivery into host cells during *Agrobacterium* transformation (Jurado-Jacome 2011), to our knowledge, no published

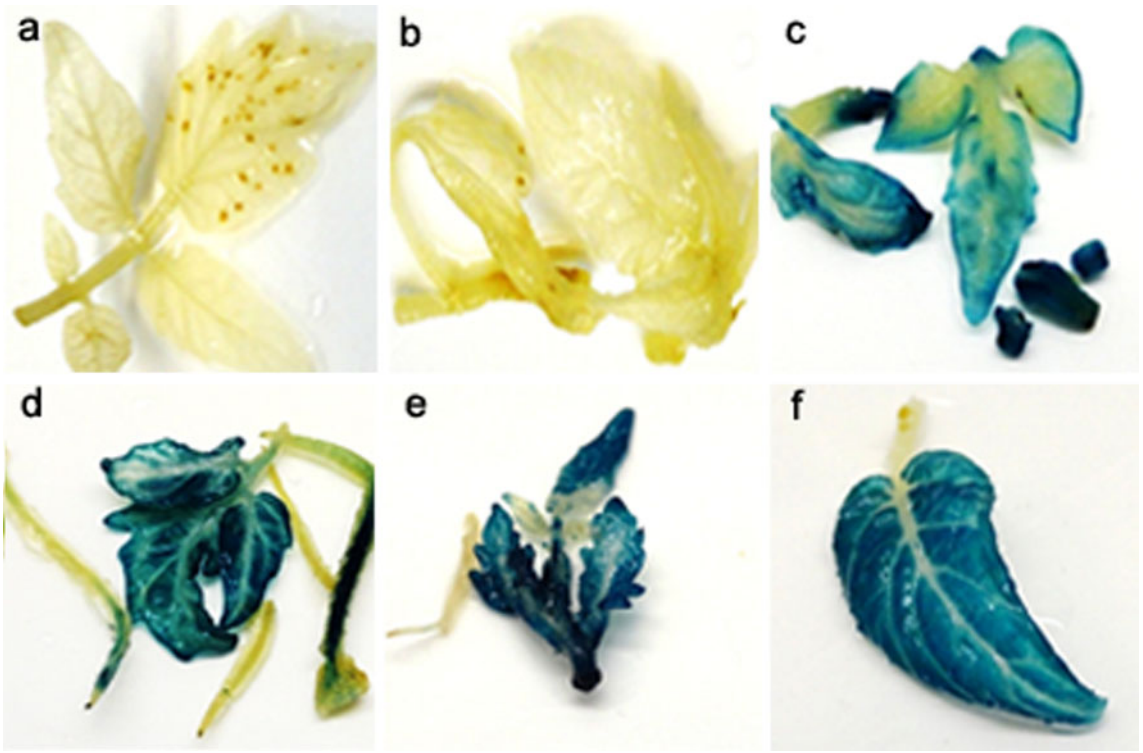

Fig. 3 Histochemical detection of GUS expression in leaves of transgenic Micro-Tom plants transformed with four different *Agrobacterium* strains harboring the plasmid pBI121. **a** Wild type (untransformed control plant); **b** PCR negative plant; **c** Strain AGL1, plant #4; **d** Strain EHA105, plant #3; **e** Strain GV3101, plant #7; and **f** Strain MP90, plant #2

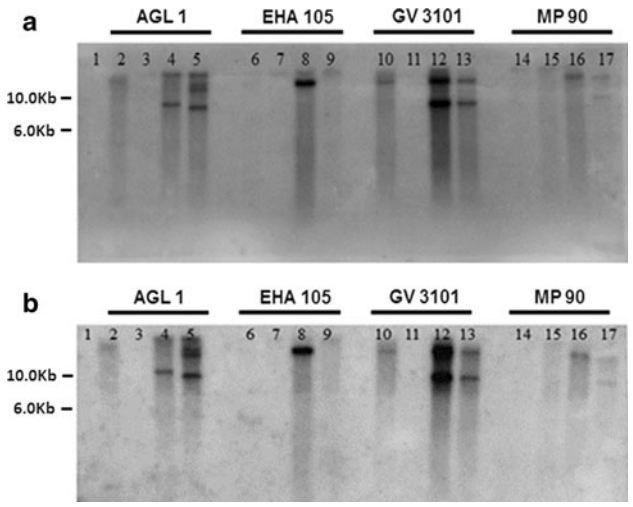

Fig. 4 Southern blot analysis of putative transgenic Micro-Tom plants regenerated after transformation with *Agrobacterium* strains AGL1 (2–5), EHA105 (6–9), GV3101 (10–13), MP90 (14–17), and wild-type, untransformed control tomato plant (1). 5 µg DNA was digested with *Pme*I and hybridized with a radiolabeled *npt*II (**a**) or *uidA* (**b**) probe (see "Materials and methods")

study exists comparing the effect of different *Agrobacterium* strains on the insertion of specific copy number of transgenes in host plant-cells. The results presented here indicate that *A. tumefaciens* strains differ not only in their ability to transform tomato Micro-Tom, but also in the number of transgene copies that these strains integrate in the tomato genome. The super virulent *A. tumefaciens* EHA105 strain appeared to possess an optimal combination of high transformation efficiency and ability to generate single copy transgenic plants. The results of this study should help establish a platform for large-scale genetic manipulation of Micro-Tom and further improve the transformation methodology. The qPCR method developed in this study can be exploited for the estimation of transgene copy number using a high throughput platform.

References

Chen X, Raymie E, Baxter H, Berk K, Hen J, Agarwal S, Zale J (2010) High throughput transient gene expression system for switch grass (*Panicum virgatum* L.) seedlings. Biotechnol Biofuels 3:9. doi:10.1186/1754-6834-3-9

Cruz-Mendivil A, Rivera-Lopez J, German-Baez LJ, Lopez-Meyer M, Hernandez-Verdugo S, Lopez-Valenzuela JA, Reyes-Moreno C, Valdez-Ortiz A (2011) A simple and efficient protocol for plant regeneration and genetic transformation of Tomato cv. Micro-Tom from leaf explants. Hort Sci 46:1660–1665

Dan Y, Yan H, Munyikwa T, Dong J, Zhang J, Armstrong CL (2006) Micro-Tom—a high-throughput model transformation system for functional genomics. Plant Cell Rep 25:432–441. doi:10.1007/s00299-005-0084-3

Escuola C, Tripathy L, Fawole I (2011) Effects of various virulent strains of *Agrobacterium tumefaciens* on genetic transformation of Banana (*Musa* sp.) cultivar William. African J Hort Sci 5:84–91

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158. doi:10.1016/0014-4827(68)90403-5

García-Saucedo PA, Valdez-Morales M, Valverde ME, Cruz-Hernández A, Paredes-López O (2005) Plant regeneration of three Opuntia genotypes used as human food. Plant Cell Tiss Org Cult 80:215–219. doi:10.1007/s11240-004-9158-0

Hansen G (2000) Evidence for *Agrobacterium*-induced apoptosis in maize cells. Mol Plant Microbe Interact 13:649–657. doi:10.1094/MPMI.2000.13.6.649

Hellen R, Mullineaux P (2000) A guide to Agrobacterium binary Ti vectors an update. Trends Plant Sci 5(10):446–451. doi:10.1016/S1360-1385(00)01740-4

Hood EE, Gelvin SB, Melehers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218. doi:10.1007/BF01977351

Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real time PCR assay for determining transgene copy number in transformed plants. Biotechniques 31:132–140. doi:71200122

James C (2011) Global Status of Commercialized Biotech/GM Crops: 2011. ISAAA Brief No. 43, ISAAA, Ithaca, NY

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jurado-Jacome E (2011) *Agrobacterium* infection: translocation of virulence proteins and role of VirF in host cells. Dissertation. Leiden University

Khanna HK, Jean-Yves P, Harding RM, Dickman MB, Dale JL (2007) Inhibition of *Agrobacterium*—induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of banana. Mol Plant Microbe Interact 20:1048–1054. doi:10.1094

Krylod A (2012) UNCTAD http://www.unctad.info/en/Infocomm/AACPproducts/COMMODITY-PROFILE—Tomato/. Accessed on 20 June 2012

Lazo GR, Stein PA, Ludwig R (1991) A DNA transformation—competent *Arabidopsis* genomic library in *Agrobacterium*. Nature Biotechnol 9:963–967. doi:10.1038/nbt1091-963

Le QV, Belies-Isles J, Dusabenyagasani M, Tremblay FM (2001) An improved procedure for production of white spruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. J Exp Bot 52:2089–2095. doi:10.1093/jexbot/52.364.2089

Lee MK, Kim HS, Kim JS, Kim SH, Park JD (2004) *Agrobacterium* mediated transformation system for large-scale production of transgenic Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) plants for insertional mutagenesis. J Plant Biol 47:300–306. doi:10.1007/BF03030544

Mason G, Provero P, Vaira AM, Accotto GP (2002) Estimating the number of integrations in transformed plants by quantitative real time PCR. BMC Biotechnol 2:20. doi:10.1186/1472-6750-2-20

Mathews H, Clendennen KS, Caldwell GC, Liu XL, Connors K, Matheis N, Schuster KD, Menasco DJ, Wagoner W, Lightner J, Wagner DR (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703. doi:10.1105/tpc.012963

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84. doi:10.1007/BF00269239

McGurl B, Pearc G, Orozco-Cárdenas ML, Ryan CA (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. Science 225:1570–1573. doi:10.1126/science.1549783

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashir A, Elkind Y, Levy A (1997) A new model system for tomato genetics. Plant J 12:1465–1472. doi:10.1046/j.1365-313x.1997.12061465.x

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with Tobacco tissue culture. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87. doi:10.1126/science.163.3862.85

Ostergaard L, Yanofsky MF (2004) Establishing gene function by mutagenesis in *Arabidopsis thaliana*. Plant J 39:682–696. doi:10.1111/j.1365-313X.2004.02149.x

Park SH, Morris JL, Park JE, Hirschi KD, Smith RH (2003) Efficient and Genotype independent *Agrobacterium*-mediated tomato transformation. J Plant Physiol 160:1253–1257. doi:10.1078/0176-1617-01103

Pereira A (2000) A transgenic perspective on plant functional genomics. Transgenic Res 9:245–260. doi:10.1023/A:1008967916498

Qiu D, Diretto G, Tavarza R, Giuliano G (2007) Improved protocol for *Agrobacterium mediated* transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene CsZCD. Sci Hort 112:172–175. doi:10.1016/j.scienta.2006.12.015

Radchuk W, Klocke E, Radchuk RI, Neumann M, Blume YaB (2000) Production of transgenic rape plants (*Brassica napus* L) using *Agrobacterium tumefaciens*. Genetika 36:932–941. PMID:1099449

Sato S, Tabata S, Hirakawa H, Asamizu E, Shirasawa K et al (2012) The tomato genome sequence provides insight into fleshy fruit evolution. Nature 485:635–641. doi:10.1038/nature11119

Scott JW, Harbaugh BK (1989) Micro-Tom—a miniature dwarf tomato. Florida Agricultural Experimental Station Circular 370:1–6

Suma B, Keshavachandran R, Nybe EV (2008) *Agrobacterium tumefaciens* mediated transformation and regeneration of ginger (*Zingiber officinale* Rosc.). J Trop Agric 46:38–44

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol 47:426–431. doi:10.1093/pcp/pci251

Torregrosa L, Iocco P, Thomas MR (2002) Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. Am J Enol Viticult 53:183–190

Tyagi AK, Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Sci 158:1–18. doi:10.1016/S0168-9452(00)00325-3

van Roekel JSC, Damm B, Melchers LS, Hoekema A (1993) Factors influencing transformation frequency of tomato (*Lycopersicum esculentum*). Plant Cell Rep 12:644–647. doi:10.1007/BF00232816

Weng H, Pan A, Yang L, Zhang C, Liu Z, Zhang D (2004) Estimating number of transgene copies in transgenic rapeseed by real time PCR assay with HMG I/Y as an endogenous reference gene. Plant Mol Biol Rep 22:289–300. doi:10.1007/BF02773139

Yang L, Ding J, Zhang C, Jia J, Weng H, Liu W, Zhang D (2005) Estimating the copy number of transgenes in transformed rice by real time quantitative PCR. Plant Cell Rep 23:759–763. doi:10.1007/s00299-004-0881-0