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Application of graphene oxide in *Agrobacterium*-mediated genetic transformation and construction of a novel DNA delivery system for watermelon

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Graphene oxide (GO) is widely used in biotechnology. The purpose of this study was to improve the efficiency of genetic transformation by constructing a delivery system based on GO. First, GO was applied in the traditional genetic transformation scheme for watermelons. We used hydroponics and tissue culture methods to determine the optimal concentration of GO for watermelon plant growth, we then used this concentration of GO for watermelon genetic transformation and found that GO can inhibit the growth of *Agrobacterium tumefaciens* and promote the growth of explants. This discovery can simplify the replacement of various culture media after explant infection, improve the regeneration rate of transgenic plants, and reduce experimental costs. To improve the efficiency of genetic transformation, a polymer-functionalized graphene oxide nanoparticle (GO-PEG-PEI) nanodelivery system was constructed, and the results showed that GO-PEG-PEI can transfer pCAMBIA1300-GFP plasmids into intact plant cells. We found that sheet-like GO-PEG-PEI can effectively load GFP and form small GO-PEG-PEI-GFP complexes, which can deliver pCAMBIA1300-GFP plasmids into plant cells. This research provides a new technique for molecular breeding.

Keywords GO, Watermelon plants, Genetic transformation, Growth promotion, Delivery system

Watermelon (*Citrullus lanatu*) is an important horticultural crop that is widely grown worldwide and is one of the most important tropical fruits in the world[1]. Watermelon plant breeding is a key technology, due to the narrow genetic background of watermelon plants, it is difficult to cultivate innovative new varieties[2]. By introducing genes, new crop varieties can be generated via molecular breeding, and this method is applicable to watermelons[3]. Among commonly used transgenic techniques, the pollen-tube pathway method is difficult to implement and has low transgenic efficiency, while the gene gun method is expensive and can damage cells[4], Therefore, the genetic transformation of watermelon plants is mainly based on *Agrobacterium tumefaciens* (*A. tumefaciens*)-mediated methods[5]. The transgenic process faces problems related to *A. tumefaciens* contamination and difficulty in plant rooting. The traditional method involves increasing the concentration of antibiotics such as cefotaxim sodium salt (Cef) and Timentin to inhibit bacteria. However, raising the concentration of antibiotics to a certain level can inhibit plant growth[6]. However, in the genetic transformation of watermelon based on *A. tumefaciens*, plant regeneration is challenging, and the transformation efficiency is low; these problems urgently need to be addressed.

With the development of nanotechnology, nanomaterials have become widely used in medicine, agriculture, biomedicine, and other fields[7,8]. Graphene oxide (GO) exhibits chemical properties that are more active than many of those of graphene[9]. GO is hydrophilic because it contains many oxygen-containing functional groups, such as hydroxyl and carboxyl groups[10]. In addition, GO has great potential for use in the delivery of biomolecules such as DNA or RNA, current research focuses mainly on animal studies. It can serve as a carrier to transport DNA or RNA, playing a synergistic role in inhibiting tumour cell proliferation[11]. For example, the polyethylene glycol (PEG)- and polyethylene imine (PEI)- biofunctionalized graphene oxide (GO-PEG-PEI) complex can be used to transmit Stat3 siRNA and inhibit the growth of mouse malignant melanoma cells[11–13]. However, there

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is still little research on the use of GO as a delivery system in plants, mainly due to the large size of untreated GO, which makes it difficult for it to penetrate plant cell walls. Research on GO applications in agriculture has revealed that it can affect seed germination and plant growth and development[14–16]. GO has significant effects on the growth and development of olive, rape, *Arabidopsis*, tomato and other crops[17–19]. Different concentrations of GO have different effects on plant growth and development, specific concentration of GO can promote the growth and development of plants, but high concentrations of graphene inhibit plant growth[20]. Furthermore, GO has a strong inhibitory effect on bacteria and fungi[21–23].

This study focused on the following two aspects. First, the effects of GO on the growth and development of watermelon plants and the inhibition of *A. tumefaciens* were investigated. This is the first study to use GO to replace antibiotics that inhibit *A. tumefaciens* in a genetic transformation system. Second, GO has a large specific surface area, can be loaded with substances, and has the ability to aggregate and stabilize nucleic acids after polymerization with PEG and PEI. It can also retain the biological activity of coupled nucleic acid molecules. Instantaneous transformation of plants has been achieved by injecting spherical GO nanoparticle (GON) siRNA complexes into tobacco leaves[24]. This study confirmed the effective genetic transformation of watermelon plants by GO-PEG-PEI using molecular biology methods such as GFP fluorescence and PCR, which is of great significance for improving the genetic transformation system of watermelon and promoting the development of watermelon molecular breeding.

Materials and methods

Materials

The "Ke xi" watermelon plants (2n = 2x = 22) were homozygotes inbred for eight generations at the experimental farm of the Henan University Genetics and Breeding Base in Kaifeng, China. GO was purchased from Guangdong Jiazhaoye New Materials Co., Ltd. in China (product name, TNGO; product number, 7782-42-5). The purchased GO powder had a thickness of 2 nm, lamellar diameter of 0.4 ~ 10 μm, tamping density of 600 g/L, starch content of 96%, carbon content of 46.9%, oxygen content of 55.8%, sulfur content of less than 3.6%, ash content of less than 2.8%, and particle size of less than 85%. The powder was prepared into a GO stock solution for plant treatment as follows. GO powder (0.1 g) was slowly added to 50 ml of ultrapure water. Ultrasonication was applied for five minutes per session, for a total of half an hour of ultrasound. Afterwards, the sample was sterilized under high pressure, and the resulting suspension had a pH of 5–6. Then, the hydroponic culture medium was prepared, an appropriate amount of the prepared GO solution was added to Murashige and Skoog (MS) liquid culture medium, and the mixture was stirred to form a uniformly dispersed liquid. The following components were used: sucrose and boric acid (Kermel Chemical Reagent Co., Ltd., Tianjin, China); agar, kanamycin sulfate (Kan) and Cef (Solarbio Technology Co., Ltd., Beijing, China); and 6-benzylamino purine (6BA) and kinetin (KT) (Biosharp Biotechnology Co., Ltd., Hefei, China).

Hydroponic GO–plant experiment and plant tissue culture conditions

First, a hydroponics experiment was conducted. Seedlings of Ke xi watermelon were grown in media supplemented with 0 mg/L, 75 mg/L, 150 mg/L or 300 mg/L MS liquid medium at 25 °C for 30 days. The same size culture bottle and the same intensity of light were used. Each concentration was repeated 7 times. The watermelon plants were all cultured in incubators at 28 °C for 16 h of light and 25 °C for 8 h of darkness with 70% humidity. Second, watermelon tissue culture experiments were conducted. Prepared callus tissue from watermelon plants infected with *A. tumefaciens* was clamped with tweezers and washed in sterile water until no adhered *A. tumefaciens* was visible on the surface. The watermelon plants were washed three times with sterile water and then transferred to a sterile conical flask containing culture medium. MS medium containing different concentrations of GO was used in this tissue culture experiment, after culture for a designated period, the level of *A. tumefaciens* contamination of the tissue-cultured seedlings was assessed, and the optimal GO concentration for plant tissue culture was determined. The hydroponics experiment simulated natural growth in an incubator to facilitate future practical application in agriculture. Tissue culture was carried out under sterile conditions, and *A. tumefaciens* growth was monitored during the transformation process.

Detection of phytoplankton in the MS liquid culture medium

Hydroponic production mainly cultivates crops in open environments, using nutrient solutions rich in various nutrients, which creates favorable conditions for the growth of phytoplankton such as algae, and can easily trigger explosive reproduction of phytoplankton in nutrient solutions. The massive reproduction of phytoplankton will bring a series of negative effects: on the one hand, it will consume nutrients in the nutrient solution, reduce dissolved oxygen, and release toxic metabolites, thereby reducing water quality; On the other hand, it also increases the risk of plants being attacked by pests and diseases[25]. To investigate the effect of GO on the growth of phytoplankton in hydroponic culture medium, MS culture medium samples containing different concentrations of GO were collected for testing. One hundred microlitres of liquid was dropped onto a plankton counting board. Photos of different phytoplankton were obtained with a Ze Xi biological analysis instrument (MAS-HL, Zhejiang, China), and then the phytoplankton types and their quantities in each sample were calculated via automatic data analysis software.

Plant transformation

The binary vector pCAMBIA1301 with GFP reporter gene was constructed as previously described[26]. The candidate genes were inserted between the *Sal*I and *Spe*I sites in the vector with the primers 1301-F and 1301-F (Supplementary materials Table 2). The resulting recombinant vectors were inserted into *A. tumefaciens* strain GV3101 cells.

Ke xi watermelon explants were transformed according to a modified method[26,27]. In particular, after surface treatment, seeds were sown in the dark for 2 days in 6% agar solid culture medium (Supplementary materials Table 3) and for 1 day under a 16-h/8-h light/dark photoperiod. *A. tumefaciens* strain GV3101 harbouring the binary vector was used for transformation. The transformed watermelon explants were placed on SAM coculture medium and incubated under dark conditions at 28 °C for three days. The cotyledon explants were cultivated in different shoot induction media (SIMs) for 4 weeks and transferred onto different shoot elongation media (SEMs) for 3 weeks. The SIMs included two types: the first type included MS, 1.0 mg/L 6BA, 30 mg/L Kan and 300 mg/L Cef; in the second type, cephalosporin was replaced with GO, and the components were MS, 1.0 mg/L 6BA, 30 mg/L Kan and 150 mg/L GO. The SEMs included two types: the first type included MS, 0.2 mg/L kinetin (KT), 30 mg/L Kan and 300 mg/L Cef; in the second type, cephalosporin was replaced with GO, and the components were MS, 0.2 mg/L KT, 30 mg/L Kan and 150 mg/L GO. Plantlets with well-developed roots were taken from the culture medium and placed in plastic cups containing vermiculite. The growth status of the explants was examined. After four weeks of growth in the SIMs, the number of adventitious buds in each group of explants was counted, with ten explants per type, and this process was repeated three times. After three weeks of growth in the SEMs, the number of adventitious roots in each group of explants was counted, with ten explants per type, which was repeated three times. At the same time, DNA was extracted from the leaves of each explant, and PCR validation was performed using the primers GFP-F and GFP-R (Supplementary materials Table 2) to determine the positive transformation efficiency under different treatments. The transgenic seedlings were observed under fluorescence observation (LUYOR-3415RG, China, Shanghai).

Preparation of GO-PEG-PEI-GFP nanomaterials

The nanomaterial preparation method was based on a previously described method, with slight modifications as follows[11]. One hundred milligrams of GO was added to 100 mL of sterile water, and a uniform transparent colloidal solution was prepared via ultrasonication. Then, 25 mmol NaOH and 8 mmol monochloroacetate were added to the GO solution, which was sonicated for 2 h. Then, 0.4 mmol EDC and 0.4 mmol NHS were added to 0.5 mg/ml GO-COOH solution and sonicated for 1 h. PEI was added to the GO solution at a mass ratio of 1:80, stirred for 24 h and then dialyzed for three days in a 100 kDa dialysis bag. Then, 20 mg of PEG was added to 10 ml of 0.5 mg/ml GO-PEI solution and stirred overnight. The mixture was then dialyzed in a 100 kDa dialysis bag for three days. The pCAMBIA1300-GFP plasmid was mixed with GO-PEI-PEG at a mass ratio of 1:0.5 and incubated at room temperature for 30 min. The prepared nanomaterials were stored at low temperature until use.

Characterization of the nanomaterials

To characterize the GO material, particle size analysis, zeta potential analysis, and electron microscopy imaging were performed. A Zetasizer Nano ZS nanoparticle size potential analyser was used to measure the nanoparticle size and zeta potential of the dispersion. The analyser measures samples on the basis of the principle of electrophoretic light scattering, and an excitation wavelength of 633 nm was applied. The specific steps were as follows: the machine was preheated for 20 min, the Zetasizer software was opened on the computer, "dispersant dispersant, 25 °C, 120 s" was selected, the prepared sample dispersion was transferred to a colorimetric dish and a zeta potential sample cell, the sample cell was placed in the instrument for particle size and zeta potential measurement, and the dispersion stability of the product was determined on the basis of the measured values.

TEM was conducted using a Sevier HT7700 transmission electron microscope. The specific steps were as follows: a pipette was used to extract 20 μl of the nanomaterial suspension, which was dropped onto a carbon film copper mesh and incubated for 3–5 min, after which filter, paper was used to remove excess liquid. 2% phosphotungstic acid was dropped onto the carbon-supported copper mesh and allowed to sit for 1–2 min. Filter paper was used to remove excess liquid, and the mixture was dried at room temperature. Images were obtained for analysis under a transmission electron microscope.

Effect of nanomaterials on pollen tube elongation

Mature pollen was dropped onto a slide with forceps, and pollen tubes were induced using pollen tube germination BM (Supplementary materials Table 3). After 30 min, 0, 12, 32, 64 or 93 mg/ml nanomaterial carrier was added, and then the growth of the pollen tubes was observed under a microscope (Nikon ECLIPSE Si, Japan) after 1 h and 2 h.

Construction of a delivery system based on GO-PEG-PEI

To improve the conversion efficiency of the pollen-tube pathway method, a GO-PEG-PEI-GFP delivery system was constructed. The pollen-tube pathway method involves injecting a DNA solution containing the target gene into the ovary after pollination and integrating it into the genome to complete the transformation[28]. The first step involved self-pollination of homozygous watermelon. Two hours later, the GO-PEG-PEI-GFP nanomaterials were applied to the stigma of the female flowers of watermelon. After the infected watermelon was mature, the watermelon seeds were peeled out, the fluorescence was observed using a laser gun, and the DNA was extracted. The transgenic efficiency was detected using the primers GFP-F and GFP-R (Supplementary materials Table 2).

Results

GO affects the growth of watermelon plants

To verify the effect of GO on watermelon plants, hydroponic experiments were performed, and observations were conducted once a week. When the concentration of GO was 75 mg/L or 300 mg/L, GO treatment had a significant inhibitory effect on the growth of roots, and the average root lengths were 5 cm and 8 cm, respectively. However, when the concentration reached 150 mg/L, GO treatment significantly promoted the growth of watermelon roots, and the average root length was 11.3 cm, approximately 2.9 cm longer than that of the control.

The results showed that 150 mg/L GO promoted the growth of watermelon roots. The changes in the watermelon plants were similar to those in the roots. Treatment with 75 mg/L or 300 mg/L GO had a significant inhibitory effect on the growth of watermelon plants. When the concentration of GO was 150 mg/L, the watermelon plants were the longest at approximately 20 cm long, 1.7 cm longer than that in the control. Therefore, 150 mg/L GO significantly promoted the growth of watermelon plants. However, GO treatment had no significant effect on the growth of the aboveground stems of watermelon plants (Fig. 1).

GO affects the growth of *A. tumefaciens*and phytoplankton

To test the inhibitory effect of GO on *A. tumefaciens* carrying transgenic vectors, watermelon genetic transformation experiments were conducted. Figure 2A a, b, c, and d show images of MS solid culture medium supplemented with 300 mg/L Cef and 75 mg/L, 150 mg/L and 300 mg/L GO. Microscopic examination revealed that the culture media containing 75 mg/L GO and 300 mg/L Cef both contained *A. tumefaciens*. The addition of 150 mg/L and 300 mg/L GO to the culture medium significantly inhibited *A. tumefaciens* (Fig. 2B, a). To assess the natural growth of phytoplankton in MS liquid medium with GO, a biological analyser was used to detect the species and quantity of phytoplankton. The results showed that the number of algae significantly decreased in the culture medium supplemented with GO (Fig. 2B, b). *Chlorella vulgaris* (*C. vulgaris*) and *Chlamydomonas* were the most abundant green algae. The number of these two algae in the culture medium containing GO was relatively small, and GO effectively inhibited the growth and reproduction of these algae.

GO promotes the regeneration of explants

To determine the impact of GO on explants in genetic transformation, the optimal GO concentration was selected for genetic transformation experiments. As shown in the example figure, watermelons are genetically transformed using different media (Fig. 3A, B). After three weeks of growth in elongation media, the seedlings in the GO treatment began to take root. By counting the number of adventitious buds and roots of plants in different culture media, it was shown that 150 mg/L GO significantly promoted the growth of adventitious buds and roots of the explants (Fig. 3C, D). Observe the fluorescence status of plants grown in the culture medium after two and three weeks of growth. PCR revealed that the positive conversion rate under normal culture conditions was 22.7%, whereas under GO culture conditions, the positive conversion rate was 25.9% (Fig. 3E).

Effect of GO-PEG-PEI-GFP on pollen tubes

To determine the effect of GO-PEG-PEI-GFP on pollen tubes, the toxicity of different concentrations of GO-PEG-PEI-GFP to pollen tubes was tested. After 30 min in pollen tube induction medium, the pollen tubes developed well (Fig. 4A, a–e). Subsequently, different concentrations of nanomaterials were added; after 1 h, the higher the concentration of nanomaterial was, the more wrinkled the pollen grains became, but the effect on the pollen tubes was not significant (Fig. 4A, f–j). After 2 h, there was still no significant effect on the pollen tubes (Fig. 4A, k–o). Calculation of the pollen germination rate at different concentrations revealed that different concentrations of GO-PEG-PEI-GFP had no significant effect on the pollen tube germination rate (Fig. 4B, a). Calculation of the length of pollen tubes at different concentrations and times indicated that after 2 h, GO-PEG-PEI-GFP at concentrations of 32, 64, and 93 mg/ml had a significant effect on the length of pollen tubes (Fig. 4B, b). The pollen toxicity experiment proved that the most suitable concentration of GO-PEG-PEI-GFP was 12 mg/ml.

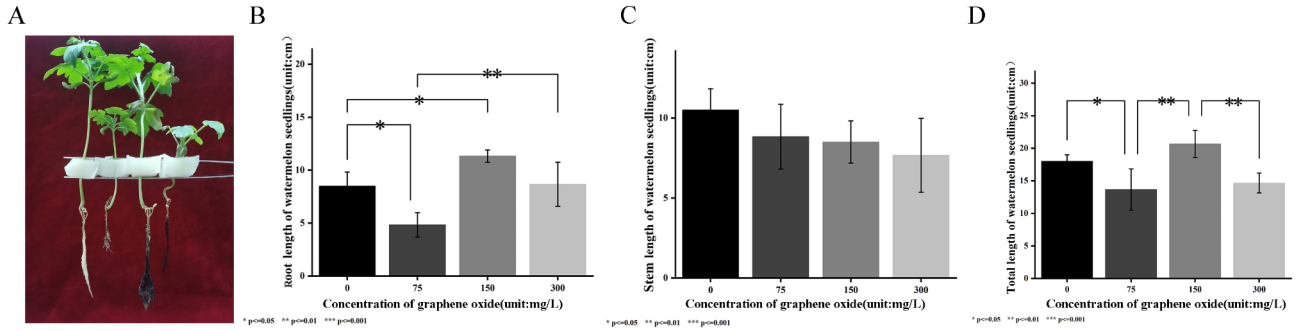

Fig. 1. Hydroponic growth chart. **(A)** Comparison of the main roots of watermelon plants after two weeks of growth. **(B)** Effects of GO on the main root; 0, 75, 150, and 300 refer to different concentrations of GO. The error lines on the columns represent the standard deviation, and the "*" annotations on the chart represent significant differences between different treatments. **(C)** Effects of GO on stem length; 0, 75, 150, and 300 refer to different concentrations of GO. The error lines on the columns represent the standard deviation, and the "*" annotations on the chart represent significant differences between different treatments. **(D)** Effect of GO on overall length; 0, 75, 150, and 300 refer to different concentrations of GO. The error lines on the columns represent the standard deviation, and the "*" annotations on the chart represent significant differences between different treatments. (* $p <= 0.05$ ** $p <= 0.01$ *** $p <= 0.001$).

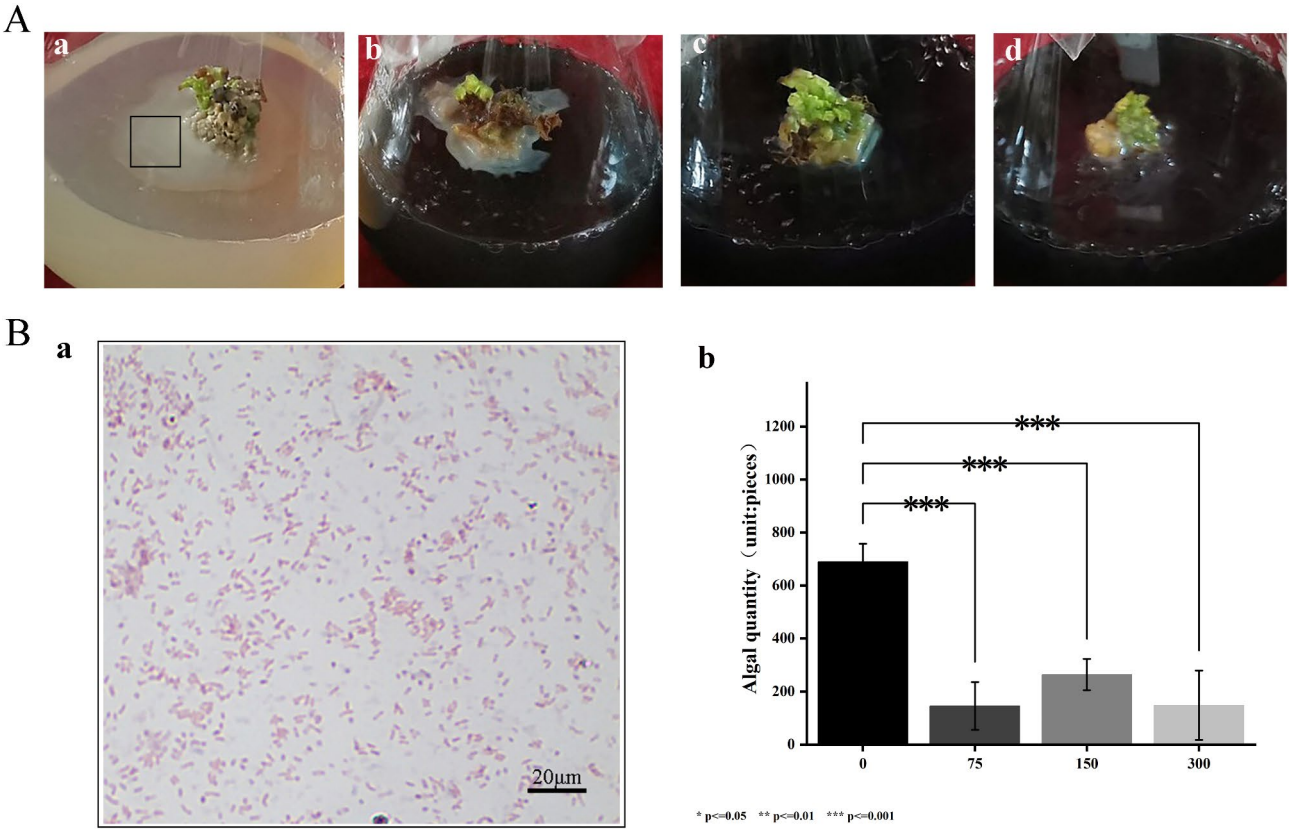

Fig. 2. Hydroponic culture medium and its microscopic changes. **(A)** a. b, c, d show images of MS solid culture medium supplemented with Cef and 75 mg/L, 150 mg/L and 300 mg/L GO. **(B)** (a), Results of Gram staining of *A. tumefaciens* picked from the culture medium. (b), Statistics on the number of algae in culture media with different concentrations of GO added, with different letters on the graph indicating significant differences between different treatments (* $p <= 0.05$ ** $p <= 0.01$ *** $p <= 0.001$).

The nanodelivery system promotes genetic transformation efficiency

In accordance with the design process, we prepared the nanomaterial GO-PEG-PEI-GFP and delivered the pCAMBIA1300-GFP plasmid into watermelon (Fig. 5A). The pCAMBIA1300-GFP plasmid was loaded onto the prepared GO-PEG-PEI by electrostatic adsorption to form a GO-PEG-PEI-GFP nanomaterial complex. pCAMBIA1300-GFP and GO-PEG-PEI were mixed at a mass ratio of 1:0.5 for 30 min at room temperature. The results of gel electrophoresis showed that pCAMBIA1300-GFP was fully loaded at this time (Fig. 5B, a). Zeta potential analysis showed that after GO was modified with PEG and PEI, the potential increased from − 11.5 mV to 31.5 mV. After loading with the pCAMBIA1300-GFP plasmid, the potential did not differ from that of GO-PEG-PEI alone, indicating that the pCAMBIA1300-GFP plasmid had no significant effect on the carrying capacity of GO-PEG-PEI (Fig. 5B, b). Interestingly, after loading the pCAMBIA1300-GFP plasmid, the particle size of the GO-PEG-PEI-GFP complex significantly decreased and was significantly smaller than that of GO-PEG-PEI (Fig. 5B, c). The nanomaterials were ultrasonically dispersed to form a uniform yellow transparent liquid, and electron microscopy revealed an irregular layered structure (Fig. 5C, a). There was no difference between the electron microscopy images of GO-PEG-PEI and GO-PEG (Fig. 5C, b), indicating that the nanomaterials are relatively stable. After the addition of the pCAMBIA1300-GFP plasmid, the plasmid was enveloped by GO-PEG-PEI (Fig. 5C, c). By using the pollen tube channel method, GO-PEG-PEI-GFP vectors were transferred into watermelons, and green fluorescence was observed in watermelons grown for 1 day, 10 days, or 30 days (Fig. 5D, a, b, c). During the experiment, we obtained three watermelons. We randomly extracted DNA from four batches of watermelon seeds, each containing 30 seeds, and analysed them via PCR. The number of positive seeds in each batch was 27, 24, 13, and 8. After calculating the positivity rate of each batch, the average was calculated as the output value. The conversion efficiency was calculated to be 59.33%.

Discussion

GO is a type of carbon nanomaterial and is biocompatible. Through hydroponic experiments, the following two conclusions were reached. First, GO significantly promotes watermelon plant growth, providing a foundation for the application of nanomaterials in watermelon plant cultivation. Research has shown that low or high concentrations of GO may inhibit plant growth due to toxic effects or oxidative stress, while the promoting effect of GO on plants is related to increased antioxidant enzyme activity and indole-3-acetic acid levels[29]. This study revealed that at lower concentrations (75 mg/mL) and higher concentrations (150 mg/mL), GO inhibited

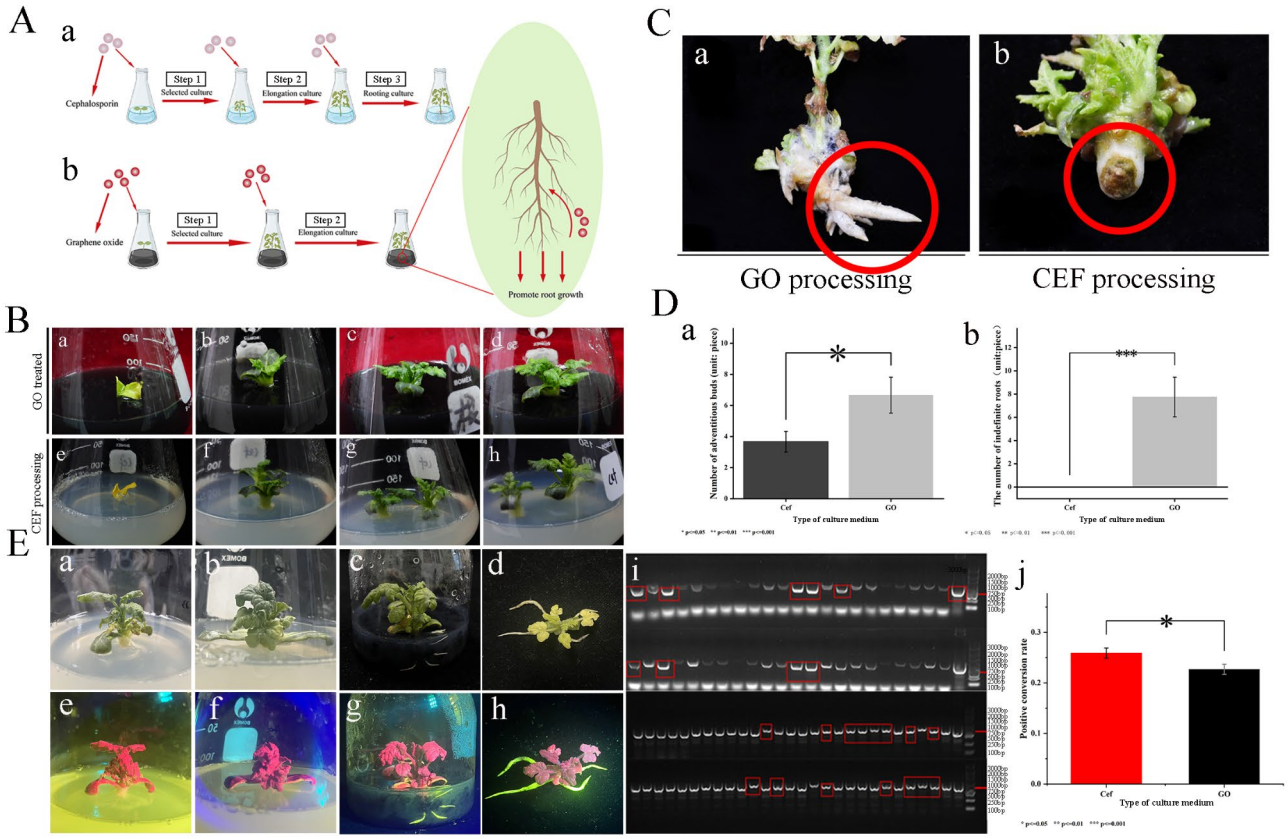

Fig. 3. Comparison of genetic transformation. **(A)** Experimental schematic of genetic transformation using GO: (a) normal genetic transformation process of watermelon; (b) genetic transformation process of watermelon with added GO; **(B)** comparison of genetic transformation at different stages with the addition of GO and cephalosporin; **(C)** comparison of root growth after three weeks of growth in elongation medium: (a) explants cultured with GO, (b) explants cultured through normal genetic transformation; **(D)** statistics on the number of adventitious buds and roots produced by explants grown in different media (* $p <= 0.05$ ** $p <= 0.01$ *** $p <= 0.001$); **(E)** (a), (c) growth in normal elongation medium after two weeks and fluorescence results, (b), (f) growth in normal elongation medium after two weeks and fluorescence results; (c), (g) growth in graphene oxide elongation medium after two weeks and fluorescence results, (h) growth in graphene oxide elongation medium after three weeks and fluorescence results, (i) PCR results for transgenic seedlings; (j) positive conversion rate of explants in different culture media (* $p <= 0.05$ ** $p <= 0.01$ *** $p <= 0.001$).

plant growth. Moreover, there is an optimal concentration for the promoting effect of GO on watermelon plants, which is similar to the phenomenon in other crops. In a previous study, GO inhibited the growth of cabbage, tomato, amaranth, and lettuce seedlings, and the degree of inhibition was positively correlated with the GO concentration[30]. However, GO treatment promoted the growth of hydroponic buckwheat seedlings. With increasing GO particle size or concentration, the overall growth indicators of buckwheat seedlings showed a trend of first increasing and then decreasing[31]. Second, GO can significantly impact the number and types of phytoplankton in liquid media, providing a foundation for the hydroponic cultivation of nanomaterials in nonsterile environments. GO has a significant inhibitory effect on *C. vulgaris* and *Chlamydomonas*, but a certain concentration of GO can increase the protein, total lipid, and carbohydrate contents in algae cells, providing energy for the algae to protect themselves and adapt to stress[32]. GO, reduced graphene oxide (RGO) and graphene, which can exist in *C. vulgaris* in their original form or be adsorbed by EPS, hinder the growth and photosynthetic activity of algae at relevant concentrations[32]. Interestingly, these previous results were consistent with the results for watermelon plant seedlings in this study. GO at low and high concentrations significantly inhibited the growth of algae in MS liquid culture medium. Graphene photocatalysis can significantly reduce species richness in the treatment area and is expected to become a new green technology for reducing harmful algae in water[34]. We are conducting molecular mechanism research to provide support for comprehensive application of GO in biology.

GO can replace antibiotics and promote plant regeneration. First, GO can inhibit genetically modified *A. tumefaciens*, suggesting that it could be applied as a substitute for antibiotics. GO inhibits the growth of gram-negative bacteria, mainly *E. coli* and *S. aureus*. RGO nanomaterials have been proven to have toxic effects on *E. coli*, in studies, *E. coli* died upon contact with nanomaterials, mainly due to the adhesion of nanomaterials to the cell surface, leading to the rupture of the cell membrane structure[35]. When GO acts on cell suspensions, the antibacterial mechanism is mainly cell capture, and large GO has good antibacterial activity[36]. The sharp nano-

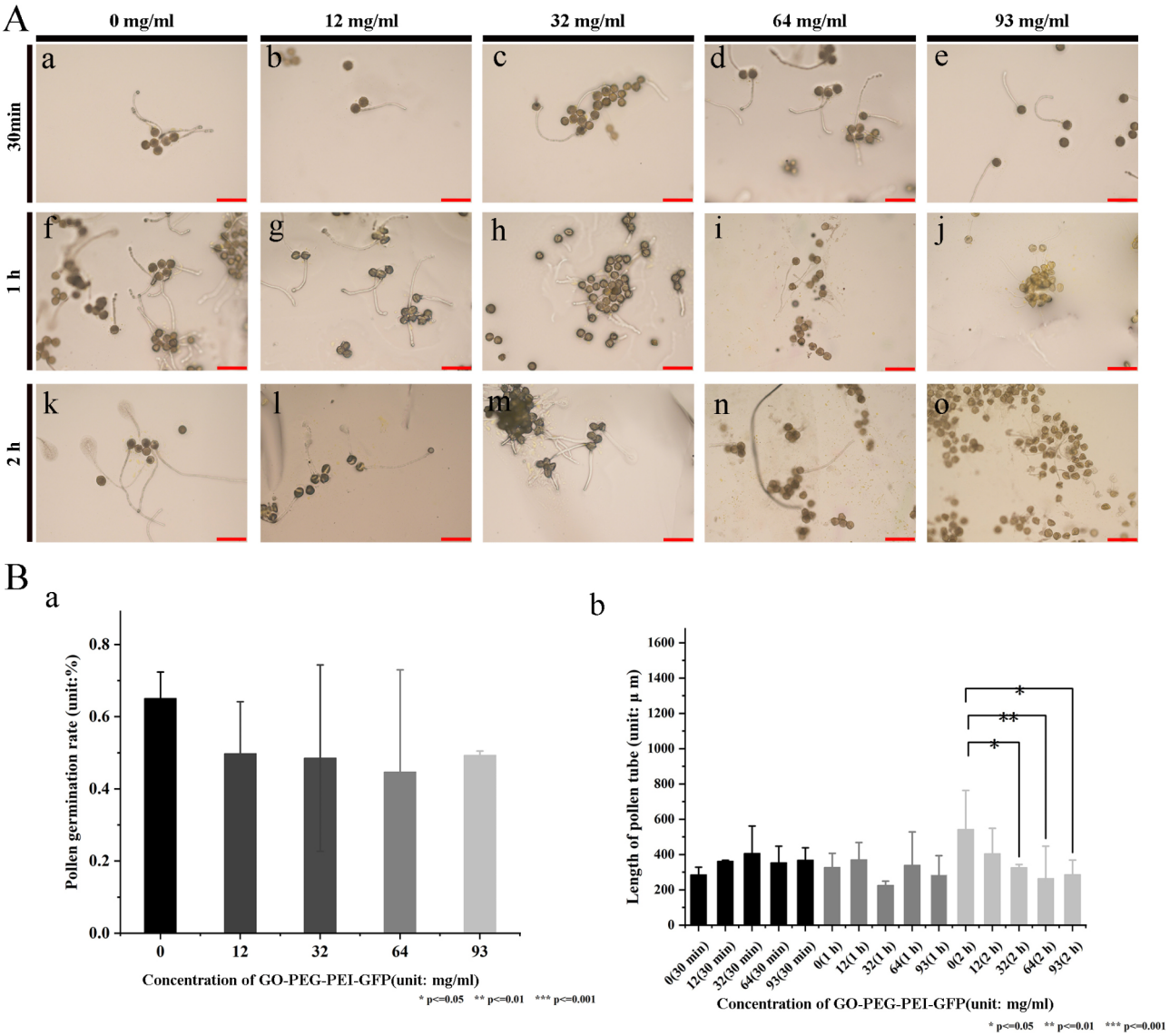

Fig. 4. Analysis of nanomaterial toxicity. **(A)** (a) (f), (k) Pollen cultured in medium without added GO; (b), (g), (l) pollen cultured in medium with 12 mg/ml GO added; (c), (h), (m) pollen cultured in medium with 32 mg/ml GO added; (d), (i), (n) pollen cultured in medium with 64 mg/ml GO added; (e), (j), (o) pollen cultured in medium with 93 mg/ml GO added (bar = 200 μm). **(B)** Pollen tube germination rate and pollen tube length: (a) germination rate at different concentrations; (b) pollen tube length at different times and concentrations (* $p <= 0.05$ ** $p <= 0.01$ *** $p <= 0.001$).

edges in GO can cut bacterial cells into fragments[37]. GO derivatives are expected to be used as supplements to clinically relevant antibiotics[38]. Forrest Nichols et al. sought to enhance the bactericidal effect by synthesizing nanocomposites to replace antibiotics[39]. Second, GO can promote the growth of adventitious buds in watermelon plants, providing a foundation for the application of nanomaterials in watermelon plant molecular breeding. Consistent with previous research, GO had a positive effect on the performance of plantain callus histiocytes at a specific concentration[40]. Third, GO can promote the rooting of genetically modified seedlings, further supporting the application of nanomaterials in watermelon plant molecular breeding. GO can not only promote the elongation of adventitious roots in cotinus coggygria tissue cultured seedlings, but also promote the growth of rice roots[6,41,42]. In addition, various indicators of raspberry tissue-cultured seedlings showed a trend of first increasing and then decreasing with increasing GO concentration[43]. These findings indicate that GO has the advantages of low price, antibacterial effects and the ability to promote the growth of watermelon transgenic plants.

In this study, considering the unique properties of pollen tubes, we constructed the GO-PEG-PEI delivery system to achieve efficient genetic transformation. First, a GO-PEG-PEI-GFP nanocomposite was constructed using the pCAMBIA1300-GFP plasmid and GO-PEG-PEI. The feasibility of the GO-PEG-PEI delivery system was validated by transferring the pCAMBIA1300-GFP plasmid into watermelon. Many studies have used GO-

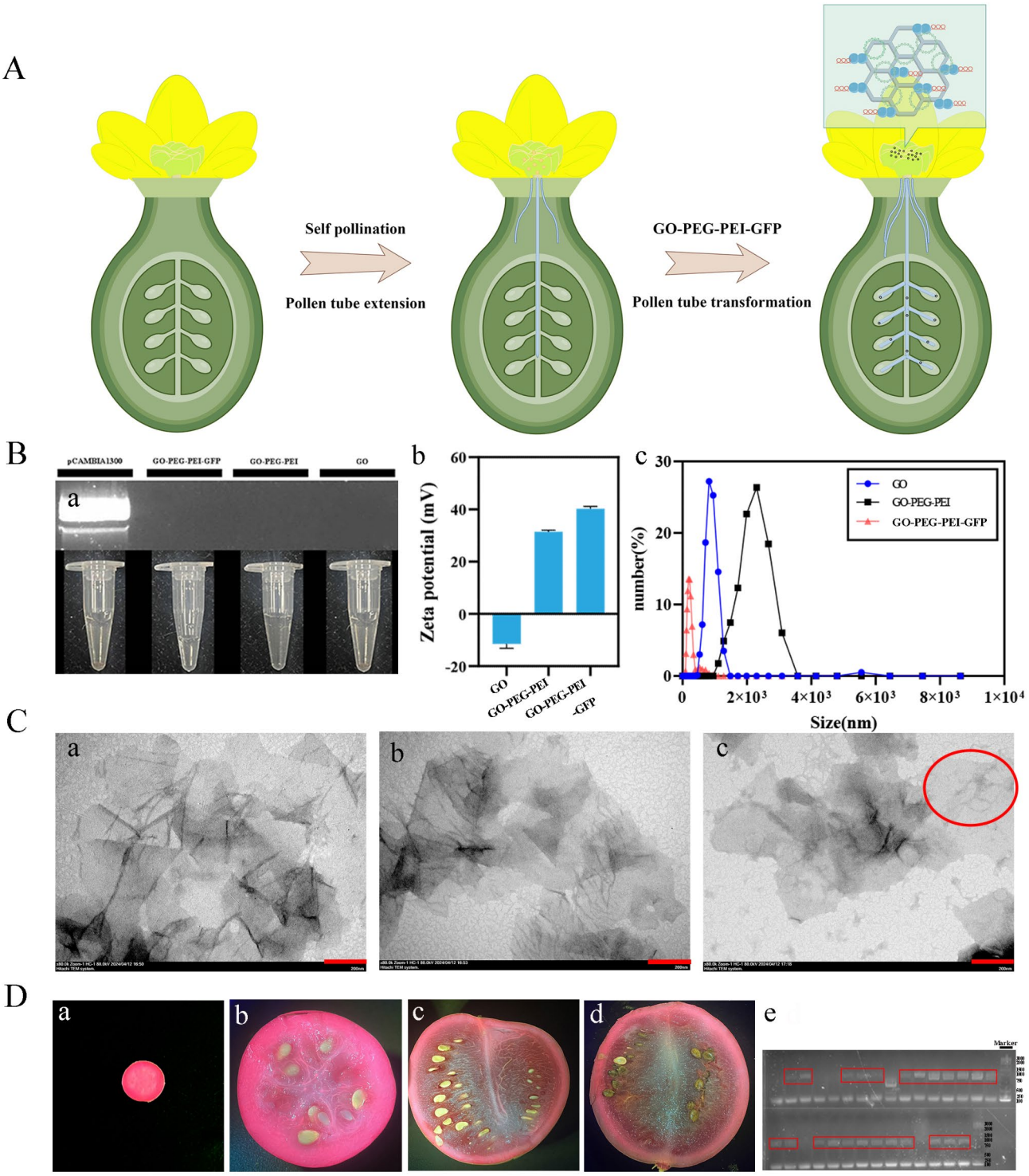

Fig. 5. The GO-PEG-PEI nanodelivery system promotes genetic transformation efficiency. **(A)** Diagram of the GO-PEG-PEI-GFP preparation process; **(B)** testing and characterization of nanomaterials; (a) electrophoresis images of the pCAMBIA1300-GFP plasmid, GO-PEG-PEI-GFP, GO-PEG-PEI, and GO; (b) zeta potential analysis results for GO, GO-PEG-PEI, and GO-PEG-PEI-GFP; (c) particle size analysis results for GO, GO-PEG-PEI, and GO-PEG-GFP; **(C)** electron microscopy images corresponding to the GO-PEG-PEI-GFP preparation process: (a) electron microscopy image of GO; (b) electron microscopy image of GO-PEG-PEI; (c) electron microscopy image of GO-PEG-PEI-GFP; (b = 200 μm); **(D)** fluorescence images of transgenic watermelons at 1 day, 10 days, and 30 days, as well as an electrophoresis image used to assess transgenic efficiency: (a) cross section of watermelon grown for one days after genetic transformation; (b) cross section of watermelon grown for ten days after genetic transformation; (c) cross section of watermelon grown for 30 days after genetic transformation; (d) cross section of watermelon grown for 30 days under normal conditions (negative control); (e) Transgenic efficiency.

PEG-PEI to deliver genes to animal cells. For example, GO-PEG-PEI loading and delivery of miR-29b promoted BMSC osteogenic differentiation and bone regeneration[44]. The extracellular vesicles of herbs can successfully bind to chitosan, PEG and GO and then bind to oestrogen receptor α-targeted siRNA for delivery to breast cancer MCF7 cells[45]. The main reason for the above results is that animal cells do not have cell walls, which might otherwise obstruct the delivery of nanomaterials. Second, this research bypasses instantaneous plant transformation and realizes stable transformation. After pollination, DNA can enter the embryo sac along the pollen tube channel and integrate into the genome to achieve stable transformation. *Arabidopsis* species have a cysteine-rich peptide (AtLURE1 peptide) that can guide pollen tubes into the ovule pore, but only pollen tubes of the same species can be effectively attracted at appropriate protein concentrations[46]. GO can efficiently and quickly transport plasmids into watermelon via the pollen tube channel method. GONs can transfer siRNA into cells to achieve transient gene silencing[24]. In addition, the genetic transformation efficiency of the pollen tube pathway method is only 2.5%, and that of Agrobacterium is approximately 6.7%[47,48], compared to the previous two methods, in this study method not only improves the efficiency of genetic transformation but also avoids the tedious steps required in aseptic operations. Thirdly, PEG modified PEG-PEI can shield the high density of positive charges in PEI, thereby reducing cytotoxicity and improving the transfection efficiency of GO-PEG-PEI on specific cells, which meets the requirements of genetic transformation[49]. And the appropriate concentration of nanomaterials will not affect pollen development or fruit growth. Research has shown that using magnetic nanoparticles loaded with exogenous DNA to transfer into pollen through the pores of plant cell membranes under the action of a magnetic field does not cause damage to pollen, and exogenous DNA can be successfully internalized in pollen grains and pollen tubes[50]. 100-fold dilution of chitosan quaternary ammonium salt dsRNA nanoparticles (HACC dsRNA NPs) has no negative effect on pollen viability, and pollen can serve as a pathway for dsRNA to enter seeds[51]. In vivo assays have demonstrated that GO-PEG-PEI-CpG and GO-PEG-PEI/siRNA complexes have low cytotoxicity and excellent therapeutic efficacy[52,53]. This study revealed that the GO-PEG-PEI nanocomplex has good biocompatibility, plasmid loading capacity, and transfection efficiency in watermelon plants.

In summary, this study simplifies the steps of genetic transformation, reduces the use of antibiotics, and reduces experimental costs, making it an effective innovation in genetic transformation experiments. In addition, a delivery system was established in watermelon plants, achieving rapid genetic transformation. The emergence of nanobiotechnology has provided unprecedented opportunities to accelerate molecular breeding.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions

W.D. designed the experiment. Y.Q. and W.D. wrote the main manuscript text. S.H., L. Z. , P. N., F. X and D. W. prepared Figs. 1 and 2. Y.Q. prepared Fig. 3. Y.Q. and C.Y. prepared Figs. 4 and 5. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Consent for publication

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