

# Rapid delivery of *Cas9* gene into the tomato cv. 'Heinz 1706' through an optimized *Agrobacterium*-mediated transformation procedure

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**Abstract:** *Solanum lycopersicum* 'Heinz 1706' is a pioneer model cultivar for tomato research, whose whole genome sequence valuable for genomics studies is available. Nevertheless, a genetic transformation procedure for this cultivar has not yet been reported. Meanwhile, various genome editing technologies such as transfection of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) ribonucleoprotein complexes into cells are in the limelight. Utilizing the *Cas9*-expressing genotype possessing a reference genome can simplify the verification of an off-target effect, resolve the economic cost of *Cas9* endonuclease preparation, and avoid the complex assembly process together with single-guide RNA (sgRNA) in the transfection approach. Thus, this study was designed to generate *Cas9*-expressing 'Heinz 1706' lines by establishing an *Agrobacterium tumefaciens*-mediated transformation (ATMT) procedure. Here, we report a rapid and reproducible transformation procedure for 'Heinz 1706' by fine-tuning various factors: *A. tumefaciens* strain, pre-culture and co-culture durations, a proper combination of phytohormones at each step, supplementation of acetosyringone, and shooting/rooting method. Particularly, through eluding subculture and simultaneously inducing shoot elongation and rooting from leaf cluster, we achieved a short duration of three months for recovering the transgenic plants expressing *Cas9*. The presence of the *Cas9* gene and its stable expression were confirmed by PCR and qRT-PCR analyses, and the *Cas9* gene integrated into the T<sub>0</sub> plant genome was stably transmitted to T<sub>1</sub> progeny. Therefore, we anticipate that our procedure appears to ease the conventional ATMT in 'Heinz 1706', and the created *Cas9*-expressing 'Heinz 1706' lines are ultimately useful in gene editing *via* unilateral transfection of sgRNA into the protoplasts.

## Introduction

The *Agrobacterium tumefaciens*-mediated transformation (ATMT) was firstly applied in tobacco plants (Herrera-Estrella *et al.*, 1983). Since then, the ATMT technique has been used most efficiently in the field of plant genetic engineering (Ellul *et al.*, 2003; Firsov *et al.*, 2020; Van Eck *et al.*, 2019). Recently, the ATMT technique is also successfully applied to deliver clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated

(Cas) expressing cassette to precisely edit a gene of interest in several crops, including tomato, accelerating functional genomics studies as well as genetic engineering in tomato (Chen *et al.*, 2019; Veillet *et al.*, 2019; Wang *et al.*, 2019; Zhang *et al.*, 2020). However, CRISPR/Cas9-mediated gene editing can often lead to unintended mutation (off-target editing) at non-specific homologous and/or mismatch tolerant sites, which may mask the true phenotype of the edited plants (Cardi *et al.*, 2017; Lee and Kim, 2018; Zhang *et al.*, 2018). As a result, it demands to conduct the genome-wide sequencing of the gene-edited plant, and then the result has to be compared with the whole genome sequence of the wild type counterpart to ascertain an off-target effect. In this aspect, the unveiled whole genome sequence information of a given cultivar is critical to increasing the

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detection efficiency of off-target editing in addition to the effectiveness of candidate single guide RNA (sgRNA). The ‘Heinz 1706’ used in this study is a pioneer model cultivar, whose whole genome was firstly sequenced in tomato and frequently referenced in various studies (Aoki *et al.*, 2013; Cambiaso *et al.*, 2020; Rezzonico *et al.*, 2017; The Tomato sequencing Consortium, 2012; Tranchida-Lombardo *et al.*, 2018). Thus, the informative genomic background of ‘Heinz 1706’ helps to ease the detection of a possible off-target edit in the mutants.

The transfection of preassembled CRISPR/Cas9 ribonucleoprotein (RNP) complex into cells is an alternative to reduce the frequency of off-target editing, which is caused by the consecutive expression of the foreign genes in the edited plant genome. Woo *et al.* (2015) mentioned that the transfected RNP complex is rapidly degraded after cleaving the target site, which might reduce the frequency of mosaicism and off-target effects. However, the preparation of RNP is time-consuming and expensive (Anders and Jinek, 2014). Therefore, creating Cas9-expressing ‘Heinz 1706’ lines will have some advantages in the transfection of either sgRNA alone or the plasmid vector unilaterally expressing sgRNA. In short, applying the Cas9-expressing lines in transfection helps to resolve the cost to prepare a purified Cas9, shortens the steps of the RNP complex assembly process, and simplifies the structure of the plasmid vector by recombining sgRNA-expression cassette alone.

The success of ATMT is often influenced by several factors in different stages: (1) during the introduction of a T-DNA into a plant cell and its integration into the plant genome, and (2) in the course of selection and regeneration of transformants (Altpeter *et al.*, 2016; Basso *et al.*, 2020; Hwang *et al.*, 2017; Sun *et al.*, 2015). Some crucial features affecting the aforementioned stages of ATMT include phytohormones concentration and combination, presence and absence of acetosyringone in co-culture media, durations of pre- and co-culturing, *A. tumefaciens* strain, plant tissue type, and plant genotype (Chetty *et al.*, 2013; Fuentes *et al.*, 2008; Guo *et al.*, 2012; Nonaka *et al.*, 2019; Stavridou *et al.*, 2019). The other persistent challenge in ATMT of tomato is the frequent occurrence of ploidy alterations in regenerated transformants (Bednarek and Orłowska, 2020; Touchell *et al.*, 2020; Ultzen *et al.*, 1995), which might be caused by long culture period coupled with improper use of phytohormones for cell proliferation and differentiation (Niedz and Evens, 2016; Ochatt *et al.*, 2011). Changes in ploidy level affect the genetic fidelity and phenotypes of the regenerated transformants, which means that a newly established/revised ATMT method needs to be assured of the ploidy normality of transformants.

Previous reports have shown that considerable efforts were made to optimize the ATMT procedures for the model tomato cultivars: ‘Moneymaker’ (Frary and Earle, 1996; Ho-Plágaro *et al.*, 2018; Shah *et al.*, 2015), ‘Micro-Tom’ (Chetty *et al.*, 2013; Guo *et al.*, 2012; Nonaka *et al.*, 2019), ‘Rio Grande’ (Shah *et al.*, 2015), and ‘M82’ (Gupta and Van Eck, 2016; Van Eck *et al.*, 2019). However, we could not find any available report so far regarding the ATMT using the model cultivar ‘Heinz 1706’. Although various ATMT procedures for tomato cultivars have been documented, the procedure

for a given cultivar may not necessarily work for other genotypes (Stavridou *et al.*, 2019). Therefore, it is imperative to establish a working procedure for ‘Heinz 1706’ to achieve a successful transformation.

Here, we report a rapid and reproducible ATMT procedure for tomato ‘Heinz 1706’ by optimizing various factors, and the development of Cas9-expressing ‘Heinz 1706’ lines that will be used for future studies on gene editing through sgRNA transfection into their protoplasts.

## Materials and Methods

### *Plant materials and in vitro culture conditions*

The tomato ‘Heinz 1706’ (accession No. LA4345) was provided from the CM Rick Tomato Genetics Resource Center (TGRC) in the University of California, Davis, while the seeds of ‘Moneymaker’ and ‘Rubion’ were introduced from the Kansas State University. The seeds were surface sterilized by submerging in 70% (v/v) ethanol for 60 s, washing in sterile distilled water three times, immersing in the 12.5% (v/v) YUHANROX (commercial bleach containing 4% sodium hypochlorite; Yuhan-Clorox, Hwaseong, Gyeonggi, Korea) diluted with 0.2% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, Missouri, USA) with stirring at 200 rpm for 45 min, and lastly cleaning in sterile distilled water five times. The surface-sterilized seeds were sown on the seed germination medium (Tab. 1) and placed in a dark growth room for 3 days (d). The germinating seeds were exposed to a 16-h photoperiod for 6 d to develop the seedlings with fully expanded cotyledons. The cotyledonary explants were prepared by removing one-third of the distal parts from the detached cotyledons. The core components of the media and solutions used during *Agrobacterium*-mediated transformation (ATMT) processes are summarized in Tab. 1. The pH of the media and solutions was adjusted to 5.7 prior to autoclaving at 121°C for 30 min. Unless otherwise mentioned, the growth room was conditioned at 25 ± 2°C, 60 ± 5% relative humidity (RH), and a 16-h light rendered by fluorescent lights (100 μmol m<sup>-2</sup> s<sup>-1</sup>).

### *Plasmid vector construction, bacterial strain, ATMT, and studied factors*

The Cas9-containing vector pHAtC (GenBank accession number KU213971.1, Kim *et al.*, 2016) was transformed into *A. tumefaciens* strains LB4404 and GV3101 using the freeze-thaw method (Holsters *et al.*, 1978). The expression of Cas9, including the nuclear localization sequence (NLS) and human influenza hemagglutinin (HA)-tag, was regulated by the *Cauliflower mosaic virus* 35S promoter. The pHAtC vector has the hygromycin resistance gene (*Hyg-R*), conferring resistance to the antibiotics hygromycin B, at the *LB*-flanking region of the T-DNA, and its expression is controlled by the nopaline synthase promoter (NOS) (Fig. 1).

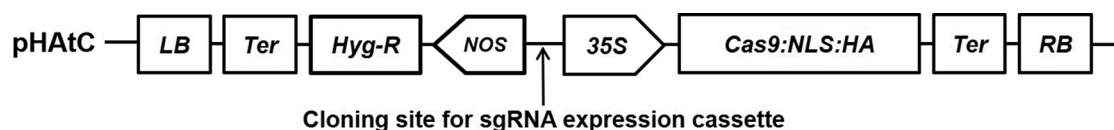
Solid and liquid yeast extract peptone (YEP) media were made to prepare bacterial inoculums (Supplementary Tab. 1). The two *A. tumefaciens* strains harboring the vector pHAtC were first cultured by streaking on the solid YEP medium and kept in the dark at 28°C for 19 h. Then the isolated colonies of each *A. tumefaciens* strain were picked up and

TABLE 1

The components of the optimized media and solutions used during *Agrobacterium*-mediated transformation trials

Components	Seed germination	Pre-culture	Inoculum	Co-culture	Washing	Leaf cluster induction	Shoot elongation/ rooting
MS salts <sup>1</sup> (strength)	half	full	full	full	full	full	full
Vitamins <sup>2</sup> (strength)	–	full	full	full	full	full	full
Sucrose (g/L)	30	30	30	30	30	30	30
Plant agar (g/L)	8	8	–	8	–	8	8
6-benzylaminopurine (mg/L)	–	1	1	1	–	–	–
$\alpha$ -naphthalene acetic acid (mg/L)	–	0.1	0.1	0.1	–	–	–
Acetosyringone ( $\mu$ M)	–	–	100	100	–	–	–
Aminoethoxyvinylglycine (mg/L)	–	–	–	0.001	–	–	–
trans-zeatin (mg/L)	–	–	–	–	2	2	0.2
Indole-3-acetic acid (mg/L)	–	–	–	–	0.1	0.1	–
Indole-3-butyric acid (mg/L)	–	–	–	–	–	–	2
Carbenicillin disodium (mg/L)	–	–	–	–	500	500	500
Clavamox <sup>®</sup> (mg/L)	–	–	–	–	250	250	250
Hygromycin B (mg/L)	–	–	–	–	10	10	10

<sup>1</sup>Murashige and Skoog (1962); <sup>2</sup>Constituents of vitamins (100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 0.1 mg/L thiamine HCl)



**FIGURE 1.** Schematic diagram of T-DNA of binary vector pHAtC used in the study. *LB*, left border; *Ter*, terminator sequence; *Hyg-R*, hygromycin resistance gene; *NOS*, nopaline synthase promoter; *35S*, *Cauliflower mosaic virus (CaMV) 35S* promoter; *Cas9*, Cas9 endonuclease gene; *NLS*, SV40 nuclear localization signal sequence; *HA*, influenza hemagglutinin epitope tag; *RB*, right border.

inoculated in 50 mL of liquid YEP medium. The cultures were incubated in a shaker at 200 rpm and 28°C for 9 h up to OD<sub>600</sub> = 0.8 to 1.0. The bacterial cells were pelleted by centrifuging with 4000×g at 4°C for 7 min, followed by washing in the inoculum solution (Tab. 1). The re-pelleted bacteria were diluted in 50 mL of inoculum solution and incubated in a shaker (200 rpm, 28°C) in darkness for 1 h. These bacterial suspensions were used to infect the explants for assaying the various factors affecting ATMT.

The cotyledonary explants were infected independently with the *A. tumefaciens* strains (LB4404 and GV3101) according to the previous report (Park *et al.*, 2003) to choose the physiologically compatible strain with 'Heinz 1706'. To ascertain the influence of pre-culture and co-culture durations on transformation, the explants were pre-cultured for 1 or 2 d on the pre-culture medium (Tab. 1) before infected by the bacteria. All the pre-cultured explants were placed into the bacterial inoculums and stirred at 200 rpm for 20 min. Then the infected explants were blotted dry on sterilized filter paper and co-cultured in the dark for 2, 3, or 4 d. The co-culture media were supplemented with various amounts of acetosyringone [3',5'-dimethoxy-4'-hydroxy acetophenone (Sigma-Aldrich, Co.); 50, 100, or 200  $\mu$ M] to investigate its contribution to the efficiency of transformation.

The co-cultured explants were washed twice with the washing solution (Tab. 1), blotted dry, transplanted to the leaf cluster induction medium (Tab. 1), and kept in the growth room. The regenerated leaf clusters were cut and transferred to the shoot elongation/rooting (SER) media with different concentrations of IBA (Sigma-Aldrich, Co.) (0 to 4 mg/L) and trans-zeatin (Sigma-Aldrich, Co.) (0–0.2 mg/L).

#### *Acclimatization of regenerated plantlets, generation of T<sub>1</sub> plants, and heritability estimation*

Elongated plantlets with roots (putative T<sub>0</sub> plantlets) were thoroughly washed with tap water, transferred to the plastic pots filled with clean commercial compost, put into a translucent plastic container, and then placed in a growth chamber conditioned at 25°C, 60% RH, and a 16-h light for a week. The acclimatized plantlets were transplanted into a 4-L plastic pot containing commercial compost, then placed in a greenhouse at day/night temperatures of 23/18 ± 5°C and 60 ± 5% RH. For estimating the heritability of the *Cas9* gene, randomly selected three PCR-positive T<sub>0</sub> plants with normal ploidy were allowed to self-pollinate. Matured T<sub>1</sub> seeds were collected from the fruits of each T<sub>0</sub> plant, which were used to develop T<sub>1</sub> seedlings for determining the transmission of the *Cas9* gene from T<sub>0</sub> plants.

### Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from the newly emerging leaves using Solg<sup>TM</sup> Genomic DNA Prep Kit (SolGenet Co, Ltd., Daejeon, South Chungcheong, Korea) according to the manufacturer's instructions. The fragment (502 bp) of the *Cas9* gene in genomic DNA was amplified with the primer set (Kim *et al.*, 2016; Supplementary Tab. 2). The PCR was performed in a 50- $\mu$ L reaction mixture containing 0.1  $\mu$ g genomic DNA, 10  $\mu$ M each of forward and reverse primers, and 25  $\mu$ L of 2X PCR BIO Taq Mix Red (PCR Biosystems Inc., Wayne, Pennsylvania, USA). Amplification consisted of 40 cycles of 95°C for 30 s, 63°C for 45 s, and 72°C for 15 s in the Super Cycler<sup>TM</sup> SC-200 (Kyratrec life sciences, Mansfield, Queensland, Australia). The PCR product was electrophoresed in 1% agarose (LonsaSeakem<sup>®</sup>LE agarose; Rockland, Maine, USA) gel and stained with ethidium bromide (Sigma-Aldrich, Co.). The amplicons were visualized and photographed on an ultraviolet trans-illuminator equipped with a molecular imaging system (Kodak Image Station 4000 MM Pro; Carestream Health, Inc., New Haven, Connecticut, USA).

### Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was first extracted from the newly emerging leaves of PCR-positive plant using RNeasy<sup>®</sup> Mini Kit (QIAGEN GmbH, Hilden, North Rhine-Westphalia, Germany) to examine the transcription of the *Cas9* gene in the transformants. The RNA sample was then treated with DNase I (RNase-Free DNase Set; QIAGEN GmbH) once more to remove any contaminated genomic DNA. The first-strand cDNA was synthesized from 100 ng of purified RNA using SuperScript<sup>TM</sup> III First-Strand Synthesis System (Invitrogen, Carlsbad, California, USA). The real-time qPCR was performed in a 20- $\mu$ L reaction mixture containing 1  $\mu$ L of the synthesized cDNA, 0.8  $\mu$ M each of forward and reverse primers for *Cas9* and *Actin* mRNAs (Coker and Davies, 2003; Hahn *et al.*, 2017; Supplementary Tab. 2), and 10  $\mu$ L of 2X qPCR BIO SyGreen Blue Mix (PCR Biosystems Inc.) using Roter-Gene Q real-time PCR cyler (QIAGEN GmbH). The assay was run in triplicates by using the reaction mixture without cDNA as a negative control and *Actin* as an internal control. Reaction consisted of 40 cycles of 95°C for 10 s, 65°C for 10 s, and 72°C for 10 s. The expression fold change was estimated by using the comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method (Schmittgen and Livak, 2008).

### Ploidy analysis

The ploidy of putative transgenic plantlets was determined using a ploidy analyzer (PAII; Partech, Münster, North Rhine-Westphalia, Germany) in comparison with the wild type seedling. Each young leaf was chopped with a razor blade in 0.5 mL of nuclei extraction buffer (Sysmex Partech GmbH, Am Flugplatz, Görlitz, Germany). The suspension was filtered through a 30- $\mu$ m nylon mesh (Sysmex Partech GmbH), which was supported by a 1.6-mL tube, and kept at room temperature for 1 min. Then, 2 mL of the staining buffer (Sysmex Partech GmbH) was added to the filtrate and incubated for about 30 s. Lastly, the ploidy of the sample was measured after inserting the tube at the probe of the ploidy analyzer.

### Data collection and analysis

Overall, seven separate experiments were conducted to establish the ATMT procedure for 'Heinz 1706'. Unless otherwise noted, the experiments were replicated at least three times, and more than 100 explants were assayed each time. The percentage of transformation was calculated by counting the number of PCR-positive plantlets and divided by the total number of co-cultured explants. The collected data were subjected to ANOVA using SAS v. 9.4 (SAS Institute Inc., Cray, North Carolina, USA). Similarly, comparisons of significant means were conducted by Student's *t*-test (*t*-test), Chi-square ( $\chi^2$ ) test, Duncan's multiple range test (DMRT), or the least significant difference (LSD) test at  $p < 0.05$ . Mean  $\pm$  standard error (SE) was used to present the data.

## Results

### Selection of suitable explant type, hormone combination, and concentration of selectable agent

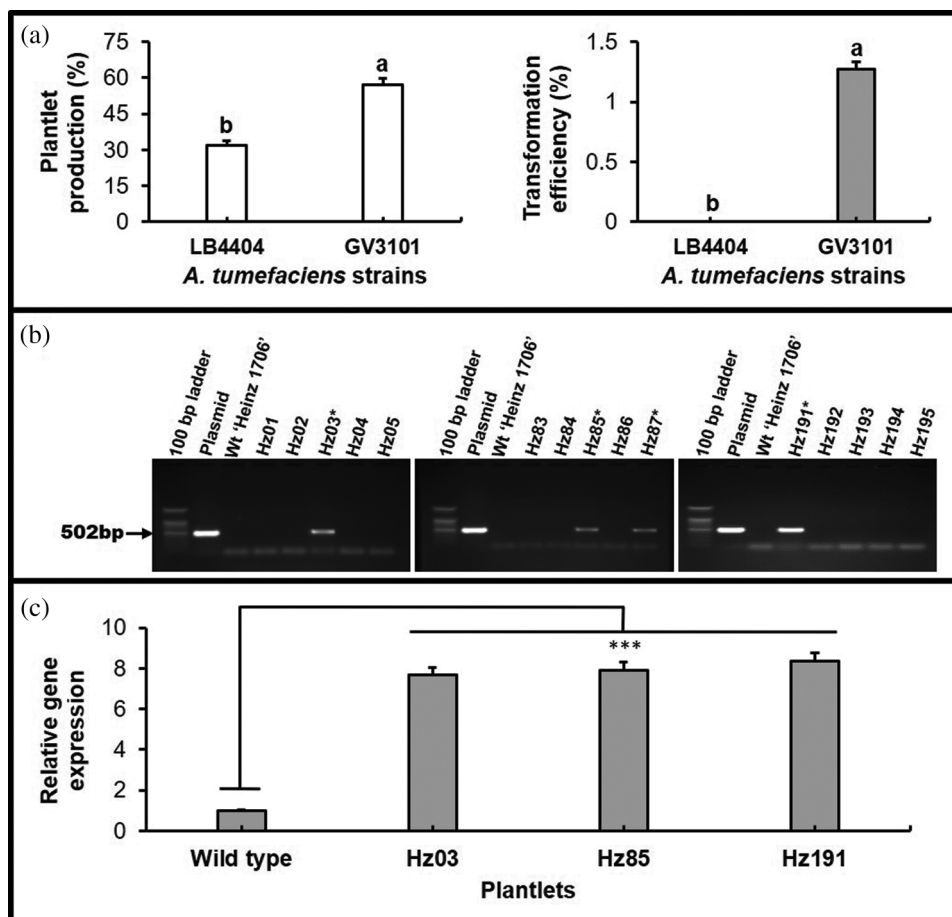
Prior to *Agrobacterium tumefaciens*-mediated transformation (ATMT) of 'Heinz 1706', the suitable explant type and phytohormone combination for leaf cluster induction in addition to the lethal dose of hygromycin B for later transformant-selection were screened. These preliminary studies showed that the cotyledonary explants were superior for leaf cluster induction to hypocotyl explants (Supplementary Fig. 1), which pushed through with cotyledon as the explant source for the following experiments. The results also indicated that the phytohormone combination of 2 mg/L trans-zeatin and 0.1 mg/L indole-3-acetic acid (IAA) was effective for leaf cluster induction from cotyledonary explants (Supplementary Fig. 2), and an addition of 10 mg/L hygromycin B was appropriate to select putative transgenic plantlets (Supplementary Fig. 3). Furthermore, the bacteria, which completed their role, was eliminated through the combined application of 500 mg/L carbenicillin (Sigma-Aldrich, Co.) and 250 mg/L Clavamox<sup>®</sup> (GlaxoSmithKline, Raleigh, North Carolina, USA) (Supplementary Tab. 3). Consequently, cotyledonary explants, together with the mentioned combination of phytohormones and concentration of antibiotics, were used for further ATMT studies.

### ATMT efficiency in 'Heinz 1706' was dependent on *A. tumefaciens* strain

To identify the more virulent strain of *A. tumefaciens* to 'Heinz 1706', a total of 1677 (1362 for strain LB4404 and 315 for strain GV3101) 1-d pre-cultured cotyledonary explants were co-cultured with the strains. Based on PCR results, four transformants were obtained only from the explants infected by GV3101. However, no transformants were recovered from LB4404 treatment (Fig. 2a and 2b). Furthermore, the results of the qRT-PCR analyses confirmed that the *Cas9* transgenes were stably expressed in the T<sub>0</sub> transgenic plants (Fig. 2c). Thus, strain GV3101 was chosen for examining other factors affecting ATMT.

### ATMT efficiency in 'Heinz 1706' was influenced by pre-culture and co-culture durations

The findings of this study demonstrated that pre-culture and co-culture durations were other important factors affecting



**FIGURE 2.** Suitability assessment of *A. tumefaciens* strain for the transformation of tomato 'Heinz 1706' and expression analysis of the *Cas9* gene. (a) Effect of strains on plantlet regeneration and transformation efficiency, Bars indicate SE, Values with different letters are statistically different ( $p < 0.05$ , by *t*-test); (b) Detection of *Cas9* gene in the transgenic plantlets generated using the bacterial strain GV3101 by PCR analysis, \* Indicate positive plantlets; (c) The transcription level of *Cas9* gene in transgenic  $T_0$  plants using the comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method (Schmittgen and Livak, 2008), Data represent the mean of five biological and three technical replicates, Bars indicate SE, \*\*\* indicate a significant difference between the wild type and  $T_0$  plants ( $p < 0.001$ , by LSD test).

transformation efficiency (Tab. 2). All the non-pre-cultured explants were finally necrotized without regenerative response regardless of co-culture duration, while the pre-cultured explants for 1 or 2 d successfully generated the plantlets with different regeneration and transformation frequencies (Tab. 2). Among the combinations of pre-culture and co-culture durations, significantly higher ( $p < 0.001$ ) number of transformants (three to four) were

obtained from the explants treated for 2 d pre-culture followed by 4 d co-culture (Tab. 2).

#### *Addition of acetosyringone enhanced the efficiency of ATMT in 'Heinz 1706'*

Various amounts of acetosyringone were supplemented to the co-culture medium to examine their contribution to transformation efficiency. The result showed that the

**TABLE 2**

#### Impacts of pre-culture and co-culture durations on transformation efficiency

Duration (days)		Plantlet regeneration (% $\pm$ SE) <sup>1</sup>	Transformation efficiency (% $\pm$ SE) <sup>2</sup>
Pre-culture	Co-culture		
0	2	0.0 $\pm$ 0.0 <sup>e</sup>	0.0 $\pm$ 0.0 <sup>f</sup>
0	3	0.0 $\pm$ 0.0 <sup>e</sup>	0.0 $\pm$ 0.0 <sup>f</sup>
0	4	0.0 $\pm$ 0.0 <sup>e</sup>	0.0 $\pm$ 0.0 <sup>f</sup>
1	2	6.0 $\pm$ 1.0 <sup>d</sup>	0.7 $\pm$ 0.6 <sup>e</sup>
1	3	10.8 $\pm$ 1.1 <sup>c</sup>	1.3 $\pm$ 0.7 <sup>cd</sup>
1	4	5.1 $\pm$ 1.3 <sup>d</sup>	0.3 $\pm$ 0.2 <sup>de</sup>
2	2	12.1 $\pm$ 1.3 <sup>b</sup>	1.9 $\pm$ 0.8 <sup>bc</sup>
2	3	12.7 $\pm$ 1.1 <sup>b</sup>	2.2 $\pm$ 0.6 <sup>b</sup>
2	4	14.2 $\pm$ 1.7 <sup>a</sup>	3.5 $\pm$ 1.1 <sup>a</sup>

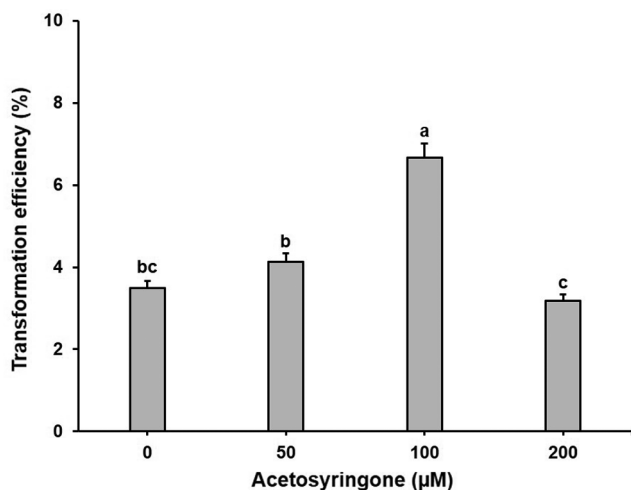
<sup>1</sup> Estimated from the number of plantlets developed from co-cultivated explants; <sup>2</sup> Estimated from the ratio of PCR-positive plantlets to the number of co-cultivated explants; Values with different letters in a column are statistically different (DMRT,  $p < 0.05$ )

addition of acetosyringone in the co-culture medium significantly influenced ( $p < 0.001$ ) the production of transgenic plantlets (Fig. 3). About a two-fold rise in transformation efficiency (6.7%: Seven transformants) was obtained with the addition of 100  $\mu\text{M}$  acetosyringone when compared to the acetosyringone-free medium. However, it appeared that the addition of 200  $\mu\text{M}$  acetosyringone has an adverse effect on the transformation (Fig. 3).

#### Rapid recovery of transgenic plantlets is possible through avoiding repeated subculture

On the leaf cluster induction medium, the leaf clusters were directly differentiated mainly from the basal cut-edges of the cotyledonary explants within three to four weeks (Fig. 4a). Interestingly, instead of shoot elongation, the leaf clusters became necrotic and replaced with new leaf clusters from the explants whenever subcultured on the fresh leaf cluster induction medium. Consequently, the firstly emerged leaf clusters were excised (Fig. 4b) and cultured on the shoot elongation/rooting (SER) media with different combinations of indole-3-butyric acid (IBA, Sigma-Aldrich, Co.) and trans-zeatin (Sigma-Aldrich, Co.) concentrations (Fig. 5). This combined addition of phytohormones to the SER media dramatically facilitated the elongation of shoots (Fig. 4c) and the subsequent development of plantlets with roots (Fig. 4d).

A significant variation ranging from 23.2% to 91.7% ( $p < 0.01$ ) in shoot elongation/rooting frequency was observed among the different SER media (Fig. 5). Compared with the frequencies in other SER media that reached a maximum at eight weeks of culture, the maximum frequency in SER10 medium was accomplished at six weeks of culture (Fig. 5). This result indicates that the SER10 medium containing 0.2 mg/L trans-zeatin and 2 mg/L IBA can reduce the duration for plantlet regeneration by two weeks. In addition, the SER10 medium was effective to recover a higher number of plantlets (Fig. 5). Meanwhile, some elongated shoots without roots and non-responsive leaf clusters were sub-cultured one more time on the respective fresh SER media, which also let them develop plantlets with different durations and frequencies. The



**FIGURE 3.** Effect of acetosyringone on transformation efficiency in tomato 'Heinz 1706'. Bars denote SE, Values with different letters are statistically different ( $p < 0.05$ , by LSD test).

acclimatized plantlets (Fig. 4e) exhibited a normal growth, flowering, and fruiting in a greenhouse (Fig. 4f).

#### The optimized ATMT procedure for 'Heinz 1706' can be adaptable to other cultivars

We applied the optimized ATMT procedure against cultivars 'Moneymaker' and 'Rubion' to evaluate its adaptability (Fig. 6). The PCR analyses of the regenerants showed high frequencies of transformation in the cultivars [15.2% (sixteen transformants) in 'Moneymaker' and 20.0% (twenty-one transformants) in 'Rubion'] (Supplementary Fig. 4; Fig. 7), indicating the applicability of the current procedure to other tomato cultivars.

#### Flow cytometric analysis revealed a low frequency of ploidy changes in regenerants

To check the frequency of ploidy changes in the regenerants derived through the present optimized procedure, all the regenerated plantlets were analyzed and compared with their respective *ex-vitro* developed seedlings. The peaks of relative DNA contents measured by flow cytometry indicated that only 5% of the regenerants showed an altered ploidy level (Supplementary Fig. 5). Thus, the minor somaclonal variation observed in the regenerants further strengthened the effectiveness and reproducibility of our ATMT procedure.

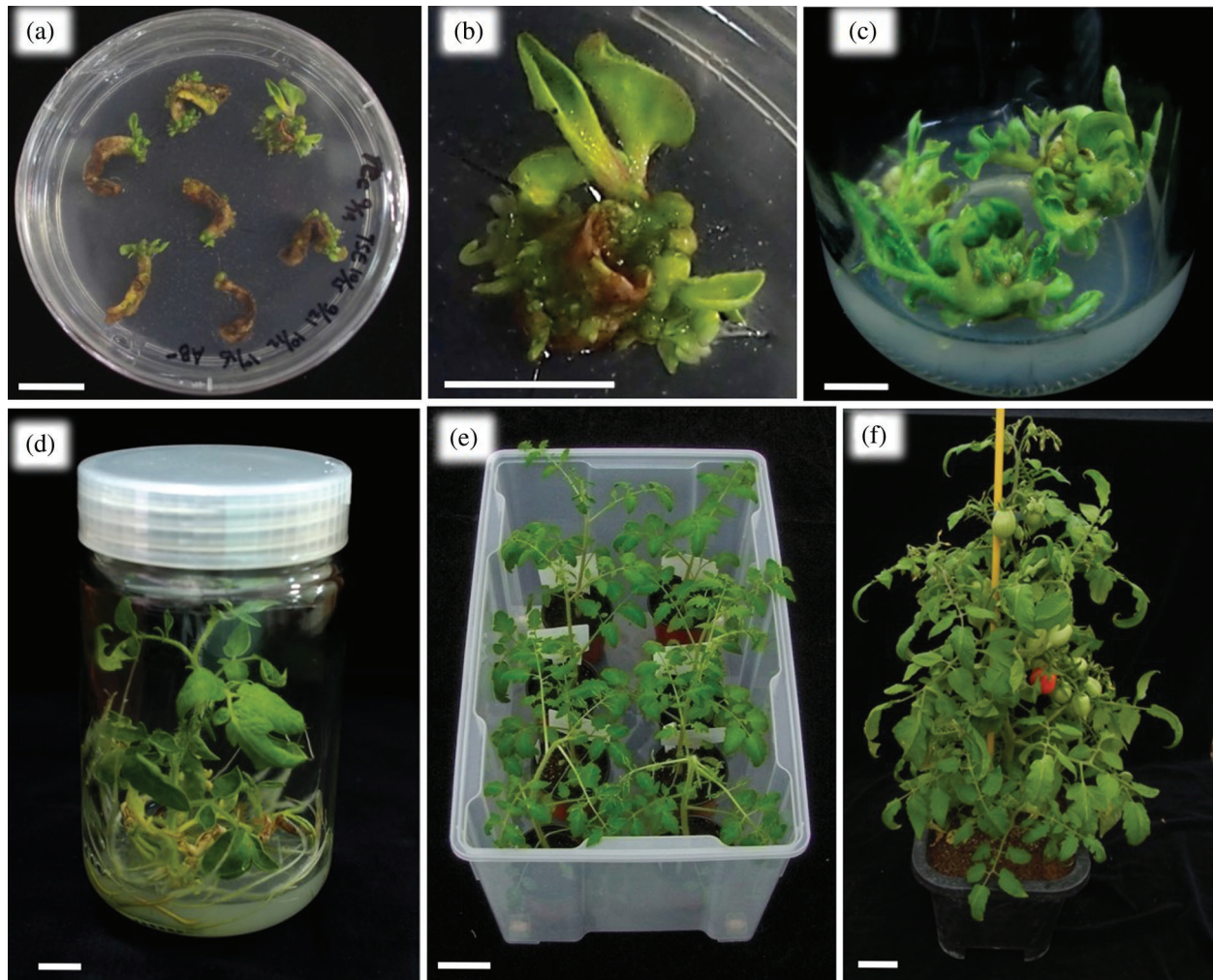
#### The Cas9 genes were stably transmitted to the next generation

Successful transmission of a foreign gene to the next generation is vital for maintaining the transgenic lines. In this regard, forty  $T_1$  seedlings were developed from each  $T_0$  plant and analyzed by PCR amplification. The PCR results revealed that the *Cas9* genes of  $T_0$  plants were typically transmitted to  $T_1$  generation with 3:1 ratio (Tab. 3).

## Discussion

Previous studies have described that the *Agrobacterium tumefaciens*-mediated transformation (ATMT) system is one of the widely employed genetic modification tools to support plant molecular studies and crop improvements (Arshad *et al.*, 2014; Hayut *et al.*, 2017; Lacroix and Citovsky, 2019; Nonaka *et al.*, 2019; Wang *et al.*, 2019; Zhao *et al.*, 2015). Nevertheless, an effective ATMT procedure optimized for one specific cultivar may not necessarily ensure a similar efficacy in other cultivars (Prihatna *et al.*, 2019; Stavridou *et al.*, 2019). Even in our initial attempt of the transformation study, the broad-spectrum tomato ATMT procedure of Park *et al.* (2003) gave rise to the extremely low frequency of transformation (0 to 1.3%) in cultivar 'Heinz 1706' (Fig. 2a), which is at least 18.7% lower than their average 20% transformant recovery rate. This result indicates that the optimization of factors affecting ATMT is imperative for achieving higher transformation efficiency in the recalcitrant 'Heinz 1706'.

The influences of various *A. tumefaciens* strains on the transformation of tomato 'Micro-Tom' have been described by Chetty *et al.* (2013) and Nonaka *et al.* (2019), who demonstrated the differential transformation efficiencies ranging from 15% to 72%, illustrating that the



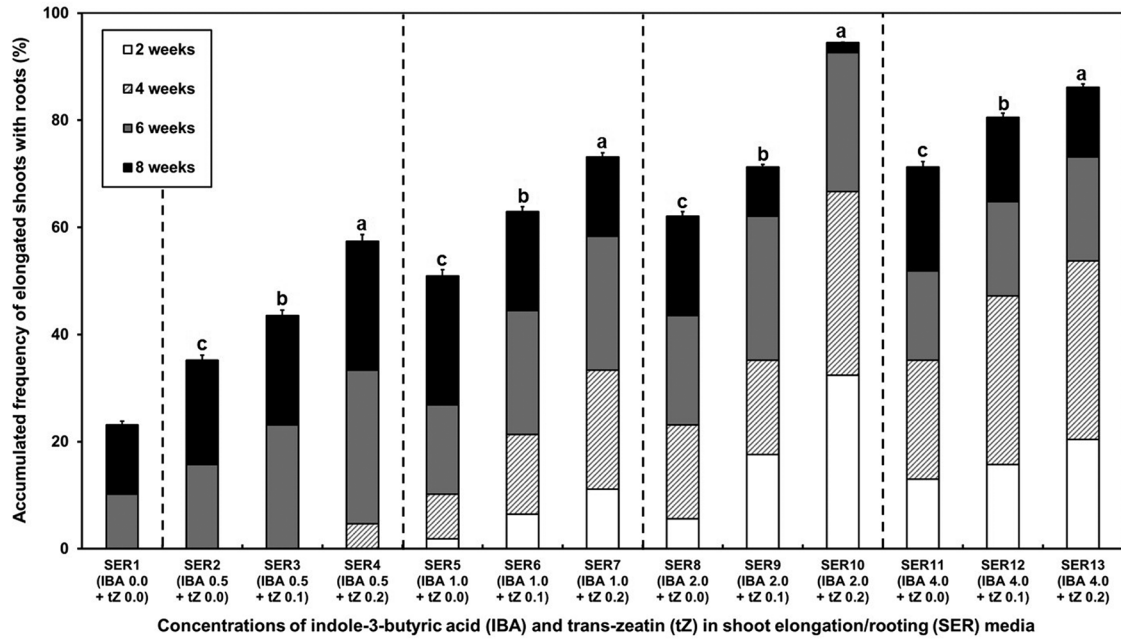
**FIGURE 4.** *In vitro* organogenesis and *ex vitro* growth stages of transgenic tomato 'Heinz 1706'. (a) Regenerated hygromycin-resistant leaf clusters after four weeks culturing on the leaf cluster induction medium; (b) A magnified view of a 4-week-old leaf cluster; (c) Well elongated shoots on the shoot elongation/rooting (SER) medium; (d) A vigorously rooted plantlet on the SER medium; Scale bars in (a–d) = 1 cm; (e) Soil-acclimatized putative transgenic plantlets; (f) An adult transgenic plant at fruiting stage in a greenhouse; Scale bars in (e–f) = 5 cm.

transformation efficiency is *A. tumefaciens* strain-dependent. On the contrary, Sharma *et al.* (2009) showed tomato cultivar-dependent transformation efficiencies (22% for 'Arka Vika' to 41% for 'Sioux') using the same *A. tumefaciens* strain AGL1. These examples implicate the significance of physiological compatibility between host and bacterial genotypes for successful ATMT in tomato. Indeed, the extremeness of ATMT efficiency depending on *A. tumefaciens* strain that ranged between 0% for LBA4404 (Fig. 2a) and 6.7% for GV3101 (Figs. 3 and 7) was observed in our study using 'Heinz 1706'. Thus, it is possible to suggest that 'Heinz 1706' might be more compatible with GV3101 than LBA4404.

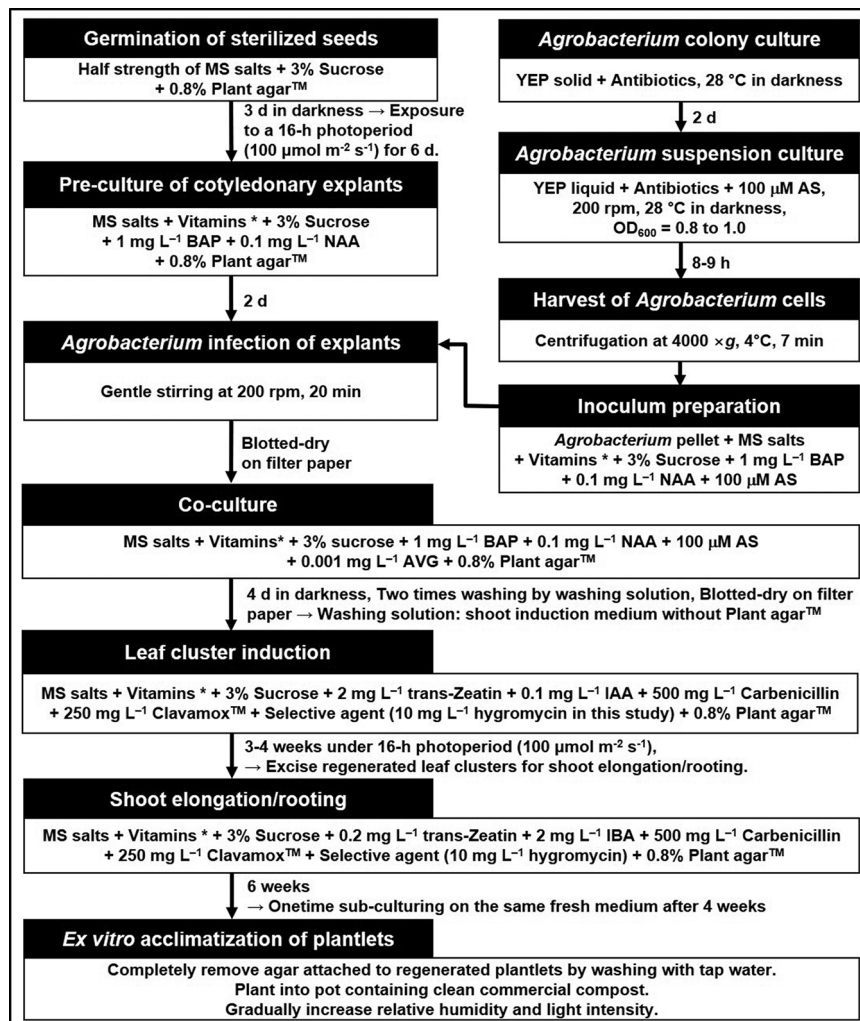
Various studies have indicated the necessity of using a feeder layer during pre- and co-cultures in tomato ATMT (Fillatti *et al.*, 1987; Gupta and Van Eck, 2016; Rai *et al.*, 2012). However, it is not easy to approach the method due to its complexity. Thus, we employed the pre- and co-culture media of Park *et al.* (2003) with the modifications in the culture duration and acetosyringone concentration. Several researchers have described that the pre- and co-

culture durations are crucial factors to increase the efficiency of transformation in tomato (Park *et al.*, 2003; Sharma *et al.*, 2009; Stavridou *et al.*, 2019), and they showed that 1-d pre-culture/3-d co-culture, 2-d pre-culture/3-d co-culture, and 1-d pre-culture/2-d co-culture was optimum, respectively. However, their observations were different from the findings of our study, in which a 2-d pre-culture/4-d co-culture was the best combination to obtain the highest efficiency of ATMT in 'Heinz 1706' (Tab. 2). Thus, our study illustrated that the longer pre-culture/co-culture duration (2/4 d) might be favorable to the bacteria to perform a series of T-DNA transfer process into the recalcitrant 'Heinz 1706' cells.

It is eminent that the supplementation of exogenous acetosyringone is effective in stimulating *A. tumefaciens* attachment on the explant cells and encouraging the bacterial *vir* genes expression (Nonaka *et al.*, 2008; Stachel *et al.*, 1986; Wu *et al.*, 2006). Our study proved that the addition of 100  $\mu$ M acetosyringone in the co-culture medium was optimum to improve the transformation efficiency in 'Heinz 1706' (Fig. 3). Even though the concentrations of acetosyringone up



**FIGURE 5.** Time-series comparison of various combinations of IBA (mg/L) and trans-zeatin (mg/L) added to the shoot elongation/rooting (SER) medium on transgenic plantlet development from leaf cluster explants of tomato 'Heinz 1706'. Values with different letters in the domain with the same concentration of IBA are statistically different (by DMRT,  $p < 0.05$ ).



**FIGURE 6.** A flowchart of the optimized *Agrobacterium tumefaciens*-mediated transformation procedure for tomato 'Heinz 1706'. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; BAP, 6-benzyl-aminopurine; NAA,  $\alpha$ -naphthalene acetic acid; AVG, aminoethoxyvinylglycine; AS, acetosyringone; \* vitamins: 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 0.1 mg/L thiamine HCl.



TABLE 3

Segregation pattern of the *Cas9* gene in T<sub>1</sub> progenies of 'Heinz 1706' as detected by PCR

T <sub>0</sub> events	Analyzed T <sub>1</sub> seedlings	PCR-positive T <sub>1</sub> seedlings	PCR-negative T <sub>1</sub> seedlings	$\chi^2$ value
Hz003	40	29	11	0.13 <sup>ns</sup>
Hz085	40	33	7	0.17 <sup>ns</sup>
Hz191	40	27	13	1.20 <sup>ns</sup>

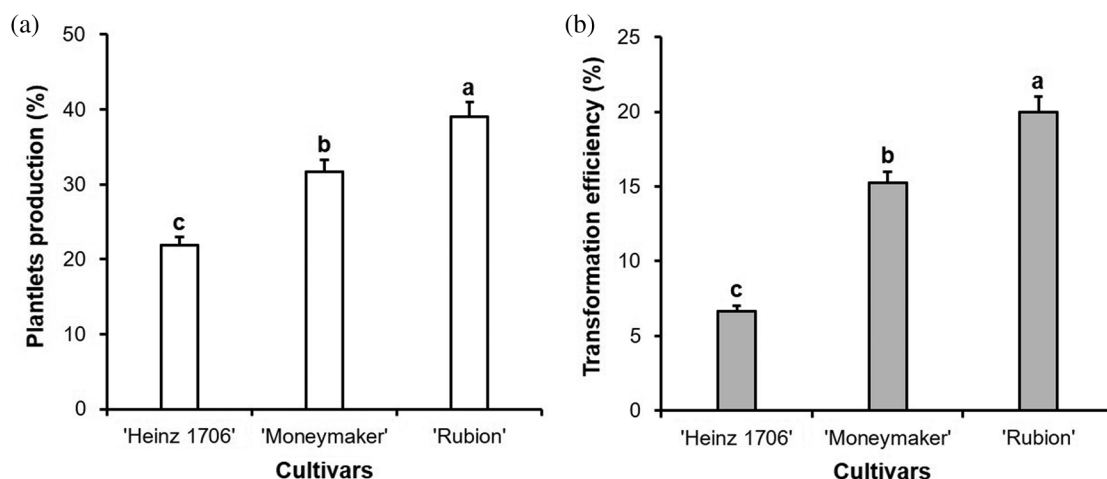
<sup>ns</sup> Indicate the segregation patterns of the *Cas9* gene are in accordance with the expected 3:1 Mendelian inheritance ratios ( $\chi^2$ ,  $p < 0.05$ )

FIGURE 7. Plantlets production (a) and transformation efficiencies (b) of three tomato cultivars when applying the ATMT procedure optimized for 'Heinz 1706' in this study. Bars denote SE. Values with different letters are statistically different ( $p < 0.05$ , by LSD test).

to 500  $\mu$ M have been applied to increase the success of transformation in tomato (Fuentes *et al.*, 2008; Khuong *et al.*, 2013; Nonaka *et al.*, 2019; Raj *et al.*, 2005; Stavridou *et al.*, 2019), the addition of 200  $\mu$ M acetosyringone adversely affected the transformation efficiency in our study (Fig. 3). The response variation in the efficiency of transformation by the supplementation of acetosyringone is evident owing to the difference in co-culture duration, the competence of the tissue, plant species, and explant type (Shrawat *et al.*, 2007), which might have a differential effect on the secretion of endogenous phenolic compounds.

Reduction in transformation duration and steps have been described as another important factor in assessing the utility of the ATMT procedure (Cruz-Mendivil *et al.*, 2011; Gupta and Van Eck, 2016; Park *et al.*, 2020; Sun *et al.*, 2015; Xu *et al.*, 2020). The whole ATMT in our procedure only required about three months, starting from seed sowing to attaining acclimated transgenic plantlets (Fig. 6). In contrast, longer durations between four months (Cruz-Mendivil *et al.*, 2011; Van Eck *et al.*, 2019) and ten months (Ellul *et al.*, 2003) were reported. The short transformation cycling period shown in our study is achieved through amalgamating the independent shoot elongation and rooting activities in a single step, avoiding the frequent subculture in each step, and optimizing phytohormonal condition (0.2 mg/L trans-zeatin and 2 mg/L IBA) in the shoot elongation/rooting (SER) medium.

Interestingly, our rapid procedure resulted in fewer regenerants with abnormal ploidy (5%, Supplementary

Fig. 5c) compared to the previously reported high frequencies (from 24.5 to 80 %) (Den Bulk *et al.*, 1990; Ellul *et al.*, 2003; Fillatti *et al.*, 1987; Ling *et al.*, 1998). The low frequency of ploidy changes shown in this study might be associated with the less frequent subculture and the shorter culture duration. In agreement with the findings of our study, Bidabadi and Jain (2020) and Sun *et al.* (2013) suggested that rapid regeneration of a plantlet by shortening the *in vitro* culture period is important to reduce the rate of somaclonal variations in regenerants. Likewise, the elevated occurrence of ploidy changes during long *in vitro* culture was also reported (Niedz and Evens, 2016; Ochatt *et al.*, 2011). Therefore, we strongly recommend the ploidy measurement of transformants before drawing conclusive remarks on the role of a transgene in tomato ATMT.

The qRT-PCR analysis revealed that our ATMT procedure was successful to create *Cas9*-expressing 'Heinz 1706' lines (Fig. 2c). The lines would hold a merit in the case of protoplast-transfection of the *in vitro* transcribed single guide RNAs (sgRNAs) or plasmid vectors expressing sgRNAs for targeted gene editing (Ali *et al.*, 2015; Svitashv *et al.*, 2016; Yin *et al.*, 2015). In CRISPR/*Cas9*-based gene editing, unexpected mutations at a non-specific site (off-target effects) frequently occur (Cardi *et al.*, 2017; Lee and Kim, 2018; Zhang *et al.*, 2018), which requires the genome-wide sequencing on the edited plants. Thus, the 'Heinz 1706' with an unveiled whole-genome sequence is very useful to simplify the detection of off-target edits. On this account, the *Cas9*-expressing 'Heinz

1706' lines, which were created in this study, can be an important plant material for detecting the off-target edits.

## Conclusion

In this study, a rapid and reproducible *Agrobacterium tumefaciens*-mediated transformation (ATMT) procedure was established for the recalcitrant tomato (*Solanum lycopersicum* cv. Heinz 1706) for the first time by optimizing the factors relevant to ATMT. All the studied factors were found to be basic for enhancing transformation efficiency, while the amalgamation of the shoot elongation and rooting steps distinctly leads to a rapid recovery of transgenic plants. The rapid ATMT procedure also has a merit for obtaining experimental results earlier and reducing the frequency of ploidy changes in the transgenic plants. Therefore, we expect that our procedure enriches the ATMT technique for the effective transfer of various desirable genes and contributes to advance the emerging CRISPR/Cas9-based genome editing studies in tomato. Besides, the Cas9-expressing 'Heinz 1706' lines obtained in this study might be valuable for genome editing studies such as sgRNA transfection and off-target detection.

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**Availability of Data and Materials:** All data generated or analyzed during this study are included in the manuscript. Data on preliminary tests are also provided in separate files as supplementary materials (Supplementary Tables and Figures). Thus, the readers can freely access all the published data with proper acknowledgment and citation. Besides, all the raw data sets generated or analyzed during the current study are available from the corresponding author on reasonable request.

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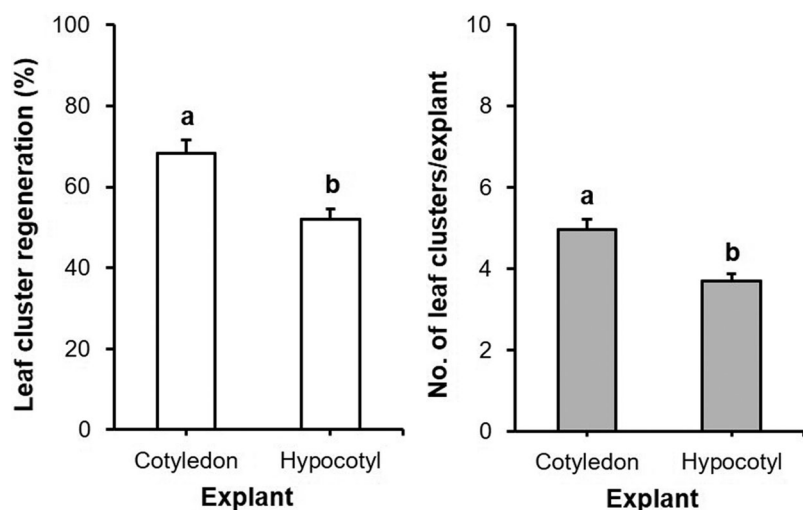
**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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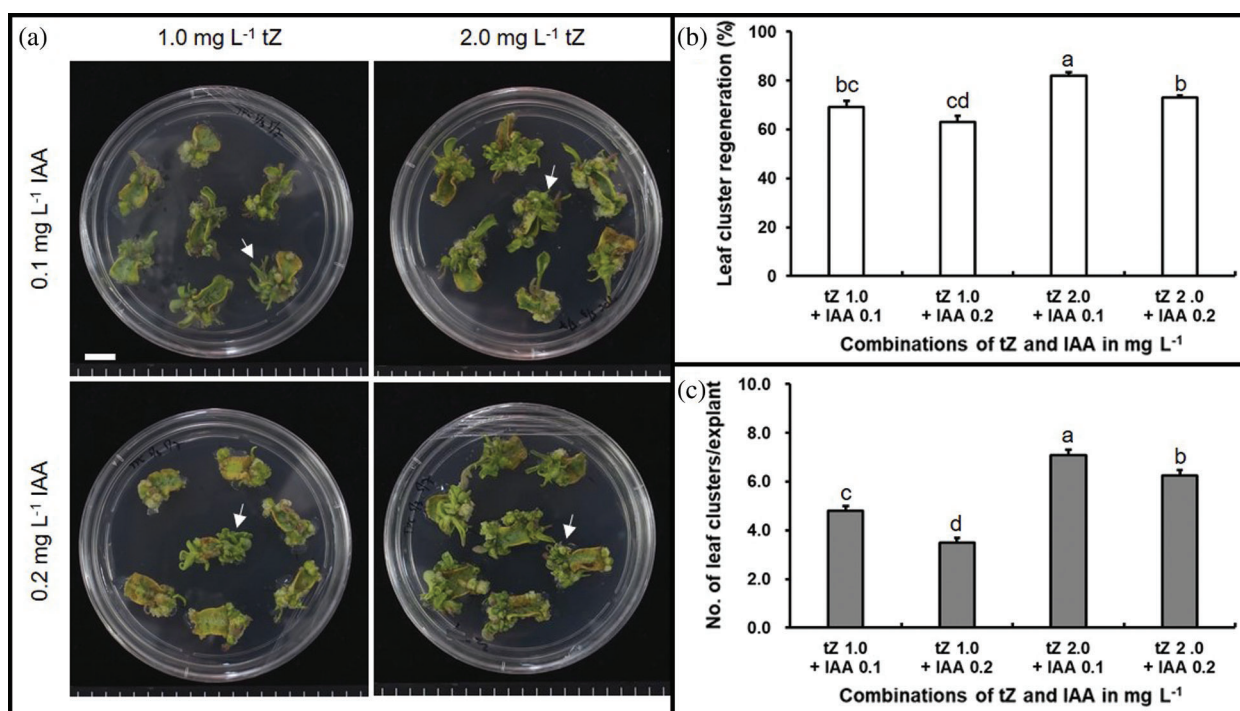
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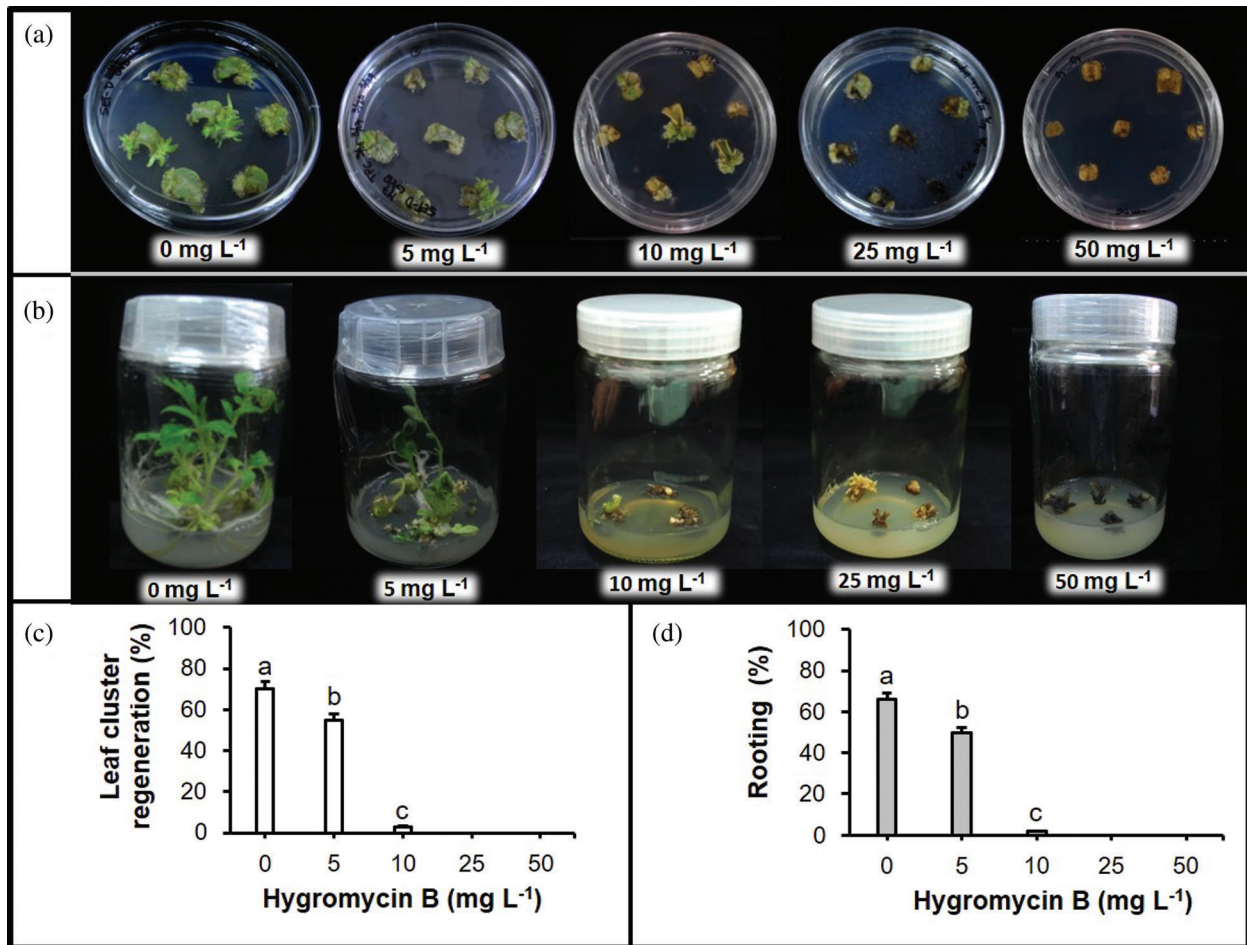
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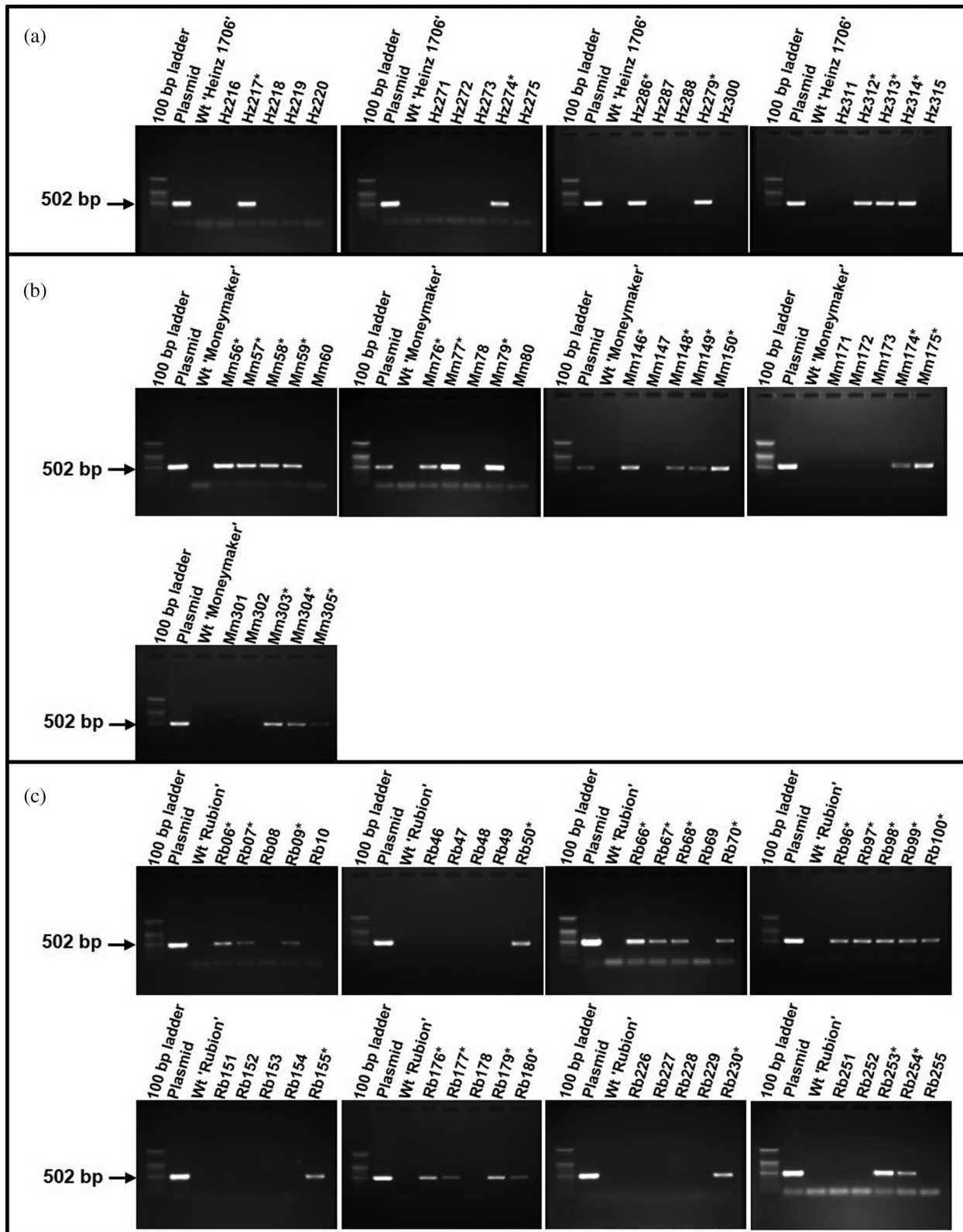
**SUPPLEMENTARY FIGURE 1.** Comparison of cotyledon and hypocotyl explants on leaf cluster induction in tomato 'Heinz 1706' on four weeks. Bars denote SE, Bars with different letters are statistically different by the Student's *t*-test ( $p < 0.05$ ).



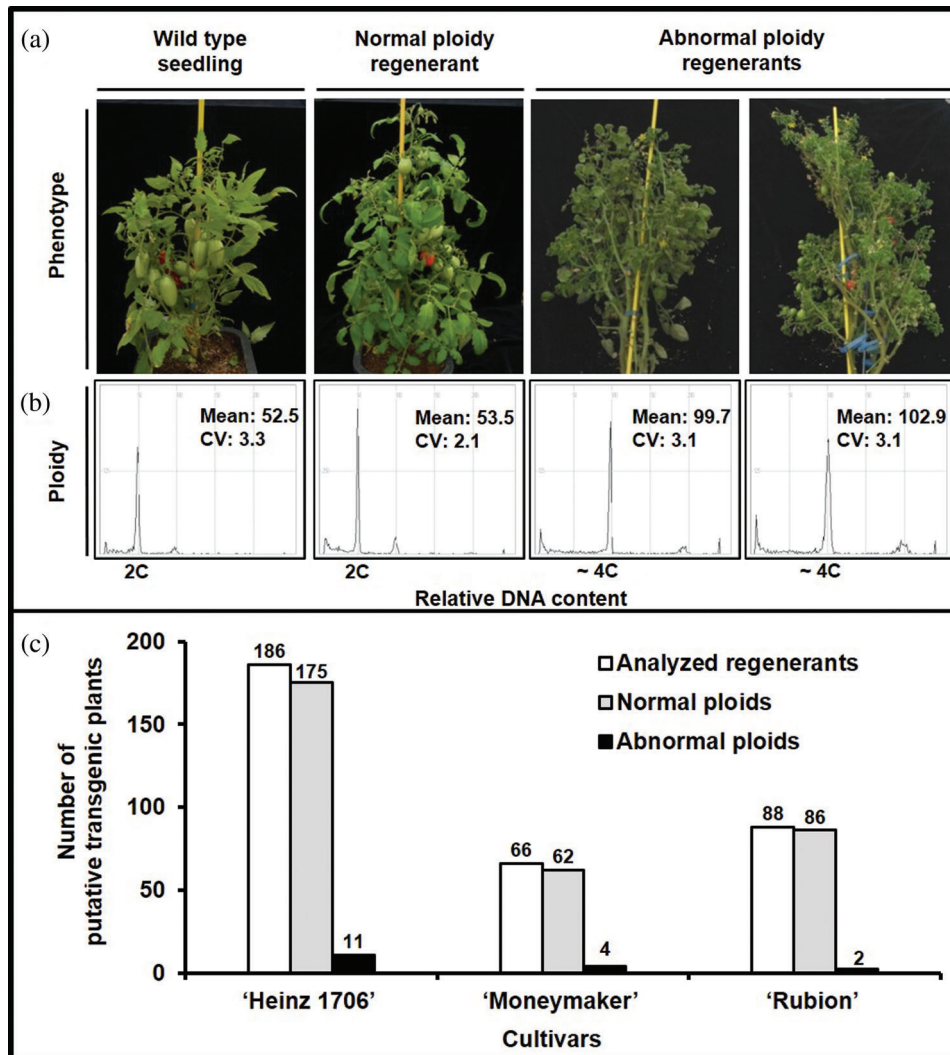
**SUPPLEMENTARY FIGURE 2.** Effects of phytohormones on leaf cluster regeneration from cotyledonary explants in tomato 'Heinz 1706' on four weeks. (a) Regenerated leaf clusters from explants on the media supplemented with different combinations of indole-3-acetic acid (IAA) and trans-zeatin (tZ), Arrows indicate leaf clusters, Scales bars indicate 1 cm; (b and c) Statistics of leaf cluster induction. Bars indicate SE ( $n = 105$ ), and bars with different letters are statistically different by the LSD test ( $p < 0.05$ ).



**SUPPLEMENTARY FIGURE 3.** Lethal effects of hygromycin B in tomato 'Heinz 1706' after four weeks. (a) On leaf cluster regeneration from cotyledonary explants; (b) On shoot elongation and rooting from leaf clusters; (c and d) Statistics of hygromycin B on organogenesis. Data delineate the average of three replicates, and Bars denote SE, and bars with different letters are statistically different by the LSD test ( $p < 0.05$ ).



SUPPLEMENTARY FIGURE 4. Detection of the *Cas9* gene in transgenic plantlets by PCR analysis. (a) 'Heinz 1706'; (b) 'Moneymaker'; (c) 'Rubion'. \* Indicate transgenic plantlets.



**SUPPLEMENTARY FIGURE 5.** Summary of changes in ploidy level of the regenerants in three tomato cultivars. The phenotype of regenerants (a) and each ploidy level (b); (c) The number of plants with normal and abnormal ploid.

**SUPPLEMENTARY TABLE 1**

Components of solid and liquid yeast extract peptone (YEP) media used to culture *A. tumefaciens* strains LB4404 and GV3101 harboring vector pHA<sub>t</sub>C

Components	LB4404		GV3101	
	Solid medium	Liquid medium	Solid medium	Liquid medium
Bacto yeast extract (g L <sup>-1</sup> )	10	10	10	10
Bacto peptone (g L <sup>-1</sup> )	10	10	10	10
Sodium chloride (g L <sup>-1</sup> )	5	5	5	5
Bacto agar (g L <sup>-1</sup> )	15	–	15	–
Spectinomycin (mg L <sup>-1</sup> )	50	50	–	–
Streptomycin (mg L <sup>-1</sup> )	50	50	–	–
Rifampicin (mg L <sup>-1</sup> )	50	50	50	50
Gentamycin (mg L <sup>-1</sup> )	–	–	50	50

The pH of both liquid and solid YEP media was rectified to 7.5 prior to autoclaving.



SUPPLEMENTARY TABLE 2

## Lists of primer sets used for PCR and qPCR analyses in this study

Genes	Analyses	GenBank accession number	Primer sequences	
			Forward (5'-3')	Reverse (5'-3')
<i>Cas9</i> gene <sup>1</sup>	PCR	KU213971.1	GAAGATCGAGAAGATCCTGA	GAAGTCCTTGCCTTGATGA
<i>Cas9</i> mRNA <sup>2</sup>	qPCR	KY080691.1	AAGAGCGAGGAGACCATC	GCAGCACCTTCTCGTTGG
<i>Actin</i> mRNA <sup>3</sup>	qPCR	NM_001330119.1	CCAACGAGAAGGTGCTGC	ACCTTGCGGTTGGTCTTG

<sup>1</sup>Kim *et al.* (2016); <sup>2</sup>Hahn *et al.* (2017); <sup>3</sup>Coker and Davies (2003).

SUPPLEMENTARY TABLE 3

## Assessment of different antibiotics for eliminating the agrobacteria completed their role

Antibiotics	Overgrowth status of agrobacteria (rating scale)					
	Strain LB4404			Strain GV3101		
	Week 2	Week 3	Week 4	Week 2	Week 3	Week 4
Cefotaxime (500 mg L <sup>-1</sup> )	+	++	++++	+	+++	++++
Timentin (300 mg L <sup>-1</sup> ) + Cefotaxime (200 mg L <sup>-1</sup> )	-	+	++	-	+	++
Carbenicillin (500 mg L <sup>-1</sup> ) + Clavamox (250 mg L <sup>-1</sup> )	-	-	-	-	-	-

## Rating scales

Rating	Description*
-	0% overgrowth of bacteria observed on explants
+	< 10% overgrowth of bacteria observed on explants
++	< 25% but > 10% overgrowth of bacteria observed on explants
+++	< 50% but > 25% overgrowth of bacteria observed on explants
++++	> 50% overgrowth of bacteria observed on explants

\*Indicate that more than 65 cotyledonary explants were tested at one time, and the set of experiments were repeated at least three times.