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Establishment of an *In Vitro* Propagation, Transformation, and Gene Editing System in Tomato *(Solanum lycopersicum)*

Galal Khamis [1, 2, *], Jun Chi [2], Qingshun Zhao [2, 3]

1. Department of Laser Application in Metrology, Photochemistry and Agriculture National Institute of Laser Enhanced Science, Cairo University, Egypt; E-Mail: Galal.khames@niles.edu.eg
2. Institute of Genome Editing, Nanjing YSY Biotech Company, No. 1 Amber Road, Nanjing, Jiangsu 211812, China; E-Mails: chijun@njfish.cn; qingshun@nju.edu.cn
3. Model Animal Research Center, Nanjing University, 12 Xuefu Road, Pukou High-tech Development Zone, Nanjing, Jiangsu 210061, China

* **Correspondence:** Galal Khamis; E-Mail: Galal.khames@niles.edu.eg

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Abstract

Tomato (*Solanum lycopersicum*), a family of Solanaceae, is an annual crop grown in fields and greenhouses. Tomato is the fourth-largest agricultural crop in the world, with a market value of more than $50 billion. Numerous attempts were conducted on the tomato for further improvement via *in vitro* regeneration and transformation. This study aims to establish *in vitro* multiplication, transformation, and gene editing systems in tomatoes by using cotyledon explants. Cotyledon explants were placed on the MS medium supported with 6-benzyl adenine (BA) and kinetin (Kin) at four different concentrations for shoot induction. The shoot induction from cotyledon explants significantly increased, and the regeneration rate was 45% after using BA at 8.8 µM compared to Kin. For root induction, indole-3-butyric acid (IBA) at 4.8 µM concentration revealed the highest number of explants producing root (55%). Three *Agrobacterium tumefaciens* strains (EHA105, GV3101, and LBA4404) were used to establish and develop a tomato transformation method. Each *Agrobacterium* strain harbors the plasmid pCAMBIA2301, including the *nptII* marker. *Agrobacterium* strain LBA4404 showed the lowest number of dead explants, besides increasing the number of explant-produced shoots

(30%) and the number of shoots per transformed explants compared to GV3101 and EHA105 strains. Our results revealed that the success of tomato transformation depends on selecting a suitable *A. tumefaciens* strain. Finally, the tomato gene editing system was successfully established by transferring the pCAMBIA1300-pCas9-sgRNA-U3 binary vector into the cotyledon using the selective *Agrobacterium* strain LBA4404, and the transformation effectivity was confirmed by PCR analysis.

Keywords
Tomato; cotyledon explants; regeneration; *Agrobacterium tumefaciens;* CRISP/Cas9

1. Introduction

Tomato, a diploid plant with 24 chromosomes and a genome of 950 Mb [1], is a commercial crop with a market value of over $50 billion [2, 3]. It is a model plant for studying recombinant genes and plant biology and is a valuable source of minerals, vitamins, and phytochemicals [4, 5]. Due to the high commercial value, numerous attempts were made to improve the tomato's quality *via in vitro* regeneration and transformation [6]. [4] revealed the first report on tomato transformation, and it was reported that the efficiency of tomato plant regeneration and transformation is influenced by factors such as explant type, growth regulators, and bacterial density, with rapid improvement over the past three decades. Moreover, the first study used anther culture to produce tomato haploid culture, with naphthalene acetic acid and kinetin-supported growth media [7]. Then, modified for microspores [8], which were converted into haploid embryoids with an increase in the anther-produced callus percentage by 38% [9].

Furthermore, tomato protoplast isolation and root culture have been conducted since 1934, but shoot induction in tomatoes is lower than in *L. esculentum* roots [10, 11]. Furthermore, tomato mass propagation has been attempted through shoot tip culture [12], somatic embryogenesis [13], and direct organogenesis using callus originating from hypocotyls or cotyledon explants; for instances, Aroshko et al. (2023) were presented a method for shoot induction through callus formation in tomato Micro-tom cultivar by using cotyledon explant placed on MS media supplemented with different concentration of Zeatin and Indol 3 Acetic Acid [14]. Thus, successful propagation depends on genotype, explant type, and plant growth regulator. Researchers need an efficient genetic transformation protocol for understanding functional genomics and gene functions, as tomato regeneration efficiency ranges from 1.8-37%, highlighting the need for improved protocols [2, 15].

Agrobacterium tumefaciens is a crucial transformation method for plant growth, offering advantages like gene integration, low copy number, and fertile transgenic plants [16]. Factors affecting transformation efficiency include explant type, Agrobacterium strain, infection time, and infection and incubation time. Although there are several studies on the plant regeneration of tomatoes, the efficiency of such a transformation protocol is generally low due to the inability of many leaf/cotyledon cells to regenerate into the shoot and root [3].

Over the past two decades, molecular and genomic biotechnology techniques have significantly advanced gene function research [17]. Genome editing methods, such as zinc finger nucleases [18] and TALENs [19], have enabled studies in reverse genetics and targeted gene parts in DNA

sequences. Whereas the third method, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) endonucleases [20], is based on an RNA-guided DNA endonuclease mechanism. These modified nuclease systems improve double-strand breaks (DSB) to induce site-directed mutations [20]. The CRISPR/Cas9 system is faster due to its high specificity and single guide RNA design, which is complementary to the gene of interest in the DNA sequence [21]. The CRISPR/Cas9 gene editing technique has been successfully applied in mutagenesis applications in plant varieties like Arabidopsis, *Nicotiana benthamiana*, and tomato [22, 23]. Tomatoes are ideal for investigating gene editing in dicot crops [20]. Furthermore, several studies showed methods for gene editing in tomatoes [24, 25], but more research is needed to establish the technique.

The study of fruit fleshy species is crucial due to the regulation of ripening mechanisms, which involve irreversible physiological and biochemical changes affecting fruit features like color, flavor, aroma, and size [26, 27]. Several studies reveal that the ripening-inhibitor gene (RIN) masters tomato fruit ripening, promoting changes like tomato red pigmentation and ethylene biosynthesis [21]. In several studies, The RIN locus mutation inhibits ripening, preventing ethylene production, preserving flesh firmness, and preventing red color conversion [28].

In this context, our study aimed to establish a regeneration and efficient transformation system to facilitate the capability to explore tomato's ripening mechanism through the Rin gene's knockout mechanism by CRISPR/Cas9 technique. We will establish an *in vitro* regeneration system in tomatoes using BA and Kin plant growth regulators at different concentrations. Also, a transformation system in tomato was established by using three strains of *A. tumefaciens* (GV3101, EHA 105, and LBA 4404) to select the most efficient Agrobacterium strain. After that, the *A. tumefaciens* strain that will show the highest transformation efficiency will be used to transfer the CRISPR/Cas9 construct containing sgRNA to target the genomic sequence of the Rin gene to explore further the mechanism of ripening in tomatoes through CRISPR/Cas9 knock-in mutation.

2. Material and Methods

2.1 Culture Conditions

The tomato seeds (*Solanum lycopersicum*) Agiad 16 cultivar were obtained from the Vegetables Research Institute, El-Doqi, Giza, Egypt. 10% commercial bleach (9.4% active chlorine) was used for 15 min. for seed sterilization. The seeds were washed four times with sterilized distilled water. The sterilized seeds were germinated using 4.2 gm per liter of MS [29], with vitamins (Sigma-Aldrich 5893) medium fortified by sucrose 3% (w/v), 0.3% phytagel, and the pH was adjusted to pH 5.7 using 1 M NaOH. Afterward, the medium and instrument were autoclaved for 20 min at 121°C and 15 psi pressure. The cultured seeds were maintained in a growth chamber at 24°C under a 16-hour photoperiod, which was controlled through cool white, fluorescent tubes with a photosynthetic photon flux density (PPFD) of 30 mmol m^{-2} s^{-1} PAR and 70% relative humidity. After 10 days, the cotyledon explants without the meristem were taken from the regenerated seedlings.

2.1.1 Shoots Induction

The whole cotyledon was excised from an aseptic tomato seedling and used as an explant to produce multiple shoots. Cotyledon explants were placed on an MS medium with vitamins

supported with kinetin (Kin) (Sigma-Aldrich-K3378) and 6-benzyl adenine (BA) (Sigma-Aldrich-B3274), which were used separately at four concentrations (2.2, 4.4, 8.8, and 17.6 µM). 30 cotyledon explants were used with each treatment and distributed on 3 petri dishes (10 cm), where each petri dish contained 10 cotyledon explants. The experiment was performed with three repetitions. Subculturing in the same medium was performed every 2 weeks. The cultured explants were maintained in a growth chamber at 24°C under a 16-hour photoperiod, which was controlled through cool white, fluorescent tubes with a photosynthetic photon flux density (PPFD) of 30 mmol m^{-2} s^{-1} PAR and 70% relative humidity. After 6 weeks, several parameters were recorded, including the percentage of regenerated explants that produced shoots, the number of shoots per explant, and shoot length. Then, explants with multiple shoots were moved to MS medium with vitamins supplied with the optimum concentration of cytokinins and mixed with α-naphthalene acetic acid (NAA) (1.3, 2.6, and 5.2 µM) at different concentrations for shoot proliferation and elongation. Also, after 4 weeks, several parameters were recorded: the explants produced shoots (%), the number of shoots per explant, and shoot length.

2.1.2 Root Induction and Acclimatization

Shoots with 2 or 3 leaves were taken and transferred to a half-strength MS medium with vitamins supported with three concentrations of indole-3-butyric acid (IBA) (1.2, 2.4, and 4.8 µM) for 2 weeks. Then, the shoots were sub-cultured for another 2 weeks on a half-strength MS medium with vitamins. After 4 weeks of the rooting stage, several factors were recorded, such as the shoots producing roots (%), the number of roots per shoot, and root length. For the acclimatization stage, complete tomato plantlets were moved to 6 cm pots containing soil (natural clay and peat, ratio 2:1) for 2 weeks with irrigation every 2 days under greenhouse conditions at 22°C and 12 h light/dark conditions by using 350 mol m^{-2} s^{-1} sodium vapor lamps. To maintain humidity, the pots were covered with plastic bags, which were removed for another 2 weeks to complete the acclimatization.

2.2 The Preparation of Agrobacterium Tumefaciens *for Tomato Transformation*

The plasmid pCAMBIA2301 (Thermo Fisher Scientific China) was used to transform tomato. The freeze/thaw shock transformation method [30] was used for plasmid amplification using *Escherichia coli* strain DH5α (Thermo Fisher Scientific China). To check the transformation efficiency, the pCAMBIA2301 plasmid was transferred into three strains of *A. tumefaciens* (GV3101, EHA105, and LBA4404) (Thermo Fisher Scientific China). Then, each transformed *A. tumefaciens* cell was spread on a 2YT medium (16 g bacto tryptone, 10 g yeast extract, 5 g NaCl, adjusted to pH 7.0 with NaOH). The plates contained the antibiotic's kanamycin (50 mg l-1) for selection. Plasmid isolation from the transformed colonies was performed after two days of incubation to detect the presence of a 650-bp gene product of *nptII* by PCR (the sequences of primers listed below). Each *A. tumefaciens* (250 µl) was inoculated in 10 ml of 2YT medium and shaken overnight (180 rpm) at 28°C. At OD_{600} 0.9-1.0, the bacteria were diluted 10 times. Afterward, the culture was maintained at 28°C on the shaker (180 rpm) until an OD_{600} of 0.6. Then, the bacteria were harvested by centrifugation (5,000 rpm) for 10 min at 4°C. After discarding the supernatant, the pellet was taken and dissolved in 100 ml of MS medium-free hormones containing 100 µM acetosyringone and shaking (180 rpm) at 25°C until an OD_{600} of 0.5.

2.2.1 Explant Preparation and Transformation

To examine the survival rate of cotyledon explants, 24 cotyledon explants were placed on an MS medium supported with 8.8 µM BA and kanamycin at four concentrations (50, 100, 200, 300 mg l^{-1}). For transformation, cotyledon explants were co-cultivated individually for 15 min. with each strain of *A. tumefaciens* suspension (OD_{600} 0.5) (GV3101, EHA105, and LBA4404). The cotyledon explants were taken, placed on sterile filter paper, and kept under a dark condition in MS medium mixed with sucrose 3%, phytagel 0.3%, and BA 8.8 µM at 22 ± 2°C for three days. The explants were washed four times with sterilized ultrapure water, and cefotaxime solution (250 mg l^{-1}) was used in the final wash to suppress *A. tumefaciens*. With each *A. tumefaciens* strain treatment, 70 explants were used and distributed to 7 Petri dishes containing MS medium mixed with 8.8 µM BA for shoot regeneration, and each plate had 10 cotyledon explants. Sub-cultures were conducted every 2 weeks on a selective MS medium mixed with kanamycin, its concentration was increased at each subculture (50, 100, 150, and 200 mg l^{-1}). After 6 weeks, several factors were recorded, such as the survival rate, the number of explants produced shoots, the number of shoots per explant, and shoot length.

2.3 Produce of sgRNA Target Sequences and CRISPR/Cas9 Binary Vector Construction

The CC top [31] program was used to select the targeted sites in the Rin gene. According to [32], with some modifications, four oligonucleotide pairs were designed (Table 1) and annealed to detect the target sequences. The fragments were individually cloned into the pGEMT-U3-sgRNA cloning vector at the *BbsI* site of a single-guide RNA (sgRNA). The freeze/thaw shock transformation method [30] introduced the pGEMT-U3-sgRNA expression cassette into *E. coli* strain DH5α competent cells. Then it was spread on a plate containing LB-agar medium mixed with ampicillin (50 µg/ml) and maintained at 37°C overnight. Afterward, several colonies were selected to conduct PCR by using oligo-R and SP6-F primers (5'-ATT TAG GTG ACA CTA TAG-3') through the following PCR conditions: 95°C for 3 min; 95°C for 30 s; 46°C for 30 s; 72°C for 30 s for 30 cycles and 72°C for 10 min.

Table 1 The sequence of four types of sgRNA was used in the study to target the Rin gene in tomatoes.

rin-sgRNA-F1	TGGCGTGGTATCTCTCCAATGTCT
rin-sgRNA-R1	AAACAGACATTGGAGAGATACCAC
rin-sgRNA-F2	TGGCGTGTACCATAATTGTATCTG
rin-sgRNA-R2	AAACCAGATACAATTATGGTACAC
rin-sgRNA-F3	TGGCGTTTTAAGCTTCAAATACTCT
rin-sgRNA-R3	AAACAGAGTATTTGAAGCTTAAAAC
rin-sgRNA-F4	TGGCGTTTGAAGCTTAAAACAAGAG
rin-sgRNA-R4	AAACCTCTTGTTTTAAGCTTCAAAC

To produce the final plasmid pCAMBIA1300-Cas9-U3-sgRNA, the expression cassette pGEMT-U3-sgRNA, and the binary vector pCAMBIA1300-pCas9 were digested with *ApaI* and *BglII* restriction enzymes for 2 hours at 37°C. Finally, four produced fragments of U3-sgRNA with CaMV terminator (approximately 850 bP) and the linearized vector fragment pCAMBIA1300-Cas9 (approximately 14

K) were ligated to produce four pCAMBIA1300-Cas9-U3-sgRNA. The freeze/thaw shock transformation method [30] introduced pCAMBIA1300-pcas9-sgRNA-U3 into *E. coli* strain DH5α competent cells. Then, it was spread on a plate containing LB-agar medium mixed with ampicillin (50 µg/ml) and maintained at 37°C overnight. Several positive colonies were selected to conduct PCR by using oligo-R and M13-F primers, and the positive clones were examined through the following PCR conditions: 95°C for 3 min,95°C for 30 s, 48°C for 30 s, 72°C for 30 s for 30 cycles and 72°C for 10 min.

In vitro digestion of the RIN genomic DNA sequence using Cas9 nuclease was performed individually, according to [33], with each four sgRNA expression cassette. Only sgRNA guide 2 showed the ability to guide Cas9 in the cleavage of the genomic fragment of the tomato RIN gene, which was used for further analysis.

2.3.1 Introducing the Plasmid pCAMBIA1300-Cas9-U3-sgRNA into *Agrobacterium Tumefaciences*

After the amplification of pCAMBIA1300-Cas9-U3-sgRNA in *E. coli* strain DH5α, the plasmid pCAMBIA1300-Cas9-U3-sgRNA was isolated and introduced into LBA4404 strains of *A. tumefaciens* by the freeze/thaw shock transformation method [30]. The transformed cells of the LBA4404 strain were spread on 2YT (16 g back to tryptone, 10 g yeast extract, 5 g NaCl, adjusted to pH 7.0 with NaOH). The plates contained the antibiotic's kanamycin (50 mg l-1) for selection. Plasmid isolation from the transformed colonies was performed after two days of incubation to detect the presence of Rin-sgRNA, and PCR detected the kanamycin gene.

The transformed LBA 4404 strain (250 µl) containing CRISPR design was inoculated in 10 ml of 2YT medium and kept overnight shaken (180 rpm) at 28°C. At OD_{600} 0.9-1.0, the bacteria were diluted 10 times. Afterward, the culture was maintained at 28°C on the shaker (180 rpm) until an OD_{600} of 0.6. Then, the bacteria were harvested by centrifugation (5,000 rpm) for 10 min at 4°C. After discarding the supernatant, the pellet was taken and dissolved in 100 ml MS medium-free hormones containing 100 µM acetosyringone and shaking (180 rpm) at 25°C until an OD_{600} of 0.5.

2.3.2 Explant Preparation and Transformation

100 whole cotyledon explants excised from tomato 10-days-old aseptic seedlings were co-cultivated for 15 min with *A. tumefaciens* LBA4404 suspension harboring pCAMBIA1300-Cas9-U3-sgRNA. The cotyledon explants were taken, placed on sterile filter paper, and kept under a dark condition at 22 ± 2°C for three days in MS medium mixed with sucrose 3%, phytagel 0.3%, and BA 8.8 µM. The explants were washed four times with sterilized ultrapure water, and cefotaxime solution (250 mg l^{-1}) was used in the final wash to suppress *A. tumefaciens*. 100 explants were used and distributed to 10 Petri dishes containing MS medium mixed with 8.8 µM BA for shoot formation, and each plate had 10 cotyledon explants. Sub-cultures were conducted every 2 weeks on a selective MS medium mixed with kanamycin, and its concentration was increased at each subculture (50, 100, 150, and 200 mg/ l^{-1}).

2.4 Molecular Analysis

First, after two months of culture, shoots were taken for DNA extraction from plantlets transformed with three strains of *A. tumefaciens* containing plasmid pCAMBIA2301 to detect the

presence of the *nptII* gene (six samples from each treatment). The DNA extraction was performed using the Plant Nucleospin II Kit, as mentioned by the manufacturer. PCR was performed through the following conditions: 95°C 3 min, then 30 s at 95°C, 30 s at 53°C, and 40 s at 72°C for 30 cycles terminated with a 10 min final step at 72°C. The sequences of primers for PCR amplification of a 650 bp kanamycin fragment were TATATAAGCTGGTGGGAG (forward primer; NPTII-pCambia) and TGTTGCTGTCTCCCAGGTC (reverse primer; NPTII-pCambia).

Second, after 6 weeks of culture, shoots were taken for DNA extraction from plantlets transformed with *A*. *tumefaciens* LBA4404 harboring pCAMBIA1300-Cas9-U3-sgRNA to detect the presence of Rin gene, Cas9, sgRNA (guide 2), and *nptII*. The following PCR condition was used in the case of Cas9 and *nptII*, 95°C 3 min, then 30 s at 95°C, 30 s at 53°C and 40 s at 72°C for 30 cycles terminated with a 10 min final step at 72°C. In the case of the Rin gene and sgRNA2, 95°C 3 min, then 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C for 30 cycles terminated with a 10 min final step at 72°C. The amplification following primers were used: Rin gene 5‘-GTGTCACATAAGCATCAGGTGT-3‘as forward primer, and Rin gene 5‘-TGTGGTCCAATCCATGTGAA-3‘as reverse primer to amplify a 900 bp RIN PCR product. Cas9 gene 5‘-CCACGACGGAGACTACAAGG-3‘as forward primer and Cas9 gene 5‘-GGTGCTGTCCACCAGTTTCT-3‘as reverse primer to amplify a 500 bp Cas9 PCR product.

sgRNA1 5‘-GTACGTTGGAAACCACGTG-3‘as U3 promoter forward primer and 5‘-AAACAGACATTGGAGAGATAACCAC-3‘as sgRNA1 reverse primer to produce a 250 bp sgRNA2 PCR fragment. *nptII* gene 5‘-CCGCTGCGTAAAAGATACGG-3‘as forward primer and Rin gene 5‘-CATACCATTGTCCGCCCTG-3‘as reverse primer to produce a 700 bp *nptII* PCR product.

2.5 Statistical Analysis

24 cotyledon explants were used in each treatment, and all shoot induction experiments were repeated thrice (with the total number of explants = 72). The data were represented as mean ± standard error (SE) (n = 72). The number of explants produced shoots, and the number of shoots produced roots were calculated as a percentage of the total number of explants used throughout the treatment. Statistical analyses were carried out using one-way ANOVA followed by the L.S.D test. Statistical significance* was set at $p \leq 0.05$.

3. Results and Discussion

3.1 Shoots Induction and Multiplication

This study aims to develop a practical and straightforward *in vitro* regeneration system in tomatoes by forming adventitious shoots from cotyledon explants using *in vitro* raised seedlings, followed by establishing a transformation system to evaluate the transformation efficiency using three Agrobacterium strains. The selected *Agrobacterium* strain was used to develop a gene-editing system in tomatoes to explore the ripening mechanism in tomatoes through CRISPR Cas9 knock-in mechanism for the loose of the function of Rin gene that is responsible for the ripening mechanism in tomatoes to produce long life tomatoes. No developed shoots were obtained from cotyledon explants placed in a free hormone MS medium as a control. In contrast, cotyledon explants produced shoots when placed on an MS medium containing different cytokinin concentrations. Cotyledon explants showed shoot formation after 2 weeks of culture. With a second subculture for another 2 weeks, the shoots proliferated, and the growth increased.

There was a significant difference between BA and Kin on shoot induction, whereas BA was more efficient in shoot induction from cotyledon explants than Kin (Table 2). Similar to our results [34], BA is more efficient for normal shoot development than zeatin in promoting *in vitro* regeneration of tomatoes. Also, [35] showed that BA was more effective in shoot regeneration from cotyledon leaves.

Table 2 Effect of different cytokinin BA and Kin concentrations at (2.2, 4.4, 8.8, and 17.6 µM) on tomato shoot regeneration from cotyledon explants after 6 weeks of culture.

Plant growth regulators (µM)		No. of explants produced shoots (%)	No. of shoots per explants forming shootlets	Shoots length (cm)
BA	**Kin**			
0	0	0	0	0
2.2		20 ± 6^{a}	2.6 ± 0.2^{a}	0.61 ± 0.2^{a}
4.4		33 ± 8^{b}	2.6 ± 0.3^{a}	0.97 ± 0.57^{b}
8.8		45 ± 7^{c}	4.2 ± 0.3^{c}	0.7 ± 0.4^{a}
17.8		32 ± 3^{b}	3.8 ± 0.3^{ab}	0.69 ± 0.2^{a}
	2.2	39 ± 3^{ab}	2.05 ± 0.9^{a}	0.97 ± 0.3^{b}
	4.4	39 ± 6^{ab}	2.1 ± 0.9^{a}	0.98 ± 0.6^{b}
	8.8	33 ± 3^{b}	2.2 ± 0.8^{a}	0.83 ± 0.3^{ab}
	17.8	25 ± 2^{a}	2.4 ± 0.3^{b}	0.87 ± 0.4^{ab}

Values are presented as mean ± SE. Means within the same column carrying different letters are significantly different at $P \leq 0.05$.

Herein, the regenerated shoots were formed from the petiolar end of the cotyledon (Figure 1). In many plant species, adventitious shoots originating closer to the petiolar end of the leaves could develop from a single cell [36, 37]. Therefore, targeting the axillary meristem in the petiolar end part could increase the transformation efficiency.

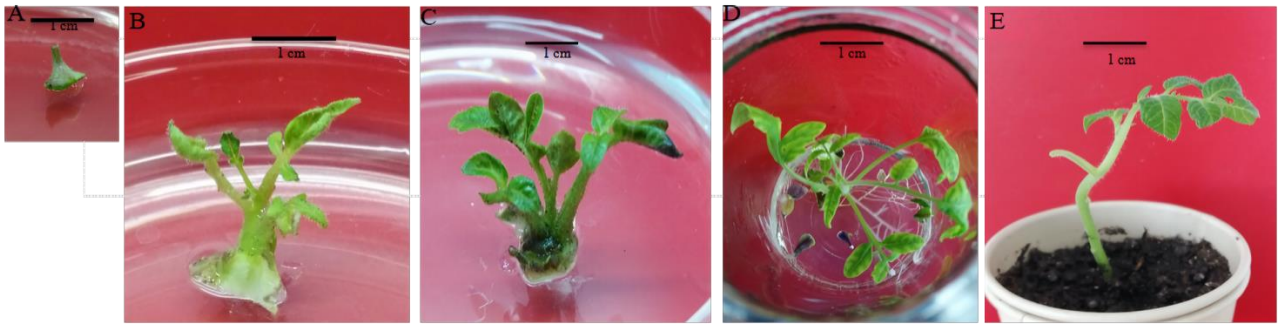

Figure 1 *In vitro* tomato propagation (A–D); A cotyledon explants on day one, B Shoots formation at 8.8 µM BA after 4 weeks of culture, C Shoots elongation and proliferation at 8.8 µM BA + 1.3 µM NAA after 8 weeks of culture, D Root induction at 1.2 µM IBA, E Complete acclimatized plantlet in soil.

There was a significant difference in the cytokinin concentrations under investigation. The highest values of the cotyledon explants producing shoots and the number of shoots per explant

forming shoots were obtained at 8.8 µM BA (45 ± 7%) and (4.2 ± 0.3), respectively. So, the cytokinin BA was more efficient than Kin in supporting the regeneration of adventitious shoots from cotyledon explants. Conversely, the cytokinin Kin was slightly more efficient than BA in promoting the shoot length. In contrast, the highest shoot length was obtained at 2.2 µM Kin, which decreased by increasing the cytokinin concentration.

Consequently, BA at 8.8 µM and 4.4 µM Kin were selected from the results of the previous culture stage and mixed individually with three concentrations of NAA (1.3, 2.6, and 5.2 µM) for further shoot proliferation (Table 3). The values of cotyledon explants-producing shoots were increased after using NAA, whereas 8.8 µM BA mixed with 2.6 µM NAA revealed the highest value of cotyledon explants-producing shoots (59 ± 5%). Also, the number of shoots per explant forming shoots increased, and the highest number was obtained at 8.8 µM BA mixed with 1.3 µM NAA (4.4 ± 0.33).

Table 3 Effect of the optimal concentration of BA and Kin mixed with different concentrations of NAA on shoots multiplication of cotyledon explants from tomato after 10 weeks of culture.

Plant growth regulators (µM)			No. of explants	No. of shoots	Shoots length
BA	**Kin**	**NAA**	**produced shoots (%)**	**per explants**	**(cm)**
8.8	-	1.3	57 ± 7^{c}	4.4 ± 0.32^{c}	0.9 ± 0.2^{a}
8.8	-	2.6	59 ± 5^{c}	2.9 ± 0.33^{b}	0.9 ± 0.2^{a}
8.8	-	5.2	46 ± 6^{ab}	3.2 ± 0.2^{b}	1.01 ± 0.4^{a}
-	4.4	1.3	48 ± 7^{ab}	3.8 ± 0.3^{ab}	1.04 ± 0.3^{a}
-	4.4	2.6	31 ± 6^{a}	2.2 ± 0.2^{a}	0.9 ± 0.2^{a}
-	4.4	5.2	42 ± 4^{b}	3.2 ± 0.3^{b}	1.03 ± 0.2^{a}

Values are presented as mean ± SE. Means within the same column carrying different letters are significantly different at $P \leq 0.05$.

In summary, mixing cytokinin BA with auxin, NAA was more effective than Kin with NAA in terms of the percentage of cotyledon explants producing shoots and the number of shoots per explant forming shoots. The highest shoot length was obtained at 8.8 µM Kin mixed with 1.3 µM NAA (1.03 ± 0.2 cm). Consistent with our results, mixin Zeatin with Indol 3-acetic acid increased the shoot induction in tomato Micro-tom [14].

3.2 Root Induction and Acclimatization

Shoots with 2-4 leaves and 1 cm length were moved to MS media supported with three concentrations of IBA (1.2, 2.4, and 4.8 µM) for root induction (Table 4). There were no significant differences in rooting rate and root length among the IBA concentrations. The highest percentage of explants-producing roots was obtained by increasing the IBA concentration to 4.8 µM (55 ± 5%). The highest number of roots per explant forming roots was obtained at 1.2 µM IBA (3 ± 1.6), whereas the most extended root length was obtained at 2.4 µM (1.0 ± 0.4). The healthy plantlets with shoots and roots were taken and moved into the greenhouse for acclimatization. After acclimatization, the survival rate of plantlets was approximately 75%. Similar to our results, several reports have reported the effectiveness of IBA in root induction in tomatoes. For instance, IBA at

0.5 mg l^{-1} concentration is effective for root induction from tomato shoot tips culture [38], where 1 mg l^{-1} concentration of IBA efficiently produced roots from tomato cotyledon [39, 40].

Table 4 Effect of different concentrations of IBA (1.2, 2.4, and 4.8 µM) on root induction of *in vitro* tomato shoots after 4 weeks of culture.

Plant growth regulators (µM) IBA	No. of shoots produced roots (%)	No. of roots per shoots forming roots	Root length (cm)
0	0	0	0
1.2	52 ± 7[a]	3 ± 1.6[b]	0.86 ± 0.1[a]
2.4	53 ± 8[a]	2.1 ± 0.9[a]	1.0 ± 0.4[a]
4.8	55 ± 5[a]	2.4 ± 0.9[a]	0.9 ± 0.1[a]

Values are presented as mean ± SE. Means within the same column carrying different letters are significantly different at $P \leq 0.05$.

3.3 Transformation of Tomato via A. Tumefaciences Harboring pCAMBIA2301

Several reports showed that the success of transformation depends on the *A. tumefaciens* strain, for instance, *Nicotiana tabacum* cv—Samsun [41], also in grapevine [42], and *Balanites aegyptiaca* [43]. Moreover, the transformation efficiency by *Agrobacterium tumefaciens* is affected by the bacterial strain in tomato besides the tomato cultivar and leafage, where the transformation efficiency by using three strains of *A. tumefaciens* in Ohio 7870 and Roma were higher than UCD82b. Also, the older genuine leaf and cotyledon were more readily transformed than the younger leaves [44].

The capability of tomato cotyledon explants to survive on MS media mixed with four different concentrations of kanamycin (50, 100, 200, and 300 mg l^{-1}) was examined for 6 weeks (Figure 2). In the presence of kanamycin, most of the cotyledon explants turned from green to yellow and could not produce shoots. Interestingly, the dead explants (%) were increased by increasing the kanamycin concentration, as follows: at 50 mg l^{-1}, 4 explants (16%) were dead out of 24; at 100 mg l^{-1} 20 explants (83%) were dead out of 24. Furthermore, at 200 and 300 mg l^{-1}, the percentage of dead explants increased to 91 and 90%, respectively. In this regard, 50 mg l^{-1} kanamycin was mixed with MS medium at the first stage of culture, and with each subculture, the kanamycin concentration was increased. In our study, *Agrobacterium* strain LBA4404 showed a higher transformation rate (30%) after two months of transformation, with the lowest mortality of cotyledon and the highest number of shoots per transformed explant followed by GV3101 strain and EHA105strain *Agrobacterium* strains. Plant survival after incubation with *Agrobacterium* is still a big challenge for plant transformation due to the defense mechanism made by *Agrobacterium* through expressing many genes in the host cell that may cause plant cell death, which was also reported in several plants such as bananas and maize [45]. Also, the infected calli with *Agrobacterium* showed a fast and hypersensitive plant cell death response, negatively affecting these plants' T-DNA transfer and transformation efficiency [45, 46]. In this regard, the cotyledon death rate due to *Agrobacterium* is an influential factor in improving the conditions of tomato transformation.

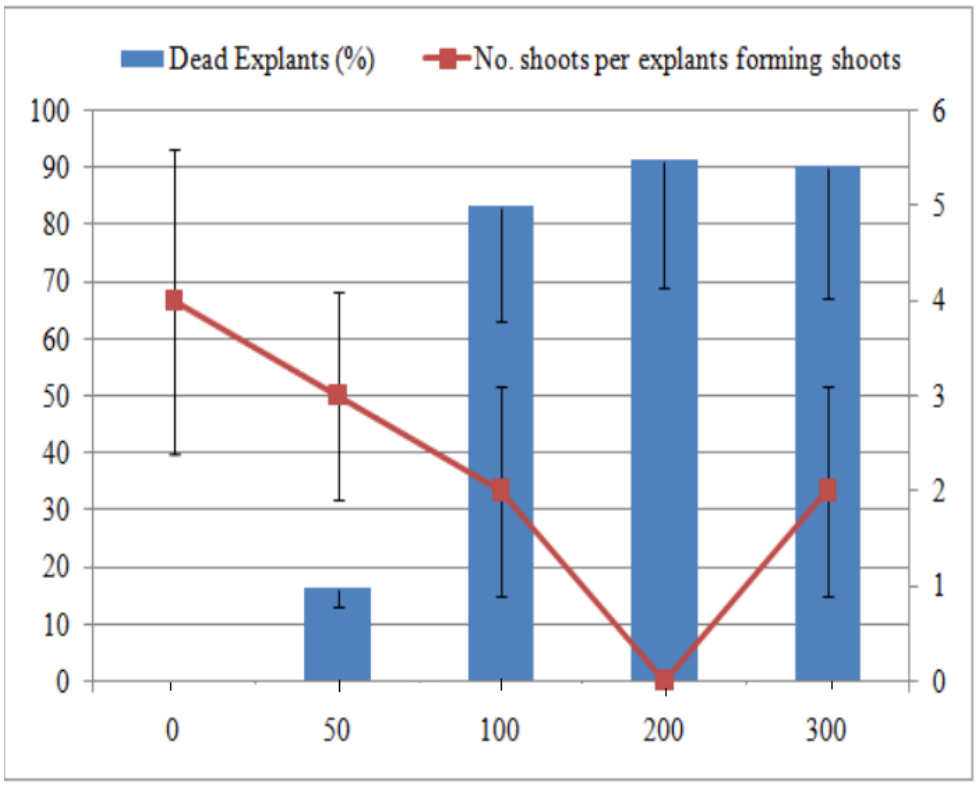

Figure 2 The effect of four different concentrations of kanamycin (50, 100, 200, and 300 mg l^{-1}) on the survival rate of tomato cotyledon explants (each treatment contains 24 cotyledon explants) after 6 weeks of culture.

Three *A. tumefaciens* strains (LBA4404, EHA105, and GV3101) were used for transformation. Each strain harboring plasmid pCAMBIA2301 contains the *neomycin phosphotransferase II* (*nptII*) as a selectable marker gene. After two months of cultivation, the presence of kanamycin in the media negatively affected the growth and shoot length of control plants, which turned yellow. In contrast, transformed plants were almost unaffected and remained green. The percentage of regeneration, number of shoots per transformed explant, and shoot length were recorded in each treatment (Table 5). The *A. tumefaciens* strain LBA4404 gave the highest regeneration percentage of transformed plants (30%), the highest number of shoots per explant (2.6 ± 0.9), and the highest shoot length (1.01 ± 0.2 cm). Leaves from six cotyledon explants were taken to detect the existence of the *nptII* gene through PCR (Figure 3). Each *A. tumefaciens* strain (LBA4404, EHA105, and GV3101) gave six positive PCR fragments. Based on these results, the highest values of regenerated transformed explants (%), number of shoots per transformed explants, and shoot length were obtained using *A. tumefaciens* strain LBA4404 compared to GV3101 and EHA105 strains. Similar to our results, [47] investigated the transformation efficiency in tomato (*Solanum lycopersicum* L.) cv. Micro-Tom uses four A. tumefaciens: AGL1, EHA105, GV3101, and MP90, harboring the plasmid pBI121.

Table 5 Transformation efficiency in tomato after two months of transformation with different strains of *A. tumefaciens* (LBA4404, EHA105, and GV3101 strains).

A. tumefaciens strain	**No. of transformed explants produced shoots (%)**	**No. of shoos per transformed explants**	**Shoots length (cm)**
LBA4404 strain	30 ± 5	2.6 ± 0.9	1.01 ± 0.2
EHA105 strain	14 ± 4	1.6 ± 1	0.82 ± 0.07
GV3101 strain	17 ± 6	1.9 ± 0.6	0.82 ± 0.06

Values are presented as mean ± SE. Means within the same column carrying different letters are significantly different at P ≤ 0.05.

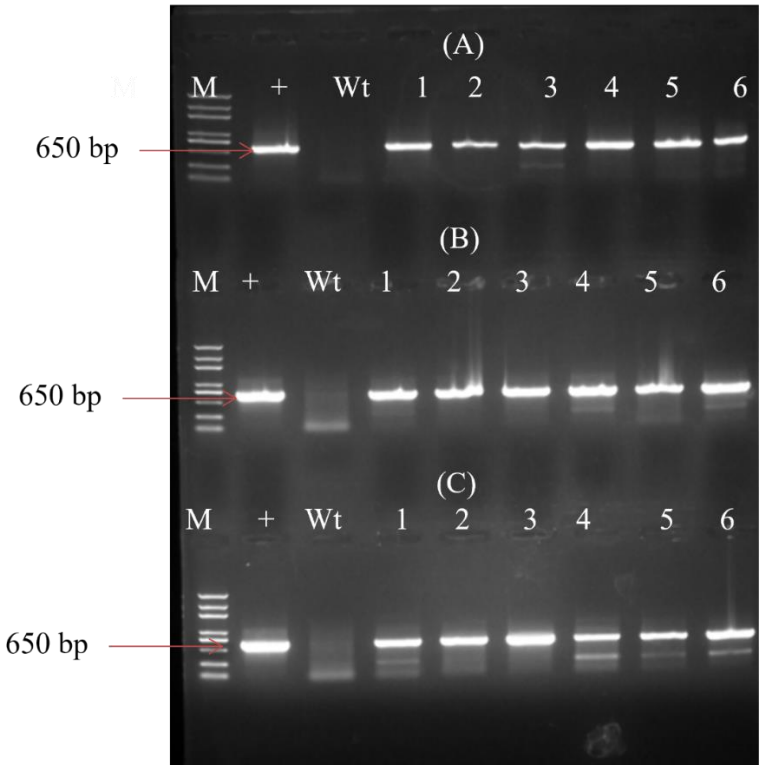

Figure 3 PCR product of *nptII* gene (650 bp) produced from the transformed shoots after 6 weeks of transformation with three different types of Agrobacterium as the following ((A–C); (M) DNA ladder; (+). Plasmid pCAMBIA2301; (Wt) control plant. (A) (1:6) Shoots excised from 6 different cotyledon explants after 6 weeks of transformation through *A. tumefaciens LBA4404* strain harboring pCAMBIA2301, (B) (1:6) Shoots excised from 6 different cotyledon explants after 6 weeks of transformation through *A. tumefaciens* GV3101 strain harboring pCAMBIA2301, and (C) (1:6) Shoots excised from 6 different cotyledon explants after 6 weeks of transformation through *A. tumefacien* EHA105 strain harboring pCAMBIA2301.

They found that the highest values of transformation rate (65%) and the lowest mortality rate of cotyledon were obtained using *Agrobacterium* strain GV3101. Several studies have revealed that the transformation efficiency was immensely varied among tomato experiments [42]. The transformation efficiency was different among the three strains of *A. tumefaciens* incubated with

Balanites aegyptiaca cotyledon explants, where *Agrobacterium* strain GV3101 showed the highest transformation efficiency by 48% compared to EHA105 and LBA4404 strains [43]. In other plants, such as grass seedlings, *Agrobacterium* strain AGL1 is more effective in delivering T-DNA than C58, GV3101, and EHA105 [48]. *Agrobacterium* strain EHA105 showed a transformation rate ranging from 4-36% in the ability to transfer T-DNA in tomato [2]. In contrast, our results showed that *Agrobacterium* strain EHA105 showed the lowest transformation rate (No. of transformed explants produced shoots (%)) and higher mortality of cotyledon compared to *Agrobacterium* strain LBA4404, which showed the highest transformation rate. Therefore, selecting the suitable *Agrobacterium* strain for transformation is an essential factor in tomato propagation and transformation, which will increase the capability of efficiency of gene editing through CRISPR Cas9 by using the most efficient Agrobacterium strain.

3.4 Transformation of Tomato via A. Tumefaciences Harboring pCAMBIA1300-Cas9-U3-sgRNA

In this study, we have used the CC top program [31] to design four sgRNA guides for the RIN gene mutagenesis to establish a gene-editing system in tomatoes. Several studies were reported on the genome editing of tomatoes [22]. CRISPR/Cas9 constructs with two gRNAs per gene target were successfully designed [23]. Also, methods were revealed for assembling CRISPR/Cas9 constructs for gene targeting and single and multiplex knockouts and for producing and identifying genome-edited tomato plants by Agrobacterium-mediated transformation in tissue culture [24]. Herein, depending on the methods above, we optimized protocols for gene editing in tomatoes. Sequences with fewer homologies to other genome sites have been chosen to avoid off-target mutagenesis. The four designed sgRNA guides were selected to target the conservative domain of the Rin gene. To establish a knock-in mutation in the Rin gene through CRISPR/Cas9 system in tomato, the selected four sgRNA guide sequences were separately cloned to finally produce four plasmids of pCAMBIA1300-Cas9-U3-sgRNA for each sg RNA guide.

The efficiency of each CRISPR/Cas9 expression cassette was examined *in vitro* to target the locus of the Rin gene and induce knock-in mutation [33]. Only sgRNA guide 2 expression cassettes could induce knock-in mutation in the RIN gene by producing three DNA fragments on the gel (1000, 750, and 250 bp) (data has not been shown). In this regard, after cloning sgRNA guide 2 into the plasmids of pCAMBIA1300-Cas9-U3-sgRNA, the plasmid was transferred *via A. tumefaciens* strain LBA4404 into the cotyledon explants of tomato to target the locus of the Rin gene and induce a knock-in mutation. After 2 weeks of transformation, leaf samples were taken for genomic DNA extraction. Four transgenic plants were used for PCR to detect the existence of the Rin gene, Cas9, sgRNA gene, and *nptII* gene, which gave a PCR product at 850, 600, 250, and 650bp, respectively (Figure 4A, B, C, and D). Finally, our study revealed that selecting the suitable *Agrobacterium* strain facilitated the transformation process and increased the capability to establish a system for gene-editing in tomatoes by transferring pCAMBIA1300-Cas9-U3-sgRNA binary vector into the cotyledon explants of tomato to target the locus of the Rin gene. However, our study introduces a new method to knock in the sequence of the Rin gene to explore the ripening mechanism in tomatoes aiming to produce long-life tomatoes. There is a demand for further analysis to examine the PCR fragment by sequencing and the presence of a *rin* mutation in the tomato fruits to finally confirm the capability of the designed CRISPR/Cas9 expression cassette to target the locus of the RIN gene and induce

knock-in mutation. That will increase our knowledge about regulating the ripening mechanism in tomatoes.

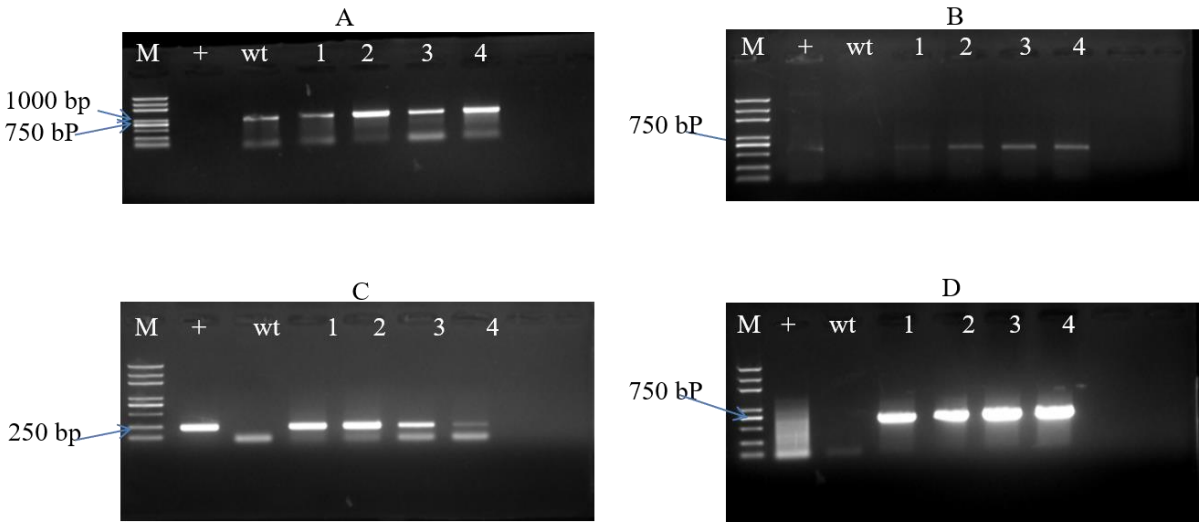

Figure 4 PCR products of leaves excised from different cotyledon explants after 2 weeks of transformation via *A. tumefaciens LBA4404 strain containing* pCAMBIA1300-Cas9-U3-sgRNA. (A) PCR product of Rin gene (850 bp) (M). 100 bp DNA ladder. (+). Plasmid (M)., (Wt) control plant (1:4) leaves excised from different cotyledon explants after 2 weeks of transformation via *A. tumefaciens LBA4404 strain containing* pCAMBIA1300-Cas9-U3-sgRNA, (B) PCR product of Cas9 gene (600 bp) (M). 100 bp DNA ladder. (+). Plasmid (M)., (Wt) control plant (1:4) leaves excised from different cotyledon explants after 2 weeks of transformation via *A. tumefaciens LBA4404 strain containing* pCAMBIA1300-Cas9-U3-sgRNA, (C) PCR product of sgRNA gene (250 bp) (M). 100 bp DNA ladder. (+). Plasmid (M)., (Wt) control plant (1:4) leaves excised from different cotyledon explants after 2 weeks of transformation via *A. tumefaciens LBA4404 strain containing* pCAMBIA1300-Cas9-U3-sgRNA. (D) PCR product of *nptII* gene (650 bp) (M). 100 bp DNA ladder. (+). Plasmid (M)., (Wt) control plant (1:4) leaves excised from different cotyledon explants after 2 weeks of transformation via *A. tumefaciens LBA4404 strain containing* pCAMBIA1300-Cas9-U3-sgRNA.

4. Conclusion

Our results showed that BA at 8.8 µM was more effective in shoot induction than Kin. Combining the optimal concentration of BA at 8.8 µM with NAA improves shoot elongation. Also, selecting a more efficient *Agrobacterium* strain played a vital role in the transformation of tomato. Our results showed that *Agrobacterium* strain LBA4404 gave the lowest number of dead explants with the highest values of transformed explant-produced shoots and the number of shoots per transformed explant. The *Agrobacterium* strain LBA4404 was successfully used to transfer pCAMBIA1300-Cas9-U3-sgRNA binary vector into tomato cotyledon to establish a gene-editing system to knock in the sequence of the Rin gene that could furtherly used to explore the ripening mechanism in tomato.

Abbreviations

BA: 6-benzyl adenine, Kin: kinetin, NAA: α-naphthalene acetic acid, IBA: indole-3-butyric acid, MS: Murashige and Skoog medium, EHA105, GV3101, and LBA4404: *Agrobacterium tumefaciens* strains, *nptII: Neomycin phosphotransferase II,* RIN: ripening inhibitor.

Author Contributions

GK designed the research study obtained the funds and wrote the first draft of the manuscript, JC helped in the bioinformatics analysis, and ZQ supervised the study and reviewed the final draft.

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Competing Interests

The authors have declared that no competing interests exist.

References

1. Sato S. The Tomato Genome Consortium, Kazusa DNA Research Institute, the tomato genome sequence provides insights into fleshy fruit evolution. Nature. 2012; 485: 635-641.
2. Sun HJ, Uchii S, Watanabe S, Ezura H. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. 2006; 47: 426-431.
3. Bhatia P, Ashwath N, Senaratna T, Midmore D. Tissue culture studies of tomato (*Lycopersicon esculentum*). Plant Cell Tissue Organ Cult. 2004; 78: 1-21.
4. McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep. 1986; 5: 81-84.
5. Uluisik S, Chapman NH, Smith R, Poole M, Adams G, Gillis RB, et al. Genetic improvement of tomato by targeted control of fruit softening. Nat Biotechnol. 2016; 34: 950-952.
6. Cortina C, Culiáñez-Macià FA. Tomato transformation and transgenic plant production. Plant Cell Tissue Organ Cult. 2004; 76: 269-275.
7. Gresshoff PM, Doy CH. Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta. 1972; 107: 161-170.
8. Varghese TM, Yadav G. Production of embryoids and calli from isolated microspores of tomato (*Lycopersicon esculentum* Mill.) in liquid media. Biol Plant. 1986; 28: 126-129.
9. Thomas P, Mythili JB. Development of cultured tomato anther to a fruit-like structure accompanied by *in vitro* ripening. Curr Sci. 1995; 69: 94-95.
10. Shahin EA. Totipotency of tomato protoplasts. Theor Appl Genet. 1985; 69: 235-240.
11. Peres LE, Morgante PG, Vecchi C, Kraus JE, Sluys MA. Shoot regeneration capacity from roots and transgenic hairy roots of tomato cultivars and wild related species. Plant Cell Tissue Organ Cult. 2001; 65: 37-44.

12. Izadpanah M, Khosh-Khui M. Comparisons of *in vitro* propagation of tomato cultivars. Iran Agric Res. 1992; 8: 37-47.
13. Kaparakis G, Alderson PG. Influence of high concentrations of cytokinins on the production of somatic embryos by germinating seeds of tomato, aubergine and pepper. J Hortic Sci Biotechnol. 2002; 77: 186-190.
14. Yaroshko O, Pasternak T, Larriba E, Pérez-Pérez JM. Optimization of callus induction and shoot regeneration from tomato cotyledon explants. Plants. 2023; 12: 2942.
15. El-Farash EM, Abdalla HI, Taghian AS, Ahmad MH. Genotype, explant age and explant type as effecting callus and shoot regeneration in tomato. Assiut J Agri Sci. 1993; 24: 3-14.
16. Sivankalyani V, Takumi S, Thangasamy S, Ashakiran K, Girija S. Punctured-hypocotyl method for high-efficient transformation and adventitious shoot regeneration of tomato. Sci Hortic. 2014; 165: 357-364.
17. Brooks C, Nekrasov V, Lippman ZB, Van Eck J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol. 2014; 166: 1292-1297.
18. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nat Rev Genet. 2010; 11: 636-646.
19. Joung JK, Sander JD. TALENs: A widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol. 2013; 14: 49-55.
20. Liu L, Fan XD. CRISPR-Cas system: A powerful tool for genome engineering. Plant Mol Biol. 2014; 85: 209-218.
21. Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Toki S. CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem Biophys Res Commun. 2015; 467: 76-82.
22. Li JF, Norville J, Aach M, McCormack D, Zhang J, Bush GM, et al. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat Biotechnol. 2013; 31: 688-691.
23. Xia X, Cheng X, Li R, Yao J, Li Z, Cheng Y. Advances in application of genome editing in tomato and recent development of genome editing technology. Theor Appl Genet. 2021; 134: 2727-2747.
24. Reem NT, Van Eck J. Application of CRISPR/Cas9-Mediated gene editing in tomato. In: Plant Genome editing with CRISPR systems: Methods in molecular biology. New York, NY: Humana Press; 2019. pp. 171-182.
25. Čermák T, Gasparini K, Kevei Z, Zsögön A. Genome editing to achieve the crop ideotype in tomato. In: Crop breeding. Methods in molecular biology. New York, NY: Humana Press; 2021. pp. 219-244.
26. Martín-Pizarro C, Posé D. Genome editing as a tool for fruit ripening manipulation. Front Plant Sci. 2018. 9: 1415.
27. Qin G, Zhu Z, Wang W, Cai J, Chen Y, Li L, et al. A Tomato vacuolar invertase inhibitor mediates sucrose metabolism and influences fruit ripening. Plant Physiol. 2016; 172: 1596-1611.
28. Robinson R. Ripening inhibitor: A gene with multiple effects on ripening. Rep Tomato Genet Coop. 1968; 18: 36-37.
29. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol. 1962; 15: 473-497.

30. Cohen SN, Chang AC, Hsu L. Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of Escherichia coli by R-factor DNA. Proc Natl Acad Sci U S A. 1972; 69: 2110-2114.
31. Stemmer M, Thumberger T, del Sol Keyer M, Wittbrodt J, Mateo JL. CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. PLoS One. 2015; 10: e0124633.
32. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, et al. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant. 2015; 8: 1274-1284.
33. Xu S, Cao S, Zou B, Yue Y, Gu C, Chen X, et al. An alternative novel tool for DNA editing without target sequence limitation: The structure-guided nuclease. Genome Biol. 2016; 17: 186.
34. Prihatna C, Chen R, Barbetti MJ, Barker SJ. Optimization of regeneration parameters improves transformation efficiency of recalcitrant tomato. Plant Cell Tissue Organ Cult. 2019; 137: 473-483.
35. Mamidala P, Nanna RS. Effect of genotype, explant source and medium on *in vitro* regeneration of tomato. Int J Genet Mol Biol. 2011; 3: 45-50.
36. Broertjes C, Haccius B, Weidlich S. Adventitious bud formation on isolated leaves and its significance for mutation breeding. Euphytica. 1968; 17: 321-344.
37. Broertjes C, Keen A. Adventitious shoots: Do they develop from one cell? Euphytica. 1980; 29: 73-87.
38. Negi PS, Joshi MC. Growing hybrid tomato in hills through tissue culture technique. Madras Agric J. 1994; 81: 649-650.
39. Vnuchkova VA. Development of a method for obtaining regenerate tomato plants under tissue culture conditions. Fiziol Rast. 1977; 24: 1094-1100.
40. Vnuchkova VA. Elaboration of methods for obtaining tomato plants by tissue culture. Fiziol Rast. 1977; 24: 1095-1100.
41. Bakhsh A, Anayol E, Ozcan SF. Comparison of transformation efficiency of five Agrobacterium tumefaciens strains in Nicotiana tabacum L. Emir J Food Agric. 2013; 26: 259-264.
42. Torregrosa L, Iocco P, Thomas MR. Influence of Agrobacterium strain, culture medium, and cultivar on the transformation efficiency of Vitis vinifera L. Am J Enol Vitic. 2002; 53: 183-190.
43. Khamis G, Winkelmann T, Schaarschmidt F, Papenbrock J. Establishment of an *in vitro* propagation and transformation system of Balanites aegyptiaca. Plant Cell Tissue Organ Cult. 2016; 125: 457-470.
44. Davis ME, Lineberger RD, Miller AR. Effects of tomato cultivar, leaf age and bacterial strain on transformation by *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult. 1991; 24: 115-121.
45. Khanna HK, Jean-Yves P, Harding RM, Dickman MB, Dale JL. Inhibition of *Agrobacterium*-induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of banana. Mol Plant-Microbe Interact. 2007; 20: 1048-1054.
46. Hansen G. Evidence for *Agrobacterium*-induced apoptosis in maize cells. Mol Plant-Microbe Interact. 2000; 13: 649-657.
47. Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. Plant Cell Rep. 2013; 32: 239-247.

48. Chen X, Raymie E, Baxter H, Berk K, Hen J, Agarwal S, et al. High throughput transient gene expression system for switch grass (*Panicum virgatum* L.) seedlings. Biotechnol Biofuels. 2010; 3: 9.