

# Study on the optimization of transformation systems in watermelon

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## Abstract

To date, the genetic transformation system of watermelon has remained inefficient. In this study, the genetic transformation system of watermelon mediated by *Agrobacterium tumefaciens* was optimized, including different seedling ages, strains of *Agrobacterium tumefaciens*, concentrations of acetosyringone infected solution, co-culture time, selection pressure of antibiotics timentin and glufosinate, and concentrations of hormones 6-benzylaminopurine, indoleacetic acid and naphthylacetic acid in the corresponding culture medium. Our results suggested that cotyledons would be used as explants, disinfected with 6% sodium hypochlorite for 12 min, cultured for 3 d, and then infected with *Agrobacterium* inoculum (*Agrobacterium* EHA105) containing 200  $\mu$ M acetosyringone and 0.02 of final OD<sub>600</sub>. The explants differentiated into adventitious shoots in the medium with 1.5 mg/L 6-benzylaminopurine and 200  $\mu$ M timentin. Positive adventitious shoots were obtained through further screening by 1.4 mg/L herbicide glufosinate-ammonium, and were induced by 0.1 mg/L naphthalene acetic acid into independent plants. Our system improves the genetic transformation efficiency of watermelon and provides a technical basis for continuous acquisition of watermelon transgenic plants.

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## INTRODUCTION

The leaf-disc method was initially created to obtain transgenic plants by infecting tobacco leaf explants with *Agrobacterium tumefaciens*, and the method was simple and effective<sup>[1]</sup>. However, considering that excellent cultivars or promising application varieties are expected as the initial material, mature and practical transgenic systems are still essential for obtaining more transgenic lines. Watermelon, one crop of the Cucurbitaceae family, plays an irreplaceable role as an economically important fruit crop worldwide. Watermelon has been studied as a model crop for fruit quality due to its unique diversity in color, shape and size, flavor, texture, aroma, and nutrient content<sup>[2]</sup>. Therefore, personalized watermelon breeding is particularly important.

Watermelon genetic transformation was first reported in 1994, meanwhile transgenic lines expressing GUS reporter gene were successfully obtained<sup>[3]</sup>. Thereafter, studies were carried out to improve the regeneration level and transformation rate of watermelon, including genotype, *Agrobacterium* strains, culture conditions, and chemical agents. Wild watermelon germplasms were more suitable for tissue culture than cultivated watermelon, and shoot regeneration rate were higher<sup>[4]</sup>. In addition, triploid was superior to diploid in watermelon adventitious bud induction<sup>[5]</sup>. Various *Agrobacterium* strains have been applied to watermelon transformation, while there was not explicit exposition regarding the optimum strain<sup>[6–10]</sup>. Adding acetosyringone (AS) in the *Agrobacterium* inoculum could improve the sensitivity of strains to watermelon explant wounds and enhance the ability of infection<sup>[6,11]</sup>. The infection efficiency and survival rate of explants was the highest after dark cultivation at 23–28 °C for 2–3 d<sup>[9,12]</sup>. Naphthalene acetic acid (NAA) and 6-benzylaminopurine (6-BA)

have been widely used for explants shooting and rooting<sup>[7–9,13,14]</sup>, while timentin (TMT), an antibacterial antibiotic, has been applied to many crops, but there was little research on watermelon. In addition, the herbicide glufosinate-ammonium (Basta) was often used as a resistance screening agent in watermelon genetic transformation studies<sup>[8,15]</sup>.

When particular genes, such as disease resistance and insect resistance genes, are introduced by means of the transformation system, the transgenic plants keep their original traits, meanwhile, new valuable agronomic traits are added. In addition, approaches for efficient gene-targeting are significant for analyzing functional genes included in the plant genome and producing genetically engineered crops<sup>[16]</sup>. Previous studies suggested that the CRISPR/Cas9 system<sup>[17]</sup> was used as a highly efficient and specific tool for accurate genome editing<sup>[18]</sup>. Because its genome sequence is available<sup>[19]</sup>, the CRISPR/Cas9 system should represent a good prospect for exploring gene functions, improving fruit quality and breeding in watermelon<sup>[20]</sup>. Transgenic lines with both Zucchini yellow mosaic virus (ZYMV) and Papaya ringspot virus type W (PRSV-W) resistance genes were obtained, and provided great potential for quality breeding of commercial watermelon varieties<sup>[7]</sup>. Similarly, transgenic lines resistant to Cucumber mosaic virus (CMV) also showed its importance in the watermelon industry<sup>[14]</sup>. By overexpressing *CITST2*, a QTL-mapped gene, the molecular mechanism of sugar accumulation in watermelon fruit was systematically elucidated<sup>[8]</sup>. In addition, gynoecious watermelon lines obtained by editing *CIWIP1*, provided a theoretical and technical basis for improving watermelon yield and breeding elite lines<sup>[21]</sup>.

Prior to this investigation, although watermelon transformation protocols have been described in several studies<sup>[13,14,21,22]</sup>,

the efficiency remained as low as 1.67%<sup>[9]</sup>, and research on the optimization of the watermelon transformation system has not yet been reported in detail. An efficient regeneration and transformation system has five characteristics: high efficiency, good reproducibility, simplicity, rapidity and wide applicability<sup>[23]</sup>. This study attempts to establish an efficient watermelon transformation system and provide a more reasonable way to introduce foreign genes into watermelon.

## RESULTS

### Effects of different seedling ages and disinfection times on seedling germination

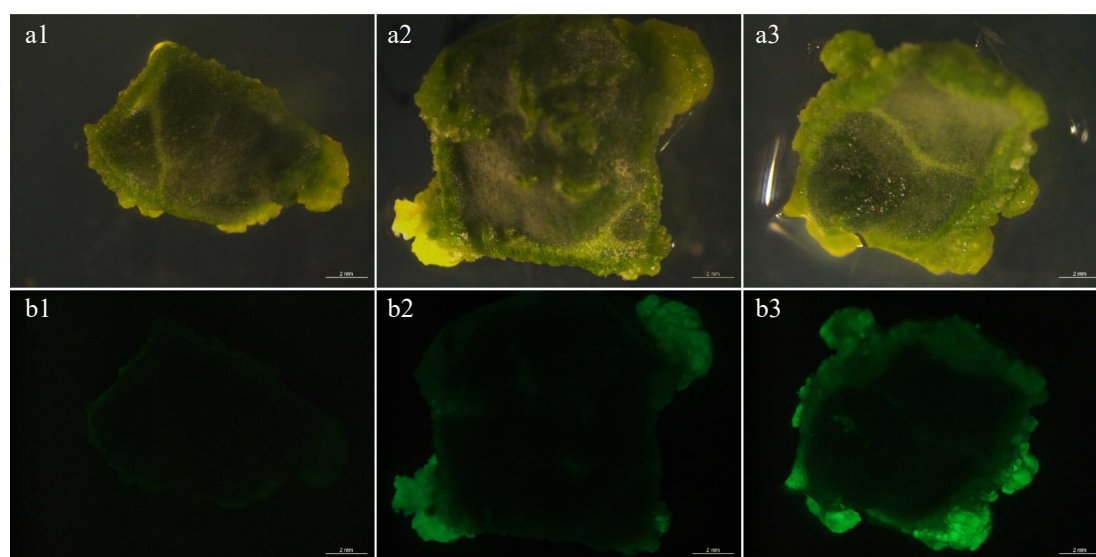
In order to improve the efficiency of seedling germination, gradient tests were carried out for the disinfection time and cultivation time in the dark. The seeds were disinfected with a mixture of 75% alcohol and 6% sodium hypochlorite. The time of disinfection with alcohol was 35 s. The time of disinfection with sodium hypochlorite was designed with three gradients: 8, 12 and 16 min. After sterilization, seeds were rinsed three times with sterile water. The cultivation time of sterilized seeds was carried out in three gradients of 2, 3 and 4 d. Combined with disinfection conditions and dark incubation time, nine different treatments were designed. The seeds were cultured in the seedling culture medium (Supplemental Table S1) at 28 °C under the same dark conditions. The results showed that average germination rates of the seeds in the nine treatment groups (Supplemental Table S2) was between 20% and 100%. There were no significant differences between the germination rates of seeds cultured for 3 d and 4 d when disinfection time was consistent. Combined with the germination states, the highest germination rate can be obtained by sterilizing with 6% sodium hypochlorite for 12 min, and then culturing for 3 d (Supplemental Fig. S1a). The reasons for poor germination rate may be due to the long disinfection time and bacterial lesions resulting from short disinfection time.

### Effects of *Agrobacterium tumefaciens* strains on transformation efficiency

According to the results of previous studies, a widely used culture condition was performed in the following experiments<sup>[9]</sup>. The explants were inoculated in *Agrobacterium tumefaciens* inoculum at 28 °C, and the initial OD<sub>600</sub> of *Agrobacterium tumefaciens* inoculum was 0.8. After 4 d dark co-cultivation, GFP transient fluorescence was observed. When *Agrobacterium* strain AGL1 was used for genetic transformation, only weak instantaneous fluorescence was observed (Fig. 1a1, b1). When strain GV3101 was used, medium fluorescence was detected at edges of the whole cotyledon, but stronger fluorescence was found at edges of the whole cotyledon by using strain EHA105 (Fig. 1a3, b3). The results showed that watermelon 'YL' was insensitive to AGL1 strain. Both GV3101 and EHA105 strains could be applicative for the genetic transformation system in watermelon.

### Effects of acetosyringone (AS) concentration in *Agrobacterium* inoculum on infection efficiency

To determine which concentration of AS in *Agrobacterium* inoculum could produce the highest genetic transformation efficiency in watermelon. The AS concentrations were separated into five gradients, which were 0, 100, 150, 200, 250 and 300 μM. In each gradient, 120 explants were inoculated. As shown in Table 1, the fluorescence efficiencies of the explants were 45.3%–85.0% due to increased concentrations of AS in the infection solution (Supplemental Table S3). It was also found that the fluorescence efficiency and differentiation efficiency of watermelon explants were significantly different. Fluorescence signals of calli from 100–200 μM AS treatments were acceptable (Fig. 2). However, the infection solution containing 250 or 300 μM AS had large negative effects on germination ability and survival rate of germinated callus, and finally led to a low transformation rate. Our results demonstrated that 200 μM AS in the infection solution was most suitable for genetic transformation of watermelon.

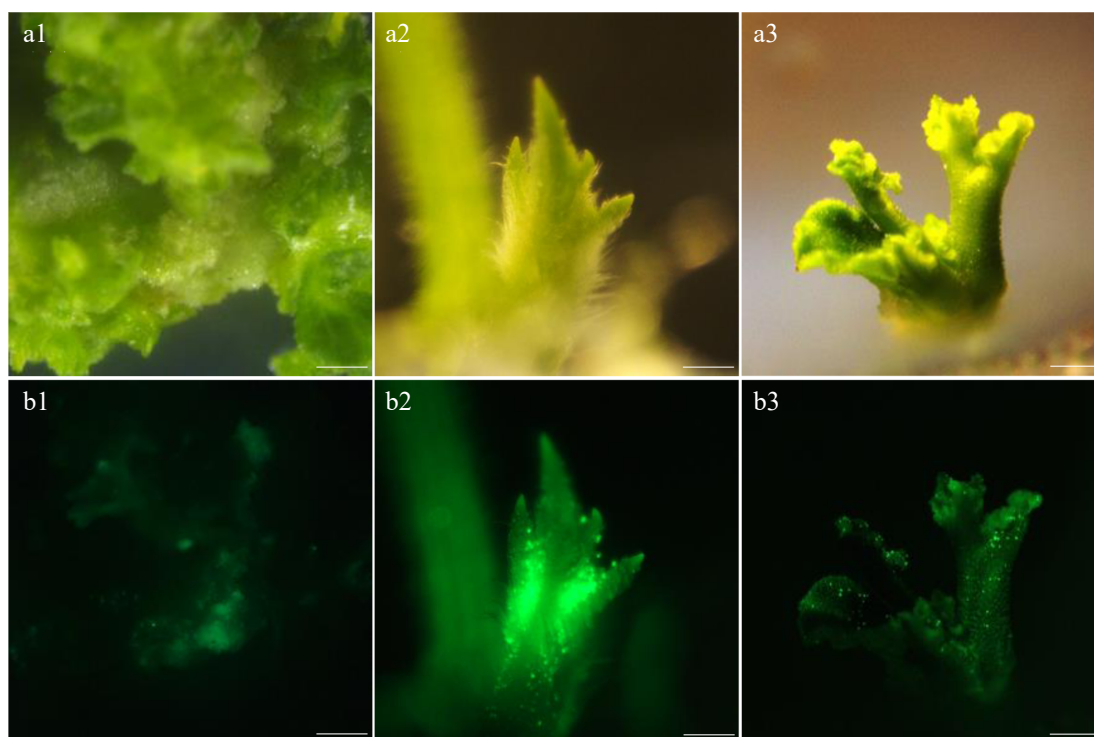


**Fig. 1** Transient fluorescence of explants. (a1)–(a3) Bright view. (b1)–(b3) GFP view. (b1) The explants showed weak fluorescence after AGL1 infection. (b2) The explants showed moderate strong fluorescence after GV3101 infection. (b3) The explants showed strong fluorescence after EHA105 infection. Bar = 2 mm.

**Table 1.** Fluorescence efficiencies, brightness and differentiation states of watermelon explants under different AS concentrations.

AS treatment ( $\mu\text{M}$ )	Fluorescence efficiency (%)	Number of fluorescent explants	Fluorescence brightness	Callus differentiation state
0	$45.3 \pm 0.05^d$	54	++	The calli were green and compact. There was no contamination and little vitrification
100	$68.8 \pm 0.03^b$	82	+++	The calli were densely arranged with dark green, fluorescent speckles and little vitrification
150	$75.0 \pm 0.02^b$	90	++++	The calli were densely arranged and appeared dark green with bright fluorescence and little vitrification
200	$85.0 \pm 0.05^a$	102	+++++	The calli were densely arranged and appeared dark green with bright fluorescence and little vitrification
250	$69.3 \pm 0.04^b$	83	++++	Most calli were densely arranged, and a few turned pale and yellowed with water stain
300	$54.7 \pm 0.05^c$	65	++++	The calli were closely arranged, partly vitrified with dark green and contaminated by bacteria

Fluorescence efficiency = Number of fluorescence explants/Total explants number  $\times$  100%. Values are means (three independent experiments)  $\pm$  standard errors (SE), and different letters indicate significant differences between treatments according to Duncan's multiple test ( $P < 0.05$ ).



**Fig. 2** Fluorescence intensity of the callus under different concentrations of AS. (a1)–(a3) Bright view. (b1)–(b3) GFP view. (a1), (b1) AS concentration at 100  $\mu\text{M}$ . (a2), (b2) AS concentration at 200  $\mu\text{M}$ . (a3), (b3) AS concentration at 300  $\mu\text{M}$ . Bar=1 mm.

### Effects of *Agrobacterium tumefaciens* concentrations in the inoculum and co-culture time on transformation efficiency

The co-cultivation stage is the key to improving infection efficiency in genetic transformation. During the middle and later periods of co-cultivation, brighter GFP fluorescence meant higher transformation success rate. Due to the browning explants auto-fluorescence, we need to ensure that explants were kept alive and free of pollution as much as possible even though bright fluorescence was found.

To improve the infection efficiency, four concentrations of *A. tumefaciens* (EHA105) infection solution were set as 0.6, 0.2, 0.02 and 0.005 at  $\text{OD}_{600}$ <sup>[7,24]</sup>. The co-culture time was set at three gradients of 2, 3 and 4 d. Therefore, 12 associations were listed in total, named A-L respectively (Table 2). Explants, which were cut into eight pieces longitudinally, were immersed in the inoculum for 10 min. The results showed that GFP fluorescence

of explants was the strongest when  $\text{OD}_{600}$  of *A. tumefaciens* infection solution was 0.6 and 0.02, but the number of fluorescent explants was more at  $\text{OD}_{600} = 0.02$ . The fluorescence efficiency of explants was relatively high when cultured in co-cultivation medium for 3 and 4 d (Fig. 1b). However, when the explants were co-cultured for 4 d, there was pollution around the explants, and the edges showed serious browning. The growth states of each combination are shown in Table 3.

### Effects of different concentrations of hormones in co-cultivation medium on callus regeneration

The direct differentiation method was used to optimize genetic transformation of watermelon, while the calli redifferentiation method was an optimization system developed on the basis of previous experience in this research<sup>[25]</sup>. According to the results of seedling germination, experiments of cotyledon dedifferentiation were conducted. The strong calli were more easily induced into adventitious shoots by 6-BA, which was

beneficial to improve the efficiency of genetic transformation efficiency. The temperatures of cotyledon differentiation ranged from 26–28 °C. Concentration treatments of hormones were divided into 12 groups (Supplemental Table S4). We further selected 3 and 4 d (named as YL-3 and YL-4 in Supplemental Table S4) to culture explants in co-cultivation medium (Supplemental Table S5). The results indicated that the combination of 1.5 mg/L 6-BA without indole acetic acid (IAA)

**Table 2.** Transient fluorescence efficiencies and brightness of watermelon explants under different concentrations of *Agrobacterium tumefaciens* infection solution and co-culture times.

Combination	<i>Agrobacterium</i> concentration (OD <sub>600</sub> )	Coculture time (days)	Fluorescence efficiency (%)	Fluorescence brightness
A	0.6	2	42.5 ± 1.5 <sup>b</sup>	+++
B	0.2	2	54.4 ± 1.4 <sup>a</sup>	++
C	0.02	2	38.5 ± 1.8 <sup>b</sup>	+
D	0.005	2	33.3 ± 0.3 <sup>c</sup>	+
E	0.6	3	38.1 ± 1.1 <sup>c</sup>	+
F	0.2	3	43.0 ± 2.0 <sup>b</sup>	++
G	0.02	3	79.1 ± 0.9 <sup>a</sup>	+++
H	0.005	3	47.1 ± 0.5 <sup>b</sup>	+
I	0.6	4	35.5 ± 1.4 <sup>c</sup>	+
J	0.2	4	44.5 ± 2.6 <sup>b</sup>	+
K	0.02	4	79.1 ± 0.6 <sup>a</sup>	+++
L	0.005	4	51.5 ± 2.5 <sup>b</sup>	++

Fluorescence efficiency = Number of fluorescence explants/Total explants number × 100%. Values are means (three independent experiments) ± standard errors (SE), and different letters indicate significant differences between treatments according to Duncan's multiple test ( $P < 0.05$ ).

**Table 3.** Growth states of watermelon explants under different *Agrobacterium tumefaciens* infection solution and co-culture times.

Combination	Growth state
A	A small part died, and the margin of the surviving explants were yellow with obvious water-stained flora
B	The edges showed traces of a watery microflora
C	The explants were well developed
D	The explants were well developed
E	Some died with serious spillage, and fluorescence explants were browning
F	A small portion were dead and the margins browned
G	The explants dilated well
H	The explants dilated well
I	Most died with serious spillage phenomenon, and virtually all the edges browned
J	Partially dead with serious spillage and serious edge browning
K	Partially edged with yellow and with spillage phenomenon
L	The explants were well developed

**Table 4.** The survival rates and growth states of callus under different concentrations of Basta.

Basta concentration (mg/L)	Callus survival rate (%)	Fluorescence rate of survival callus (%)	Callus state
0.4	100.0 ± 0.0 <sup>a</sup>	69.1 ± 4.1 <sup>c</sup>	The tissues appeared dark green and grew well
1.4	73.6 ± 0.4 <sup>b</sup>	78.7 ± 0.3 <sup>b</sup>	Partial tissues appeared brown and most grew well
2.4	73.0 ± 3.0 <sup>b</sup>	80.5 ± 1.5 <sup>b</sup>	Partial tissues were transparent and light green with serious vitrification
3.4	40.8 ± 1.2 <sup>c</sup>	92.0 ± 2.9 <sup>a</sup>	Partial tissues died with serious yellowing phenomenon and obvious spillage
4.4	0.7 ± 0.3 <sup>d</sup>	0.7 ± 0.3 <sup>d</sup>	Most tissues died, and the spillage was obvious

Callus survival rate = Survival callus number/Total callus number × 100%; Fluorescence rate of survival callus = Survival callus number with fluorescence/Total survival callus number. Values are means (three independent experiments) ± standard errors (SE), and different letters indicate significant differences between treatments according to Duncan's multiple test ( $P < 0.05$ ).

had the highest callus differentiation rate after 3 d of co-cultivation.

### Effects of different concentrations of TMT and Basta on callus regeneration

Ticarcillin, one of the active components in TMT, was used as an antibacterial agent. Based on the differentiation and growth state of callus, TMT with a concentration of 50–350 mg/L could inhibit the growth of bacteria at an early stage. However, concentrations of TMT at 350 mg/L caused more severe aetiolation in the later stages of development, especially in the rooting stage, which could affect the growth of the roots (Supplemental Table S6). The addition of TMT with a concentration at 200 mg/L had no fungal contamination. Based on the standard of high proliferation rate and normal growth state, 200 mg/L TMT was considered as the best concentration, which was added to the recovery medium (Supplemental Table S7). In addition, 200 mg/L TMT could effectively inhibit the growth of *Agrobacterium tumefaciens* and no contamination was detected after regenerated explants were transferred to a TMT-free culture medium. TMT with a concentration higher than 350 mg/L resulted in yellowing phenomenon and seedling death, which indicates that excessive TMT has some toxic effect. We named the recovery stage after the recovery process, as shown in the Supplemental Fig. S1c.

The gene editing vector used in this experiment contained a marker gene that could be resistant to Basta, and resistance screening was used to screen the positive transgenic shoot (Supplemental Table S8). At concentrations of 0.4 and 1.4 mg/L Basta, the survival rate and fluorescence efficiency of callus were higher than those of other concentration gradients, although there was no obvious difference between them (Table 4). However, when the concentration of Basta was 2.4 mg/L, the callus vitrified seriously, and the redifferentiation of calls were inhibited. At 1.4 mg/L, the well-grown callus showed high fluorescence efficiency, and the brown callus was selected as the best concentration. We named the selection stage after the selection process (Supplemental Fig. S1d). The results revealed that the concentration of Basta higher than 1.4 mg/L caused the shorter survival time of explants.

### Effects of hormone concentrations on adventitious shoot elongation

Adventitious shoot differentiation was easier in well-developed and dedifferentiated calli. In order to improve the regeneration rates of buds, three concentrations of 6-BA (0.05, 0.1, 0.15 mg/L) and two concentrations of naphthylacetic acid (NAA) (0.05, 0.1 mg/L) were applied to analyze the formation of adventitious elongated shoots (Table 5). When the concentration of 6-BA is 0.1 mg/L and NAA is 0.1 mg/L, the frequencies



**Table 5.** Adventitious shoot elongation efficiencies and shoot growth states under different concentrations of hormones.

6-BA (mg/L)	NAA (mg/L)	Shoot regeneration rate (%)	Adventitious shoot growth state
0.05	0.05	10.3 ± 1.8 <sup>c</sup>	The adventitious shoots differentiated less and had serious vitrification
0.1	0.05	22.0 ± 1.0 <sup>b</sup>	Less differentiation, and vitrification was not serious
0.15	0.05	32.4 ± 2.6 <sup>a</sup>	Less differentiation, some shoots unable to continue to elongate and showed yellowing
0.05	0.1	29.2 ± 0.7 <sup>b</sup>	Less differentiation, calli partly yellowed and vitrified
0.1	0.1	64.5 ± 5.5 <sup>a</sup>	With more differentiation, calli partly vitrified without yellowing phenomenon
0.15	0.1	45.6 ± 3.8 <sup>b</sup>	More differentiation, shoots partly yellowed with a small amount producing air roots

Shoot regeneration rate = Number of regerminated shoots/Total explants number × 100%. Values are means (three independent experiments) ± standard errors (SE), and different letters indicate significant differences between treatments according to Duncan's multiple test ( $P < 0.05$ ).

of shoot elongation was the highest (Fig. 3a). When 6-BA and NAA were higher than 0.15 mg/L and 0.1 mg/L respectively, the callus directly differentiated into aerial roots, and explants were prone to yellowing (Fig. 3b). When 6-BA and NAA were 0.1 mg/L and 0.05 mg/L respectively, shoot elongation was inhibited (Fig. 3c). The results indicated that 0.1 mg/L 6-BA and 0.1 mg/L NAA were the best concentrations to improve the elongation efficiency of adventitious shoots and keep their good growth states (Supplemental Fig. S1e). Shoot elongation medium is shown in Supplemental Table S9.

#### Effects of budding methods on positive plant rooting

In this study, it was found that adventitious buds could generate roots 7–14 d after being transferred to the rooting medium. (Supplemental Table S10). Based on these results, all optimized aspects were used in our following research to obtain an effective transformation frequency. A positive plant was obtained, which is shown in Supplemental Fig. S1f.

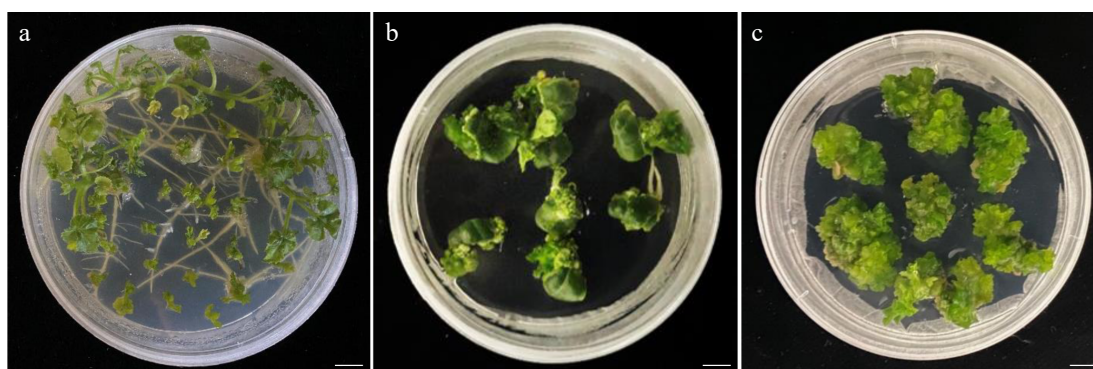
#### Verification of optimization efficiency of watermelon genetic transformation system

The above experimental results showed that the watermelon transformation system had been optimized to some extent. To verify the efficiency, we selected *CIREC8* (Cla97C07G132920),

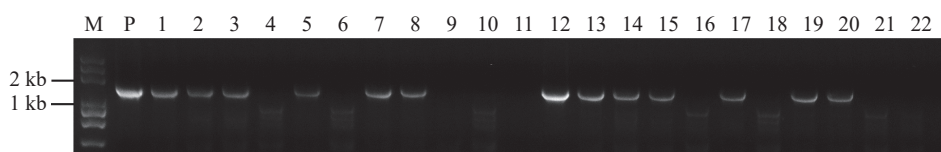
*CIACS1* (Cla97C01G017090) and *CIACS7* (Cla97C03G066110) as target genes to perform gene knockout assay in watermelon. PCR amplification was performed using the primers Cas9-F and Cas9-R, and the results revealed the transgenic plants contained exogenous T-DNA inserts (Fig. 4). Furthermore, targeted gene sequences in the transgenic plants were sequenced, and the results in three target genes were partly shown (Fig. 5, Supplemental Fig. S2). As a result, in approximately 300 inoculated explants, we obtained a total of 45 T<sub>0</sub> transgenic lines, among which 42 plants were successfully edited. The percentage of 93.3% meant that the optimized transformation system could also be used for watermelon gene editing.

#### DISCUSSION

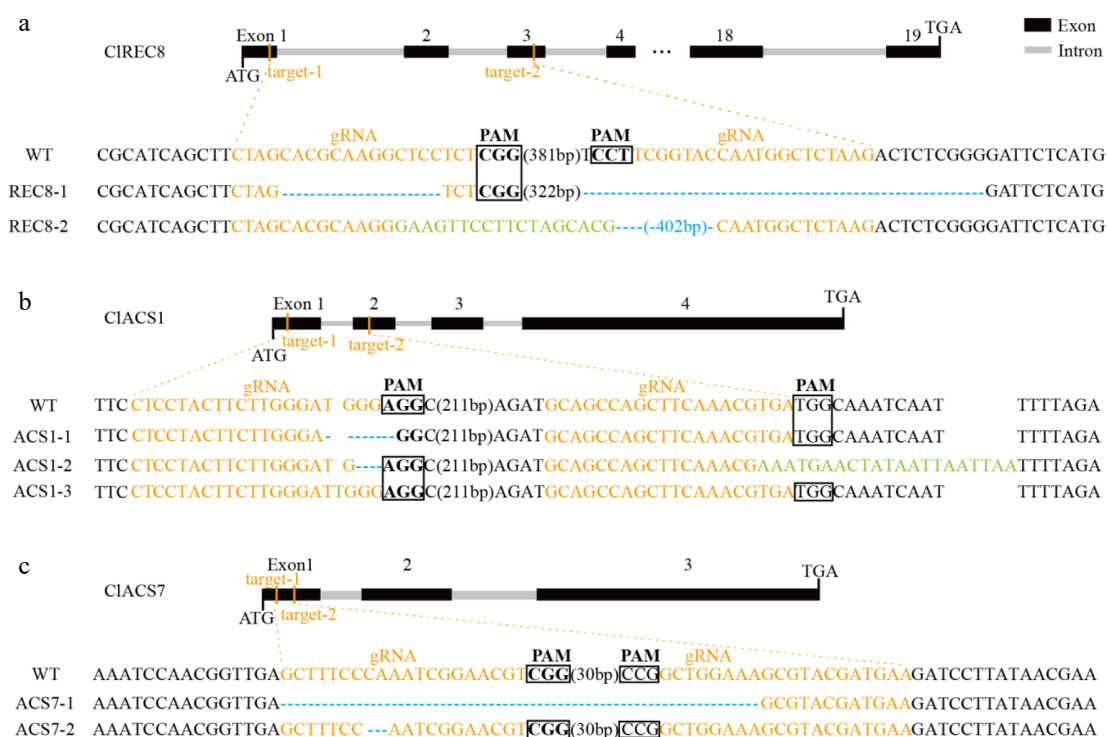
It is widely known that transgenesis and gene-editing technologies were made full use of for promoting plant research processes [25–27]. Considering the significance of the genetic stability of both transformed exogenous genes and regenerated plants, various methods have been tried for transformation, including particle acceleration [28], electroporation and polyethylene glycol permeabilization of protoplasts [29], and DNA transfer mediated by *Agrobacterium*.



**Fig. 3** Redifferentiation state of explants under different hormone concentrations. (a) The callus differentiated well under 0.1 mg/L concentration of 6-BA and 0.1 mg/L concentration of NAA. (b) The callus redifferentiated to form aerial roots under 0.15 mg/L concentration of 6-BA and 0.1 mg/L concentration of NAA. (c) The callus appeared to yellow and could not differentiate under 0.15 mg/L concentration of 6-BA and 0.05 mg/L concentration of NAA. Bar = 12 mm.



**Fig. 4** PCR analysis of the transgenic plants. Genomic DNA isolated from putative transgenic plants were subjected to PCR amplification with Cas9 primers. Lane M, Trans2K Plus DNA Marker; Lane P, positive control (plasmid); Lanes 1, 2, 3, 5, 7, 8, 12, 13, 14, 15, 17, 19 and 20, putative transgenic watermelons; Lane 4, 6, 9, 10, 11, 16, 18, 21 and 22, Non-transgenic watermelon.



**Fig. 5** Targeted mutagenesis of (a) *CIREC8*, (b) *CIACS1*, and (c) *CIACS7* in the transgenic lines. The schematic diagrams illustrate sgRNA targeting the exons. The target sequences are shown in orange with protospacer adjacent motifs (PAM) sequence highlighted with black rectangles. Nucleotide deletions are shown with blue dashes, and inserted nucleotides are shown in green.

However, previous studies indicated that only few positive lines have been obtained due to difficulties in watermelon transformation<sup>[17,30]</sup>. Extremely low transformation efficiency in watermelon transformants was largely due to the high degree of escape and chimeric shoot<sup>[14]</sup>.

In this study, we optimized the watermelon genetic transformation system to improve the genetic transformation efficiency. The growth process of young seedlings grew promisingly compared with old seedlings<sup>[31]</sup>, however, both tender aged and over aged seedlings are not suitable for better yield<sup>[32]</sup>. The watermelon seedling age, which was of great significance for callus regeneration, was the key factor as to whether induction was successful and highly effective or not. The explants state determined the impregnation efficiency of the subsequent *Agrobacterium*-mediated method. Poor explants state usually causes problems such as bacterial growth, vitrification or redifferentiating inability in the cultivation process. The experimental results showed that explants with good viability could be obtained after a disinfection time of 12 min and cultivation time of 3 d.

The findings of this study were consistent with other reports of watermelon transformation with the *Agrobacterium*-mediated method<sup>[3]</sup>. AS, a phenolic compound which was a known *vir* gene inducer especially in monocotyledon plants, plays a crucial role in the transferring process<sup>[33]</sup>. However, a high quantity of AS was toxic to the plants, which could be mainly due to the presence of alcohol as a solvent<sup>[34]</sup>. Germplasm differences showed different sensitivity to AS. In many other crops, such as wheat, cucumber and sesbania, the addition of AS to *Agrobacterium* inoculum and co-cultivation medium has been shown to improve conversion efficiency<sup>[35–37]</sup>. Previous studies indicated that the highest concentration of AS used for transformation of various plants

was between 100–200  $\mu\text{M}$ <sup>[38–40]</sup>. We selected an AS concentration at 200  $\mu\text{M}$  to improve infection efficiencies of strain and fluorescence efficiency.

Due to germplasm differences possessing diverse genotypes, efficiencies of watermelon genetic transformation were also different<sup>[41]</sup>. It was also essential to select the appropriate *Agrobacterium* strain for different watermelon germplasms to improve their ability in infecting explants. In previous studies, types of *Agrobacterium* strains used in the genetic transformation of cucurbitaceae crops were different, including AGL1, EHA105 and GV3101<sup>[6,42]</sup>. Our study indicated that watermelon germplasm 'YL' was sensitive to *Agrobacterium* strain EHA105, which was the basis for improving the transformation efficiency.

*Agrobacterium* concentration and coculture time were considered as the key to improving the genetic transformation efficiency. Too low a concentration could not infect explants smoothly, whereas too high a concentration led to bacterial overgrowth, causing explants death. Too short coculture time could not ensure T-DNA transferred into the explant cells, and integrated into the genome. Instead, bacterial overgrowth resulted in explants not being able to continue to grow, and transformation efficiency was reduced. In terms of *Agrobacterium* population density, the fluorescence efficiency of watermelon explants was higher at an OD600 of 0.02. The total time was fixed at 10 min, which was the same time reported previously for immersion in *Agrobacterium* inoculum<sup>[43]</sup>. The optimum working concentration of *Agrobacterium* used in this study was relatively low, and the fluorescence was brighter when co-cultured for 3 d. This may be related to the sensitivity of explants from different germplasms. However, if infected by lower concentrations, the efficiency cannot be improved. In contrast, co-cultivation for too long would cause bacteria colonies proliferation and explants death.

## Transformation in watermelon

Efficiencies of watermelon transformation were affected by many other factors, and certain parameters were slightly different in a variety of watermelon germplasms<sup>[44]</sup>. Taking watermelon germplasm 'YL' as the original material, this study further explored hormone concentrations for watermelon callus development. The explants differentiation rate was the highest with 1.5 mg/L 6-BA and no IAA. In addition, adventitious shoots could be induced to differentiate and even elongate by using the hormone combination of 0.1 mg/L 6-BA and 0.1 mg/L NAA, and growth states were good. The results indicated that 'YL' might not be sensitive to IAA.

Antibiotics could effectively inhibit a portion of *Agrobacterium* to ensure good growth of explants. TMT, an antibiotic agent that is a combination of ticarcillin and clavulanic acid, can positively replace other antibiotics like carbenicillin and cefotaxime in tissue culture<sup>[45,46]</sup>. Because it is stable in solid agar medium and can keep effective for longer than 70 d, TMT could be an alternative antibiotic for suppressing *Agrobacterium* growth in transformation effectively and improving regeneration potential compared with other antibiotics such as carbenicillin<sup>[47,48]</sup>. Low concentration of TMT could not arrest bacteria proliferation so that explants cannot continue to grow. In contrast, high concentrations of TMT would not only inhibit the bacterial growth but also inhibit the growth of explants, resulting in a decline in transformation efficiency. Our results demonstrated that the most appropriate concentration of TMT was 200 mg/L, which could facilitate shoot regeneration.

A codon-modified phosphinothricin acetyltransferase gene, which confers resistance to the herbicide glufosinate-ammonium, was used as the selectable marker<sup>[49]</sup>. Glufosinate-ammonium is able to block the activity of an enzyme used in the biosynthesis of amino acid glutamine, which has been reported in different species<sup>[50–52]</sup>. The study demonstrated that Basta had a pivotal effect on the screening of positive callus and shoot at 1.4 mg/L. The screening method could greatly improve the detection efficiency, and reduce the workload.

The experiments provided preparation for the subsequent obtaining of knocked lines and reduced the problems such as poor differentiation ability of callus, yellowing phenomenon, and vitrification in watermelon. Meanwhile, fluorescence efficiencies of the watermelon callus, which was conducive to the fluorescent shoots, were improved. The pH and humidity of each medium should be strictly controlled in the experiment, and the callus growth states should be observed regularly and repeatedly.

Recently, a study revealed that a mutation in the miR396 microRNA region included in *CIGRF4* gene led to efficiency up to 67.27% in watermelon, no matter which genotype was applied for transformation<sup>[53]</sup>. Nevertheless, our results suggested that watermelon transformation efficiency (from explant to transgenic plant) was increased to 12.5% using the optimized system in this study. Multigene knockouts as well as gene replacements have not yet been widely studied in watermelon and other plants<sup>[54]</sup>. Our research made it possible to optimize the watermelon multi-genetic transformation system more efficiently in the future.

## MATERIALS AND METHODS

### Plant materials

The watermelon germplasm 'YL', cultivated at 36°–39° N and 107°–111° E from a warm semi-humid climate, has strong

drought resistance and moderate resistance to *Fusarium* wilt, and was used as the original material in this experiment. The materials were planted in a greenhouse at the College of Horticulture, Northwest A&F University (Shaanxi, China).

### A. *tumefaciens* strains and binary vector construction

Three *A. tumefaciens* strains including AGL1<sup>[55]</sup>, GV3101<sup>[6]</sup> and EHA105<sup>[56]</sup> were tested in this study. The binary CRISPR/Cas9 vectors pBSE402 (carrying a *Bar* and a *GFP* genes, both of which were driven by 35S promoters) was modified from pBSE401 provided by Dr. Qijun Chen from China Agricultural University (Beijing, China), and the vectors were constructed as described<sup>[7,16]</sup>. Positive regenerated plants were detected by polymerase chain reaction. The PCR primers Cas9-F (5'-GCAG CTCTCCAAGGACACAT-3') and Cas9-R: (5'-CGTGAGTCTCTG GCCCTT-3') were designed using Primer Premier 5 software. The PCR was conducted using Taq PCR Mix (GenStar, China), and performed in an optical 96-well plate with 2720 Thermal Cycler (Applied Biosystems, USA). The positive results were further sub-cloned with pUC18 plasmids, sequenced, and analyzed according to Kaur et al.<sup>[57]</sup>.

### Vitro culture and regeneration system

Plants were regenerated from watermelon cotyledons<sup>[58,59]</sup>. Adventitious shoots formed on the proximal cut edges of the cotyledonary explants<sup>[60]</sup>. Cotyledons of sterile watermelon seedlings aged 2–5 d were cut into small pieces of 0.5 cm, then inoculated on MS medium with 6-BA to dedifferentiate into calli, on which a lot of adventitious shoots appeared after 2 weeks. Adventitious shoots were then transferred to the rooting medium for 2–3 weeks. The culture conditions were 28 °C, 16 h day, and 3,000 lx light intensity.

### Optimization of the genetic transformation system

The experiment was carried out from several aspects to optimize the transformation system, including different seedling ages, *Agrobacterium* strains, AS and *Agrobacterium* concentrations of the inoculum, concentrations of antibiotic TMT and Basta selection pressure, and concentrations of added hormones.

### Data availability

The data that support the results are included in this manuscript and its supporting information files. Other relevant materials are available from the corresponding author upon reasonable request.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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## REFERENCES

- Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, et al. 1985. A simple and general-method for transferring genes into plants. *Science* 227:1229–31
- Wechter WP, Levi A, Harris KR, Davis AR, Fei Z, et al. 2008. Gene expression in developing watermelon fruit. *BMC Genomics* 9:275
- Choi PS, Soh WY, Kim YS, Yoo OJ, Liu JR. 1994. Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumefaciens*. *Plant Cell Reports* 13:344–48
- Akashi K, Morikawa K, Yokota A. 2005. *Agrobacterium*-mediated transformation system for the drought and excess light stress-tolerant wild watermelon (*Citrullus lanatus*). *Plant Biotechnology* 22:13–18
- Suratman F, Huyop F, Parveez GKA. 2009. In vitro shoot regeneration of *Citrullus vulgaris* schrad (watermelon). *Biotechnology* 8:393–404
- Cho MA, Moon CY, Liu JR, Choi PS. 2008. *Agrobacterium*-mediated transformation in *Citrullus lanatus*. *Biologia Plantarum* 52:365–69
- Yu TA, Chiang CH, Wu HW, Li CM, Yang CF, et al. 2011. Generation of transgenic watermelon resistant to *Zucchini yellow mosaic virus* and *Papaya ringspot virus* type W. *Plant Cell Reports* 30:359–71
- Ren Y, Guo S, Zhang J, He H, Sun H, et al. 2017. A tonoplast sugar transporter underlies a sugar accumulation QTL in watermelon. *Plant Physiology* 176:836–50
- Tian S, Jiang L, Gao Q, Zhang J, Zong M, et al. 2017. Efficient CRISPR/Cas9-based gene knockout in watermelon. *Plant Cell Reports* 36:399–406
- Wang Y, Wang J, Guo S, Tian S, Zhang J, et al. 2021. CRISPR/Cas9-mediated mutagenesis of *CIBG1* decreased seed size and promoted seed germination in watermelon. *Horticulture Research* 8:70
- Godwin I, Todd G, Ford-Lloyd B, Newbury HJ. 1991. The effects of acetosyringone and PH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Reports* 9:671–75
- Vengadesan G, Anand RP, Selvaraj N, Perl-Treves R, Ganapathi A. 2005. Transfer and expression of *nptII* and *bar* genes in cucumber (*Cucumis Sativus* L.). *In Vitro Cellular & Developmental Biology - Plant* 41:17–21
- Huang YC, Chiang CH, Li CM, Yu TA. 2011. Transgenic watermelon lines expressing the nucleocapsid gene of *Watermelon silver mottle virus* and the role of thiamine in reducing hyperhydricity in regenerated shoots. *Plant Cell, Tissue and Organ Culture* 106:21–29
- Liu L, Gu Q, Ijaz R, Zhang J, Ye Z. 2016. Generation of transgenic watermelon resistance to Cucumber mosaic virus facilitated by an effective *Agrobacterium*-mediated transformation method. *Scientia Horticulturae* 205:32–38
- Zhang R, Chang J, Li J, Lan G, Xuan C, et al. 2021. Disruption of the bHLH transcription factor Abnormal Tapetum 1 causes male sterility in watermelon. *Horticulture Research* 8:258
- Xing H, Dong L, Wang Z, Zhang H, Han C, et al. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology* 14:327
- Jia H, Wang N. 2014. Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* 9:e93806
- Ren C, Liu X, Zhang Z, Wang Y, Duan W, et al. 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera* L.). *Scientific Reports* 6:32289
- Guo S, Zhang J, Sun H, Salse J, J Lucas WJ, et al. 2013. The draft genome of watermelon (*Citrullus lanatus*) and resequencing of 20 diverse accessions. *Nature Genetics* 45:51–58
- Ren Y, Li M, Guo S, Sun H, Zhao J, et al. 2021. Evolutionary gain of oligosaccharide hydrolysis and sugar transport enhanced carbohydrate partitioning in sweet watermelon fruits. *The Plant Cell* 33:1554–73
- Zhang J, Guo S, Ji G, Zhao H, Sun H, et al. 2020. A unique chromosome translocation disrupting *CIWIP1* leads to gynoeicy in watermelon. *The Plant Journal* 101:265–77
- Chang J, Guo Y, Yan J, Zhang Z, Yuan L, et al. 2021. The role of watermelon caffeic acid O-methyltransferase (*CICOMT1*) in melatonin biosynthesis and abiotic stress tolerance. *Horticulture Research* 8:210
- Guo H, Guo H, Zhang L, Fan Y, Fan Y, et al. 2019. SELTP-assembled battery drives totipotency of somatic plant cell. *Plant Biotechnology Journal* 17:1188–90
- Nanasato Y, Konagaya KI, Okuzaki A, Tsuda M, Tabei Y. 2013. Improvement of *Agrobacterium*-mediated transformation of cucumber (*Cucumis sativus* L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. *Plant Biotechnology Reports* 7:267–76
- Li Q, Wu G, Zhao Y, Wang B, Zhao B, et al. 2020. CRISPR/Cas9-mediated knockout and overexpression studies reveal a role of maize phytochrome C in regulating flowering time and plant height. *Plant Biotechnology Journal* 18:2520–32
- Azeez A, Busov V. 2021. CRISPR/Cas9-mediated single and biallelic knockout of poplar *STERILE APETALA (PopSAP)* leads to complete reproductive sterility. *Plant Biotechnology Journal* 19:23–25
- Liu Y, Wang Y, Xu S, Tang X, Zhao J, et al. 2019. Efficient genetic transformation and CRISPR/Cas9-mediated genome editing in *Lemna aequinoctialis*. *Plant Biotechnology Journal* 17:2143–52
- Sanford JC. 1990. Biolistic plant transformation. *Physiologia Plantarum* 79:206–9
- Potrykus I. 1990. Gene transfer to cereals: an assessment. *Nature Biotechnology* 8:535–42
- Vasudevan V, Siva R, Krishnan V, Manickavasagam M. 2020. Polyamines, sonication and vacuum infiltration enhances the *Agrobacterium*-mediated transformation in watermelon (*Citrullus lanatus* Thunb.). *South African Journal of Botany* 128:333–38
- Li Y, Liu Y, Wang Y, Ding Y, Wang S, et al. 2020. Effects of seedling age on the growth stage and yield formation of hydroponically grown long-mat rice seedlings. *Journal of Integrative Agriculture* 19:1755–67
- Tinni TBR, Ali MA, Mehraj H, Shiam IH, Jamal Uddin AFM. 2014. Influence of seedling age on growth and yield of brinjal (*Solanum Melongena* L.). *Bangladesh Research Publication Journal* 10:170–74
- Lin J, Zhou B, Yang Y, Mei J, Zhao X, et al. 2009. Piercing and vacuum infiltration of the mature embryo: a simplified method for *Agrobacterium*-mediated transformation of *indica* rice. *Plant Cell Reports* 28:1065–74
- de Oliveira MLP, Febres VJ, Costa MGC, Moore GA, Otoni WC. 2009. High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. *Plant Cell Reports* 28:387–95
- Wu H, Sparks C, Amoah B, Jones HD. 2003. Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. *Plant Cell Reports* 21:659–68
- Nishibayashi S, Kaneko H, Hayakawa T. 1996. Transformation of cucumber (*Cucumis sativus* L.) plants using *Agrobacterium tumefaciens* and regeneration from hypocotyl explants. *Plant Cell Reports* 15:809–14
- Padmanabhan P, Sahi SV. 2009. Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. *Plant Cell Reports* 28:31–40
- Bakshi S, Sadhukhan A, Mishra S, Sahoo L. 2011. Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration. *Plant Cell Reports* 30:2281–92
- Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, et al. 2015. *Agrobacterium*-mediated in planta genetic transformation of sugarcane setts. *Plant Cell Reports* 34:1835–48
- Hiei Y, Komari T, Kubo T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Molecular Biology* 35:205–18
- Cho SM, Oh SA, Choi YS, Park SB. 2014. Effect of plant growth regulators on regeneration from the cotyledon explants in watermelon (*Citrullus lanatus* (thunb.) Matsum. & Nakai). *Korean Journal of Plant Resources* 27:51–59



## Transformation in watermelon

42. Compton ME. 2000. Interaction between explant size and cultivar affects shoot organogenic competence of watermelon cotyledons. *HortScience* 5:749–50
43. Selvaraj N, Kasthuriangan S, Vasudevan A, Manickavasagam M, Choi CW, et al. 2010. Evaluation of green fluorescent protein as a reporter gene and phosphinothricin as the selective agent for achieving a higher recovery of transformants in cucumber (*Cucumis sativus* L. cv. Poinsett76) via *Agrobacterium tumefaciens*. *In Vitro Cellular & Developmental Biology - Plant* 46:329–37
44. Mujaju C. 2011. Local-level assessment of watermelon genetic diversity in a village in Masvingo Province, Zimbabwe: Structure and dynamics of landraces on farm. *African Journal of Agricultural Research* 6:5822–34
45. Gentry LO, Macko V, Lind R, Heilman A. 1985. Ticarcillin plus clavulanic acid (Timentin) therapy for osteomyelitis. *The American Journal of Medicine* 79:116–21
46. Nauerby B, Billing K, Wyndaele R. 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Science* 123:169–77
47. Cheng ZM, Schnurr JA, Kapaun JA. 1998. Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Plant Cell Reports* 17:646–49
48. Li ZN, Liu GF, Fang F, Bao MZ. 2007. Adventitious shoot regeneration of *Platanus acerifolia* Willd. facilitated by Timentin, an antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Forestry Studies in China* 9:14–18
49. Wu E, Lenderts B, Glassman K, Berezowska-Kaniewska M, Christensen H, et al. 2014. Optimized *Agrobacterium*-mediated sorghum transformation protocol and molecular data of transgenic sorghum plants. *In Vitro Cellular & Developmental Biology Plant* 50:9–18
50. Hoerlein G. 1994. Glufosinate (phosphinothricin), a natural amino acid with unexpected herbicidal properties. In *Reviews of Environmental Contamination and Toxicology*, ed. Ware GW. vol 138. New York: Springer, New York. pp. 73–145 [https://doi.org/10.1007/978-1-4612-2672-7\\_4](https://doi.org/10.1007/978-1-4612-2672-7_4)
51. Kutlesa NJ, Caveney S. 2001. Insecticidal activity of glufosinate through glutamine depletion in a caterpillar. *Pest Management Science* 57:25–32
52. Kang GR, Song HY, Kim DS. 2014. Toxicity and effects of the herbicide glufosinate-ammonium (Basta) on the marine medaka *Oryzias dancena*. *Fisheries and Aquatic Sciences* 17:105–13
53. Feng Q, Xiao L, He Y, Liu M, Wang J, et al. 2021. Highly efficient, genotype-independent transformation and gene editing in watermelon (*Citrullus lanatus*) using a chimeric *CIGRF4-GIF1* gene. *Journal of Integrative Plant Biology* 63:2038–42
54. Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJY, et al. 2017. A multipurpose toolkit to enable advanced genome engineering in plants. *The Plant Cell* 29:1196–1217
55. Lazo GR, Stein PA, Ludwig RA. 1991. A DNA transformation competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology* 6:963–67
56. Hood EE, Gelvin SB, Melchers LS, Hoekema A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* 2:208–18
57. Kaur N, Alok A, Shivani, Kaur N, Pandey P, et al. 2018. CRISPR/Cas9-mediated efficient editing in *phytoene desaturase* (*PDS*) demonstrates precise manipulation in banana cv. Rasthali genome. *Functional & Integrative Genomics* 18:89–99
58. Srivastava DR, Andrianov VM, Piruzian ES. 1989. Tissue culture and plant regeneration of watermelon (*Citrullus vulgaris* Schrad. cv. Melitopolski). *Plant Cell Reports* 8:300–2
59. Parveez GKA, Ghazali H, Rahmat Z, Wagiran A, Suratman F. 2010. Cotyledon with hypocotyl segment as an explant for the production of transgenic *Citrullus vulgaris* Schrad (watermelon) mediated by *Agrobacterium tumefaciens*. *Biotechnology* 9:106–18
60. Compton ME, Gray DJ. 1994. Adventitious shoot organogenesis and plant regeneration from cotyledons of tetraploid watermelon. *HortScience* 29:211–13



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