A Protocol for Agrobacterium-mediated Transformation of Cucumber (Cucumis sativus L.) from cotyledon explants

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Method Article

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Abstract

Genetic transformation is central to the discovery, delivery, and functional analysis of genes, and for associated crop improvement, and yet is technically challenging in many plant species. As an example, cucumber \(_Cucumis sativus_ L.) is a model species for the study of phloem characteristics, raffinose family oligosaccharide \(RFO) metabolism and sex determination, but methods for its genetic transformation have generally had low efficiency, thereby limiting basic and applied studies. Here, we describe a rapid and efficient protocol for _Agrobacterium_-mediated transformation of cucumber cotyledon explants, using vacuum infiltration. The transformation efficiency of the regenerated plants was as high as 54%, and the final positive plantlet transformation efficiency was approximately 26% of the total number of infected explants and this is obviously higher than previously published protocols. Transgenic plantlets can be obtained in 3 to 4 months and the transgenic T1 seeds generated in the subsequent 3 to 4 months after self-pollination. Using this protocol, we have obtained more than 600 transgenic cucumber lines. This protocol is of great importance for studies of cucumber and of other _Cucurbitaceae_ species, since it enables gene functional analysis, and so opens a pipeline for rapid cucumber variety improvement.

Introduction

Cucumber \(_Cucumis sativus_ L.), a crop with economic and nutritional importance that is cultivated worldwide, is a typical raffinose family oligosaccharides \(RFOs)-transporting plant[1-4], and a model species for studying phloem functional characteristics and sex determination. Cucumber phloem is very complicated, there is one set of vascular bundle \(VB, a bicollateral bundle including the internal and external phloem) in the petiole, stem and peduncle, but four sets of VBs in the fruit, including the peripheral, main, carpel and placental VB \(two bicollateral, two collateral). Cucumber is a monoecious species with unisexual flowers, and is a model species for the study of sex determination. It has seven types of sex phenotypes, including andromonoecious, monoecious, androecious, heterozygous-gynoecious, hermaphrodite, gynoecious and gynoecious-monoecious. In addition, it can be parthenocarpic. However, a lack of effective genetic transformation methods, especially those based on _Agrobacterium_, has severely limited cucumber gene functional analyses and remains a bottleneck for both developing cucumber as a model, and for its improvement as a crop. The transfer and stable integration of genetic material into the cucumber genome will enhance the breeding of more disease resistant cucumber \(_Cucurbitaceae_ plants or cucurbits) varieties, as well as the characterization of cucumber genes. It offers the possibility to introduce new characters into a cucumber cultivar, which is difficult to achieve via conventional breeding techniques[5]. Recently, the genome sequences of both cucumber and watermelon, another cucurbit, were released \(http://www.icugi.org/cgi-bin/ICuGI/index.cgi). Moreover, a range of studies have investigated diverse aspects of cucumber biology, such as the rapid fruit expansion that occurs after flowering, and some candidate genes that influence this important process have been identified[6,7]. The development of an efficient cucumber transformation system is therefore timely and critical for gene discovery and validation. Many studies have reported

successful cucumber transformation, but only a few have obtained a high transformation rate and T_1 generation, and there are very few reports on functional analysis using transformed plants \(Table 1). The first transformation of cucumber plants, which expressed the NPT II gene, was reported by Trulson _et al_. \(1986)[8]. This transformation was achieved by infecting cucumber hypocotyl segments with _Agrobacterium rhizogenes_ and regenerating the transformed issue into whole plants. This approach had a low plant regeneration frequency, which limited its utility \(Table 1). Other reports have described the successful regeneration of cucumber plants from cotyledon explants after transformation using _A. tumefaciens_, but transformation efficiency was still < 12%, and only a few of these studies resulted in transgenic T1 plants \(Table 1). Higher transformation rates \(16-23%) have been reported using cotyledon explants[9-11], but in these studies, the transformation rate was calculated as the identified positive shoots and not plantlets, and few of these lines resulted in a stably transformed T_1 generation. The selection of cucumber transformants using antibiotic selection with kanamycin[9,12], phosphinothricin[11,13] and hygromycin[14], have been reported; however the method with the high transformation efficiency \(23%) reported by He _et al_. \(2006) was based on mannose and sucrose resistance[10] \(Table 1). Other regeneration protocols with various culture types have also been investigated, such as leaf explants[15], petioles[15,16], hypocotyls[14,17], internodes[17], protoplasts[18] and suspension cultures[19,20], but these all showed a lower transformation efficiency than using cotyledons as explants. Until now, _Agrobacterium_-mediated transformation methods using cotyledons as explants has been the most successful approach for cucumber transformation[21]. With this approach, the transformation frequency is dependent on several factors, including plant genotype, explant source and size, _Agrobacterium_ strains and concentration, length of exposure to Agrobacterium, and selection system[22,23], among others. It was reported that dissecting half of an adaxial cotyledon using a V-shaped cut resulted in a higher transformation rate[24], while another report showed that using cotyledon nodes as explants resulted in regeneration at the junction of cotyledon and hypocotyl in the proximal regions of the cotyledons[12]. In this current protocol, we use similar explants, half adaxial cotyledons, but we have modified the protocol in that we break off the hypocotyl instead of using the V-shaped cut to detach the growing point. We also highlight the key factors in the procedure that can cause technical problems for the inexperienced researcher. Our laboratory has established a rapid and efficient _Agrobacterium_-mediated transformation system for cucumber that differs from previously reported protocols, and we have identified critical factors that influence transformation efficiency. To date, we have produced more than 600 transgenic lines and functionally analyzed a range of cucumber genes using this protocol[25-33]. The method can be widely used in cucumber breeding, variety improvement, and the characterization of gene function, and can be a reference for the transformation of other cucurbits, such as _Cucumis melo_ and _C.metuliferus_[21,23]. **Overview of the technique** \(Figure 1) This protocol consists of seven major steps, which can be briefly summarized as follows: Stage I: preparation of sterilized seeds. Stage II: explant preparation, infection and co-cultivation with _A. tumefaciens_. Stage III: shoot regeneration. Stage IV: root regeneration. Stage V: acclimation and molecular identification of T_0 plants. Stage VI: cultivation and self-crossing of T_0 plants. Stage VII: seed harvest from T_0 plants. **Key factors for

successful transformation of cucumber** _**Varieties amenable to _Agrobacterium_-mediated transformation**_ It has been reported that the genotype of transformed cucumber influences its shoot regeneration frequency[9,21], and relatively high regeneration rates have been reported for some cucumber genotypes, such as Poinsett 76, Jinyan No.7, Cengel Köy, Shinhokusei 1, cv. pickling genotype 3676, 3672, and Xintaimici \(Table 1). However, in our analyses we observed no differences in the regeneration frequency between different cucumber genotypes. We obtained a high regeneration rate \(80-97 %) for the Deltasta, Guonong25, cucumber line 3413, 3407, 3461, Chinese long: 461, 9930, R1407, R470, short 32x, and Xintaimici genotypes, and obtained transgenic plants from all these varieties. Even so, it is advisable to determine the regeneration rate before using a new cucumber genotype for _Agrobacterium_-mediated transformation, in order to optimize the chance of successful transformation. We note that homozygous varieties should be considered, since they can provide a homozygous transgenic T_1 generation for subsequent gene functional studies. _**Preventing contamination with endophytic bacteria**_ Different endophytes may be associated with different parts of the cucumber seed. Before soaking seeds in tap water for 2 hours, they should be sterilized with HCl and NaClO in a sealed container for 1-2 hours, to kill bacteria on the seed surface or inside the seed coat. Some endophytic bacterial species have been isolated from surface sterilized cucumber seeds, and most of these were located in the seedling radicles[34,35]. It is therefore important to cut off the radicles before detaching the cotyledons to prevent the contamination with endophytic bacteria, and then knocking or breaking off the hypocotyl on new sterilized filter paper. _**Explant preparation**_ The selection and preparation of explants is critical factor in the success of the transformation pipeline. In our protocol, the embryonic cotyledons are used as the explants. The cucumber seeds should be soaked in water for 2 hours to facilitate the removal of the seed coats and to obtain a high germination rate. The de-coated seeds should then be surface sterilized with 75% \(vol/vol) alcohol for no more than 1 minute. After germination for approximately 2 days on MS/B5 medium, a radicle of 1-1.5 cm and root hairs are visible, and the cotyledons can be detached from the embryos. Half of the adaxial cotyledon is then used as the explant. Seeds have a better germination rate on B5 medium than on MS medium; however, different varieties may have a difference in germination time and media requirements, as well as in how readily the cotyledons can be detached. Forceps, rather than a knife, should be used to detach the cotyledons from the radicle growing point \(Figure 1, Explant preparation) in order to obtain a higher regeneration rate. Previous studies have shown a high regeneration frequency with cotyledon nodes as explants[36], and we also found that breaking off the hypocotyl to detach the cotyledon, rather than cutting it, is important to obtain high regeneration frequency. However, care needs to be taken to cut off the growing point of the seedlings to prevent negative plants growing. _**Influence of abscisic acid \(ABA) and 6-benzylaminopurine \(BAP) levels on shoot organogenesis during shoot initiation and outgrowth**_ It has been reported that the hormones ABA and BAP can induce the regeneration of cucumber cotyledon explants[36, 38], and most of these studies focused on the influence of BAP levels on cucumber shoot regeneration. In this study we have investigated the combined effects of BAP and ABA levels on shoot regeneration from cotyledon explants \(Table 2, Supplementary Figure S1). We observed that the highest shoot regeneration rate were 76% and 86%, using a ABA/BAP concentration of 1.0/0.8-1.5 mg/l \(Table 2), and noted that the

regenerated shoots grew more slowly with higher ABA and lower BAP concentrations \(Supplementary Figure S1 A). We therefore chose an ABA/BAP concentration of 1.0/0.8-1.5 mg/l, as this resulted in healthy and fast growing plants, as well as a high regeneration rates with the _Agrobacterium_-mediated transformation system. We compared efficacy of the MS medium and B5 medium for shoot regeneration with a 1.0 mg/l ABA concentration and different BAP concentration, and found that the average number of regenerated shoots per explant in MS and B5 media was 2.5 and 1.0, respectively \(Table 3, Supplementary Figure S1 B). We concluded that use of the MS medium resulted in the production of more shoots than the B5 medium \(Table 3), while the B5 medium was more effective for seed generation than the MS medium. _**Agrobacterium concentration and infection time**_ The _Agrobacterium_ concentration and the infection time were found to be important factors in preventing explant turning necrosis and improving transformation efficiency. We determined that the optical density \(OD, at 600 nm) of the _Agrobacterium_ culture should be adjusted to approximately 0.25 \(Table 4), and that the infection time should be about 15 minutes to avoid explant necrosis, and to obtain the highest regeneration rate of 86% \(Table 4). _**Agrobacterium vacuum infiltration**_ It has been reported that vacuum infiltration can improve the _Agrobacterium_-mediated transformation of cucumber[12], and we also found this to be the case. Since we had found that the infection time and _Agrobacterium_ concentration were important in obtaining a high transformation rate, we vacuum infiltrated with _Agrobacterium_ solution and explants in beakerflask \(OD_{600}=0.25) twice for 5 min, and then move them into shaker for an additional 5 min. _**Orientation of the explants on the co-cultivation medium**_ After _Agrobacterium_ infection, placed the cotyledon explants on co-cultivation medium with the adaxial surface facing upward, so that the abaxial surface was in contact with the culture medium. We found this to be essential for high transformation efficiency. After 2 days of co-cultivation, we transferred the cotyledons to the shooting medium, and again made sure that the abaxial surface of the cotyledon was touching the medium. _**Shoot regeneration and rooting**_ We determined that when regenerated shoots have grown to 1 cm, they should be excised from the cotyledon explants and transferred to rooting medium. Embryoids that appear on the cut end surface should be detached. Regenerated shoots will then grow rapidly and the leaves will expand immediately after transfer to rooting medium. The un-rooted regenerated shoot end shoot then be re-cut and the unfolded leaves and the shoots transferred into new rooting medium once per week for the next 2-3 weeks, ensuring that the lower part of the regenerated shoots are inserted in the rooting medium. We found this to be an effective way to remove residual ABA/BAP from the shoots and to be important for efficient rooting. _**Antibiotic Selection**_ The use of kanamycin selection has previously been reported for cucumber transformation[37,38], and we found it to be more effective than hygromycin since the latter affects plant development. Kanamycin concentrations of 50-150 mg/l are commonly used[14,22,39], and it has been shown that a higher concentration will slow growth at the rooting stage and suppress regeneration[24]. Here, 50 mg/l was used during shoot initiation, shoot outgrowth and the rooting stage to encourage the regeneration of transgenic plants. In addition to antibiotic selection, PCR should be used to confirm the presence of the targeted transgene in each transformant at each generation. _**Plant growth conditions**_ Since cucumber is a warm-season crop, the tissue cultures should be kept at 25°C, and after plant acclimation in the soil, the temperature should

be maintained at 28°C/18°C with a 16 h/8 h light/dark cycle, which are typical cucumber cultivation conditions.

Reagents

REAGENTS Cucumber \(_Cucumis sativus_ L.) seeds Agrobacterium strain Sodium hypochlorite, 13% \(wt/vol) \(NaClO; Chem-Supply, CAS 7681-52-9) Hydrochloric acid, HCl, 36.0-38.0% \(Sinopharm Chemical Reagent Co., Ltd, 80070591) B5 Medium \(Waryong GT213250B) Waryong Phytagel plantcell , \(Waryong GT21026M) Agar \(Biowest, 9012-36-6) for LB plates Tryptone \(Oxoid, cat. no. 91079-40-2) Yeast extract \(Oxoid, cat. no. 8013-1-2) Sodium chloride \(NaCl; Chem-Supply, GB/T 1266-19867520, cat. no. 10019792) Sodium hydroxide \(NaOH; Chem-Supply, cat. no.1310-73-2) Sterile distilled water Ethanol \(Chem-Supply, cat. no. 10009292) Ethanol 75% \(Vol/Vol) in distilled water Sucrose \($C_{12}H_{22}O_{11}$; Chem-Supply, cat. no. 10021492) 6-Benzylaminopurine \(BAP, $C_{12}H_{11}N_{5}$l; Sigma, cat. No. B3408) Abscisic acid \(ABA, $C_{15}H_{20}O_4$; Bjxjksw, cat. no. 21293-29-8) Rifampicin \($C_{43}H_{58}N_4O_{12}$; Bjxjksw, cat. no. 13292-46-1) Kanamycin monosulfate \($C_{18}H_{36}N_4O_{11}$ H_2SO_4) Carbenicillin disodium salt \($C_{17}H_{16}N_2O_6SNa_2$; MP Biomedicals, cat. No. 195092) Murashige and Skoog basal medium with vitamins \(PhytoTechnology Laboratories, cat. No. M519) Gellan gum powder \(PhytoTechnology Laboratories, cat. No. G434) PCR Primerstar mix \(Takara Biotechnology, Cat. no. R045A) **REAGENT SETUP** **LB liquid medium for _Agrobacterium_ preparation** Dissolve 5 g of yeast extract, 10 g tryptone and 5 g sodium chloride in 1 liter of distilled water; pH 7.0 autoclave. Add 50 µl of 100 mg/ml rifampicin stock and 50 µl of 50 mg/ml kanamycin stock to 50 ml LB solid medium. **Murashige and Skoog \(MS) liquid medium for _Agrobacterium_ suspension** Dissolve 4.43 g murashige and skoog basal medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water; PH 5.8 autoclave **Murashige and Skoog \(MS) plates for seed germination \(germination medium)** Dissolve 4.43 g murashige and skoog basal medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water; PH 5.8. Add 2.5 g gellan gum powder, autoclave. Pour about 25 ml of medium into sterile Petri dishes under laminar flow hood. **B5 plates for seed germination \(germination medium)** Dissolve 30.21 g B5 medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water; PH 5.8. Add 2.5 g gellan gum powder, autoclave. Pour about 25 ml of medium into sterile Petri dishes under laminar flow hood. **Cocultivation medium** Dissolve 4.43 g murashige and skoog basal medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water, add 750 µl 2 mg/ml BAP; PH 5.8. Add 2.5 g gellan gum powder, autoclave. Add 500 µl 2 mg/ml ABA, and pour about 25 ml of medium into sterile Petri dishes under laminar flow hood. **Shooting medium** Dissolve 4.43 g murashige and skoog basal medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water, add 750 µl 2 mg/ml BAP; PH 5.8. Add 2.5 g gellan gum powder, autoclave. Add 500 µl 2 mg/ml ABA, 50 µl of 50 mg/ml kanamycin and 2.5 ml of 200 mg/ml carbenicillin. Pour about 25 ml of medium into sterile Petri dishes under laminar flow hood. **Rooting medium** Dissolve 4.43 g murashige and skoog basal medium \(with vitamins) powder and 30 g sucrose in 1 liter of distilled water; PH 5.8. Add 2.5 g gellan gum powder, autoclave. Add 50 µl of 50 mg/ml kanamycin and 1 ml of 200 mg/ml carbenicillin. Pour about 40 ml of medium into 100 ml glass beaker under laminar flow hood.

Equipment

Sterile 50 ml plastic tubes \(Corning, cat. no. 430828) Autoclave \(Cheng YI, LS-B35 Type; cat. no. 09J-3298) Controlled tissue culture rooms 25 °C/25 °C, 16/8 h light/dark regime Controlled environmental rooms \(16 h photoperiod) or glasshouse facilities for whole plant culture Orbital incubator shaker \(Pei Ting, THZ-C-1 Type) Tabletop orbital shaker Vacuum pump \(DA-60D; ULVAC, Kanagawa, Japan) was connected to a desiccator \(VM-C; AS ONE, Osaka, Japan) PH meter \(PHSJ-4A Type) Laminar flow hood for plant culture work Glassware \(100 ml glass beaker, graduated cylinders, glass Petri dish, Duran bottles and Erlenmeyer flasks) Filter paper 9 cm diameter \(15-20 μm) Parafilm \(Bemis Flexible Packaging) 230 mm pot \(230 mm in diameter nd 190 mm in height) for cucumber cultivation Forceps and scalpel Surgical blades Plant growth culture vessels Pipettes: P1000, P200, P20, P10 Sterile pipettes tips \(Axygen Scientific: 1–200 μl, cat. no. T-200Y; 1–1,000 μl, cat. no. T-1000B; 1–20 μl, cat. no. T-20Y; 1–10 μl, cat. no. T-10W) Centrifuge Spectrophotometer \(SFZ0806011544 Type) Mini Vortexer \(QL-901 Type)

Procedure

Remove the seed coats, surface sterilization and germination of cucumber seeds •TIMING 3 hours for handling, 2 days for cultivation \(seeds germination) 1. Use Cl_2 \(add 2 ml HCl to 15 ml NaClO in a seal container, please be careful) sterile the cucumber seeds for 1-2 hours, not for long. Soak cucumber mature seeds in tap water for 2 h at room temperature in Petri dish. 2. Remove the seed coats from seeds with forceps by pinch the pointed end of seeds first and then push aside the seed coats. 3. Sterilize 20-50 seeds for 30 s with 20 ml of 75% ethanol in a sterile Petri dish with a lid. Drain ethanol and rinse with 20 ml of 3% sodium hypochlorite solution. Gently shake the seeds on a shaker for 15 min. Rinse three times with sterile deionized water. From this step to step 18 should be performed in a laminar flow hood. 4. Place surface-sterilized seeds with forceps onto Petri plates containing seed germination medium, allowing 15-25 seeds per plate. Germinate the seeds in the dark at 28°C for 2 d. **Inoculum preparation •TIMING 30 min for handling, 2 days for cultivation** 5. On the day after Step 4, inoculating 2 ml of LB liquid medium containing rifampicin \(100 mg/l) and Kanamycin \(50 mg/l) with a single Agrobacterium colony carrying the appropriate construct. Culture at 28°C in a shaker at 200 rpm for 24 h. 6. Subculture 200 μl of 2 ml culture from Step 6 into 50 ml of LB liquid medium containing rifampicin \(100 mg/l) and Kanamycin \(50 mg/l). Culture at 28°C in a shaker at 200 rpm until the OD at 600 nm to 0.6-0.8. 7. Spin the Agrobacterium culture down \(4000g or 5000g) for 10 min at RT \(room temperature). Remove the supernatant and rinse the pellet in 1 ml liquid MS liquid medium. Dilute the MS medium containing _Agrobacterium_ to OD_{600}=0.25 using MS liquid medium. **Explant preparation, _Agrobacterium_ infection and co-cultivation •TIMING 4 hours for handling, 2 days for cultivation \(co-cultivation)** 8. Pull the seedlings \(from Step 5) out of the germination medium and place in an empty sterile Petri plate dishes with a stack of sterile filter paper. 9. Cut the radicle first, and then cut off 1/2-1/3 the end of cotyledon off and remove the endopleura off at the same time. Detach the first cotyledon by using the sterile scalpel to wipe the upside cotyledon off. Knocking the hypocotyl over to detach the downside

cotyledon and growing point using sterile forceps and scalpel \(Fig. 1). 10. Collect the detached cotyledons into MS liquid medium \(without _Agrobacterium_) in a 50 ml sterile glass beaker. 11. Pour out the MS liquid medium \(without _Agrobacterium_), and pour into MS liquid medium \(with _Agrobacterium_ OD_{600}=0.25 from Step 8) and shake gently. Cover the sterile glass beaker with sterile culture container sealing film and put into the desiccator which connected with the vacuum pump. After 2 sessions of vacuum infiltration were applied for 5 min, explants were leave for another 5 min in _Agrobacterium_. 12. Pour out the MS liquid medium \(with _Agrobacterium_), put a sterile filter paper onto cocultivation medium and touch the medium exactly. Place the infected explants on the filter paper and keep the adaxial surface of the cotyledons upward using forceps \(Fig. 1), allowing about 20-35 explants per Petri dish plate. Seal the plate and incubate in dark at 28°C for 2d. **Shoot initiation •TIMING 0.5 hours for handling, 2-3 weeks for cultivation \(shoot regeneration)** 13. Transfer explants onto shooting medium containing 50 mg/ml kanamycin and 500 mg/ml carbenicillin to select the transgenic shoot and inhibit the growth of Agrobacterium, allowing 5-6 explants per Petri dish plate, as the explants will grow up. Incubate under light \(150 μmol m^{-2} s^{-1}, 16 h) at 25°C for 2-3 weeks. **Regeneration of transgenic plants •TIMING 3 hours for handling, 4-6 weeks for cultivation \(root regeneration)** 14. After the shoots growth out from the explants \(Fig. 1), pull the explants out and place in an empty sterile Petri plate dishes with a stack of sterile filter paper. 15. Cut the shoots off using sterile forceps and scalpel. Remove the embryoids on the end part of the shoots. Transfer shoots to 100 ml glass Erlenmeyer flasks or glass tissue culture vessels containing rooting medium with kanamycin \(50 mg/l) and carbenicillin \(500 mg/l). Place three to four shoots per Erlenmeyer flasks. Incubate under light \(150 μmol m^{-2} s^{-1}, 16 h) at 25°C for 1-2 weeks. 16. After 1-2 weeks, take out the no rooting plants and recut the unfolded leaves and the end part of the shoots using sterile forceps and tweezers and transfer shoots to new rooting medium. Repeat this procedure one times per week in the next 2-3 weeks. Place one to two shoots per glass Erlenmeyer flasks. Incubate in under light \(150 μmol m^{-2} s^{-1}, 16 h) at 25°C for 2-4 weeks. **Plant acclimation •TIMING 3 hours for handling, 3-5 weeks for cultivation** 17. After the roots grow strongly, open the cover of the glass Erlenmeyer flasks. Put the cover on the top of the Erlenmeyer flasks loosely. Incubate in dim light \(10-50 μmol m^{-2} s^{-1}) at 25°C for 3 days. 18. Knock the bottom of the Erlenmeyer flasks gently and make the medium not firmed. Pull out the plants from the Erlenmeyer flasks gently using forceps. Wash off the medium with running tap water gently. 19. Fill the pots with wet compost mixture, place the regenerated plants into the compost by making a hole. Compact the soil and water slightly. 20. Cover the plants with zip bags and tight to the pot using rubber band to keep moisture. Incubate in good condition chamber for cucumber growth under light \(gradually from 300 μmol m^{-2} s^{-1} to 500 μmol m^{-2} s^{-1}, 16 h) at 25°C/18°C for 1-2 weeks. Add water into the tray under pots when the soil is getting dry. 21. Take the zip bags off after the regeneration plants grown in a good condition. Watering the plants when the soil is getting dry. Incubate in under light \(500 μmol m^{-2} s^{-1}, 16 h) at 25°C/18°C for 1-2 weeks. **Examination of transgenic plants •TIMING 1 day for handling** 22. Number the regeneration cucumber plants and cut a peace of the mature leaves, extract the genome DNA using the cetyltrimethy ammonium bromide \(CTAB) method[40]. 23. Confirm the transgenic plants has been inserted in the T-DNA using the specific primers designed on the T-DNA and do PCR amplification and gel electrophoresis

assay. **Seed production from transgenic plants •TIMING 1 hour for handling, 8-12 weeks for cultivation** 24. Transfer the molecular identified transgenic plants to greenhouse, to give seedlings normal environment condition with the temperature no higher than 33°C and no lower than 16°C, light intensity 500 μmol m^{-2} s^{-1}, relative humidity 70-80%. 25. The transgenic cucumber plants require normal cultivation management and artificial self-pollination when plants flowering. On the day before the female and male flower blooming, clip the both the female and male flowers on the same plants, and then do artificial pollination using the same plants male flowers to the female flowers on the second morning, and then clip the female flowers to keep away the pollen from the other plants. 26. When the epidermis of autocarps are turned yellow and indicated that the seeds are fully mature, pick them up and place under the cool, dry place for 1-2 weeks for the after-ripening of seeds. 27. Open the cucumber fruit using knife and collect the seeds. Wash the seeds clean with tap water and store in mesh bags under the cool, dry place to dry the seeds. 28. The transgenic cucumber seeds of T0 can germinated directly in soil or in cultivation containers filled soil and used for further analysis of gene biology after molecular identified the exit of T-DNA insertion. T-DNA identification refer to Step 25 and Step 26.

Timing

Steps 1–4, Remove the seed coats, surface sterilization and germination of cucumber seeds: 3 hours for handling, 2 days for cultivation \(seeds germination) Step 5-7, Inoculum preparation: 30 min for handling, 2 days for cultivation Step 8-12, Explant preparation, Agrobacterium infection and co-cultivation: 4 hours for handling, 2 days for cultivation \(co-cultivation) Steps 13, Shoot initiation: 0.5 hours for handling, 2-3 weeks for cultivation \(shoot regeneration) Steps 14–16, Regeneration of transgenic plants: 3 hours for handling, 4-6 weeks for cultivation \(root regeneration) Steps 17–21, Plant acclimation: 3 hours for handling, 3-5 weeks for cultivation. Steps 22–23, Examination of transgenic plants: 1 day for handling Steps 24–28, Seed production from transgenic plants: 1 hour for handling, 8-12 weeks for cultivation

Troubleshooting

Troubleshooting advice can be found in Table 5

Anticipated Results

The average plant regeneration rate and transformation frequencies are affected by cucumber genotypes, explants types, explants preparation methods, the _Agrobacterium_ infection methods, the concentration and ratio of ABA and BAP and culture methods \(Table 1, Table 2, Table 3, Table 4 and figure 1). Different _Agrobacterium_ species \(EHA105, C58, LBA4404 AGL1 and GV3101) contained a series of plant binary vectors \(pBI121, pCAMBIA 2301, pCAMBIA1391, pCAMBIA1305.1 and pFGC1008) has been used to transform different cucumber varieties \(Deltasta, Guonong25, cucumber line 3407, 3413, 3461, Chinses long: 461, 9930, R1407, R470, short 32x, and Xintaimici,) and obtained the transgenic plants successfully[25-33]. The pure line should be used when considering for obtain T_1 generation and for gene function analysis. This protocol has improved the frequency of transformation to 26%, and we have

successfully obtained over six hundred cucumber transgenic lines and published some papers of gene function analysis using this protocol[25-33]. We believe that this protocol is and will play important role in gene discovery, gene delivery, gene functional analyses and variety improvement for cucumber and _Cucurbitaceae_ plants \(need to be slightly modified) in the future study.

References

1 Rennie, E. A. & Turgeon, R. A comprehensive picture of phloem loading strategies. _Proc Natl Acad Sci USA_ **106**, 14162-14167 \(2009). 2 Turgeon, R. Phloem loading and plasmodesmata. _Trends Plant Sci_ **1**, 418-423 \(1996). 3 Hu, L. P. et al. Phloem unloading follows an extensive apoplasmic pathway in cucumber \(_Cucumis sativus_ L.) fruit from anthesis to marketable maturing stage. _Plant Cell Environ_ **34**, 1835-1848 \(2011). 4 Gross, K. C. & Pharr, D. M. A potential pathway for galactose metabolism in _Cucumis sativus_ L, a stachyose transporting species. _Plant Physiol_ **69**, 117-121 \(1982). 5 Gardner, R. C. Gene-transfer into tropical and tubtropical crops. _Sci Hortic_ **55**, 65-82 \(1993). 6 Wei, Q. Z. _et al_. Rapid identification of fruit length loci in cucumber \(_Cucumis sativus_ L.) using next-generation sequencing \(NGS)-based QTL analysis. _Sci Rep_ **6** \(2016). 7 Yuan, X. J. _et al_. Genetic mapping and QTL analysis of fruit and flower related traits in cucumber \(_Cucumis sativus_ L.) using recombinant inbred lines. _Euphytica_ **164**, 473-491 \(2008). 8 Trulson, A. J., Simpson, R. B. & Shahin, E. A. Transformation of cucumber \(_Cucumis sativus_ L.) plants with _Agrobacterium rhizogenes_. _Theor Appl Genet_ **73**, 11-15 \(1986). 9 Kose, E. & Koç, N. K. _Agrobacterium_-mediated transformation of cucumber \(_Cucumis Sativus_ L.) and plant regeneration. _Biotechnol Biotec Eq_ **17**, 56-62 \(2003). 10 He, Z. Q. _et al_. Mannose selection system used for cucumber transformation. _Plant Cell Rep_ **25**, 953-958 \(2006). 11 Selvaraj, N. _et al_. 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 Cell Dev Biol_ **46**, 329-337 \(2010). 12 Nanasato, Y., Konagaya, K., Okuzaki, A., Tsuda, M. & Tabei, Y. Improvement of _Agrobacterium_-mediated transformation of cucumber \(_Cucumis sativus_ L.) by combination of vacuum infiltration and co-cultivation on filter paper wicks. _Plant Biotechnol Rep_ **7**, 267-276 \(2013). 13 Vengadesan, G., Anand, R. P., Selvaraj, N., Perl-Treves, R. & Ganapathi, A. Transfer and expression of nptII and bar genes in cucumber \(_Cucumis sativus_ L.). _In Vitro Cell Dev Biol_ **41**, 17-21 \(2005). 14 Nishibayashi, S., Kaneko, H. & Hayakawa, T. Transformation of cucumber \(_Cucumis sativus_ L.) plants using _Agrobacterium_ tumefaciens and regeneration from hypocotyl explants. _Plant Cell Rep_ **15**, 809-814 \(1996). 15 Punja, Z. K., Abbas, N., Sarmento, G. G. & Tang, F. A. Regeneration of _Cucumis sativus_ var. hardwickii, C. _melo_, and C. _metuliferus_ from explants through somatic embryogenesis and organogenesis. _Plant Cell Tiss Org_ **21**, 93-102 \(1990). 16 Raharjo, S. H. T., Hernandez, M. O., Zhang, Y. Y. & Punja, Z. K. Transformation of pickling cucumber with chitinase-encoding genes using _Agrobacterium_ tumefaciens. _Plant Cell Rep_ **15**, 591-596 \(1996). 17 Lou, H. & Kako, S. Somatic embryogenesis and plant regeneration in cucumber. _Hortscience_ **29**, 906-909 \(1994). 18 Colijnhooymans, C. M., Bouwer, R., Orczyk, W. & Dons, J. J. M. Plant regeneration from cucumber \(_Cucumis sativus_) protoplasts. _Plant Sci_ **57**, 63-71 \(1988). 19 Wehner, T. C. &

Locy, R. D. In vitro adventitious shoot and root formation of cultivars and lines of _cucumis sativus_ L. _Hortscience_ **16**, 759-760 \(1981). 20 Schulze, J. _et al_. Biolistic transformation of cucumber using embryogenic suspension cultures: long-term expression of reporter genes. _Plant Sci_ **112**, 197-206 \(1995). 21 Wang, S. L. _et al_. Current status of genetic transformation technology developed in cucumber \(_Cucumis sativus_ L.). _J Integr Agr_ **14**, 469-482 \(2015). 22 Chee, P. P. Transformation of _Cucumis sativus_ tissue by _Agrobacterium_ tumefaciens and the regeneration of transformed plants. _Plant Cell Rep_ **9**, 245-248 \(1990). 23 Lin, Y. T. _et al_. In Vitro regeneration and genetic tansformation of cucumis metuliferus through cotyledon organogenesis. _Hortscience_ **46**, 616-621 \(2011). 24 Rajagopalan, P. A. & Perl-Treves, R. Improved cucumber transformation by a modified explant dissection and selection protocol. _Hortscience_ **40**, 431-435 \(2005). 25 Cheng, J. T. _et al_. Down-regulating CsHT1, a cucumber pollen specific hexose transporter, inhibits pollen germination, tube growth, and seed development. _Plant Physiol_ **168**, 635-647 \(2015). 26 Wang, H. Y. _et al_. Antisense suppression of cucumber \(_Cucumis sativus_ L.) sucrose synthase 3 \(CsSUS3) reduces hypoxic stress tolerance. _Plant Cell Environ_ **37**, 795-810 \(2014). 27 Ding, L. _et al_. HANABA TARANU regulates the shoot apical meristem and leaf development in cucumber \(_Cucumis sativus_ L.). _J Exp Bot_ **66**, 7075-7087 \(2015). 28 Chen, C. H. _et al_. The WD-repeat protein CsTTG1 regulates fruit wart formation through interaction with the homeodomain-leucine zipper I protein Mict. _Plant Physiol_ **171**, 1156-1168 \(2016). 29 Liu, B. _et al_. Silencing of the gibberellin receptor homolog, CsGID1a, affects locule formation in cucumber \(_Cucumis sativus_) fruit. _New Phytol_ **210**, 551-563 \(2016). 30 Wang, W. J. _et al_. _Cucumis sativus_ L. WAX2 plays a pivotal role in wax biosynthesis, influencing pollen fertility and plant biotic and abiotic stress responses. _Plant Cell Physiol_ **56**, 1339-1354 \(2015). 31 Wang, W. J. _et al_. Cucumber ECERIFERUM1 \(CsCER1), which influences the cuticle properties and drought tolerance of cucumber, plays a key role in VLC alkanes biosynthesis. _Plant Mol Biol_ **87**, 219-233 \(2015). 32 Zhang, Y. _et al_. A GAMYB homologue CsGAMYB1 regulates sex expression of cucumber via an ethylene independent pathway. _J Exp Bot_ **65**, 3201-3213 \(2014). 33 Lu, J. _et al_. Suppression of cucumber stachyose synthase gene \(CsSTS) inhibits phloem loading and reduces low temperature stress tolerance. Plant Mol Biol, doi:10.1007/s11103-017-0621-9 \(2017). 34 Hallmann, J., Quadt-Hallmann, A., Rodríguez-Kábana, R. & Kloepper, J. W. Interactions between Meloidogyne incognita and endophytic bacteria in cotton and cucumber. _Soil Biology and Biochemistry_ **30**, 925-937 \(1998). 35 Yan, X., Huang, L. L., Tu, X., Gao, X. N. & Kang, Z. S. Saccharothrix yanglingensis sp. nov., an antagonistic endophytic actinomycete isolated from cucumber plant. _Antonie van Leeuwenhoek_ **101** \(2012). 36 Wang, J. _et al_. _Agrobacterium_-mediated transformation of cucumber \(_Cucumis sativus_ L.) using a sense mitogen-activated protein kinase gene \(CsNMAPK). _Plant Cell Tiss Org_ **113**, 269-277 \(2013). 37 Tabei, Y. _et al_. Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold \(Botrytis cinerea). _Plant Cell Rep_ **17**, 159-164 \(1998). 38 Sarmento, G. G., Alpert, K., Tang, F. A. & Punja, Z. K. Factors influencing _Agrobacterium_-tumefaciens mediated transformation and expression of kanamycin resistance in pickling Cucumber. _Plant Cell Tiss Org_ **31**, 185-193 \(1992). 39 Ganapathi, A. & Perl-Treves, R. _Agrobacterium_-mediated transformation in _Cucumis sativus_ via direct organogenesis. _Acta Hortic_ **510**, 405-408 \(2000). 40 Lodhi, M. A. , Ye, G. N., Weeden, N. F., Reisch, B. I. A simple and efficient

method for DNA extraction from grapevine cultivars, Vitis species and Ampelopsis. _Plant Mol Biol Rep_. **12**, 6-13 \(1994).

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Figures

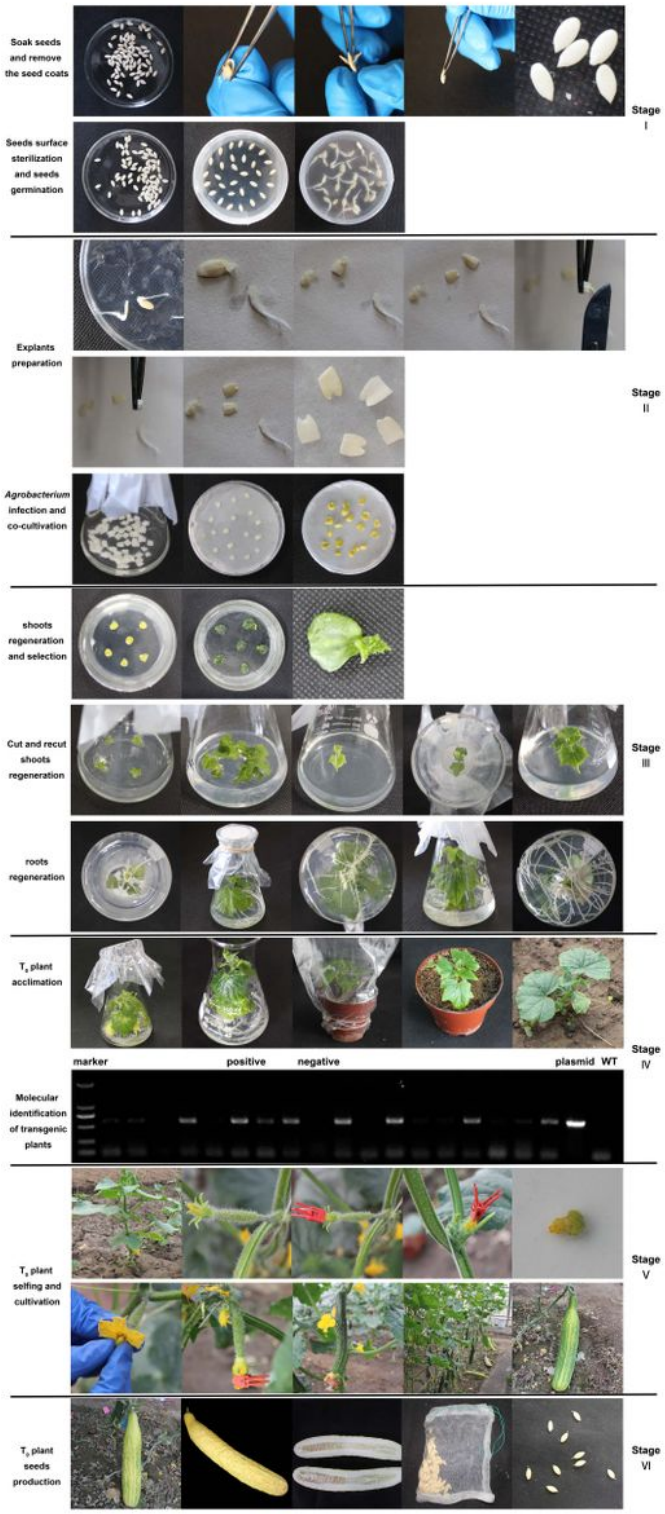

Figure 1

Overview of the seven-stage procedure of Agrobacterium-mediated transformation of cucumber. Stage I: the seeds sterilizing, germination and removing seed coat. Stage II: explant preparation, infection and co-cultivation with Agrobacterium tumefaciens. Stage III: shoot regeneration. Stage IV: root regeneration. Stage V: acclimation and molecular identification of T0 plants. Stage VI: cultivation and self-crossing of T0 plants. Stage VII: seed harvest from T0 plants.

Genotype	Explant	Resistance	T_0 transformation efficiency (%)	T_1 generation	References
cv. Jinyan no.7	Cotyledon	Mannose and sucrose	23% (shoots)	NR	He, Z. Q. *et al*. (2006)[10]
cv. Poinsett 76	Cotyledon	Phosphinothricin	21% (shoots)	Reported	Selvaraj, N. *et al.* (2010)[11]
cv. Cengel Köy	Cotyledon	Kanamycin	16% (shoots)	NR	Kose, E. & Koç, N. K. (2003)[9]
cv. Shinhokusei 1	Cotyledon	Kanamycin	11.9% ± 3.5 (plants)	Reported	Nanasato, Y. *et al.* (2013)[12]
cv. pickling genotype 3676 and 3672	Petiole and leaves	Kanamycin	9% (plants)	Reported	Sarmento, G. G. *et al.* (1992)[37]
cv. Greenlong	Cotyledon	Phosphinothricin	7.2% (plantlets)	NR	Vengadesan, G. *et al.* (2005)[13]
cv. Xintaimici	Cotyledon	Kanamycin	4.8% (plantlets)	NR	Wang, J. *et al.* (2013)[34]
cv. Straight Eight	Hypocotyl	Kanamycin	3.2% (plantlets)	NR	Trulson, A. J. *et al.* (1986)[8]
cv. Poinsett 76	Cotyledon	Kanamycin	1.7% (plantlets)	NR	Rajagopalan, P. A. & Perl-Treves, R. (2005)[24]
cv. 1021	Hypocotyl	Hygromycin	0.9-1.8% (plantlets)	Reported	Nishibayashi, S. *et al.* (1996)[14]
cv. Poinsett 76	Cotyledon	Kanamycin	Undetermined	Reported	Chee, P. P. (1990)[22]
cv. Endeavor	Petiole	Kanamycin	Undetermined	NR	Raharjo, S. H. T. (1996)[16]

Figure 2

Table 1 Published reports of Agrobacterium-mediated transformation of cucumber T_0_ Transformation efficiency is calculated based on the number of plants identified as being transformed by molecular techniques, or shoots out of the total number of infected explants. NR: not reported. T_1_ generation lines related to the T_0_ generation plants.

	Regeneration efficiency (%)					
	BAP 0	BAP 0.2	BAP 0.5	BAP 0.8	BAP 1.5	BAP 2
ABA 0.0	0.0	35.1	67.0	57.3	61.1	22.2
ABA 0.5	2.8	44.4	70.8	73.6	72.2	72.2
ABA 1.0	18.4	38.2	59.4	76.4	86.1	69.4
ABA 1.5	4.2	10.4	18.8	62.5	81.7	66.7

Figure 3

Table 2 Combined effect of abscisic acid (ABA, mg/l) and 6-benzylaminopurine (BAP, mg/l) concentration on cucumber shoot regeneration from cotyledon explants using the Xintaimici genotype.

	The number of regenerated shoots per cotyledon					
	BAP 0	BAP 0.5	BAP 1.0	BAP 1.5	BAP 2.0	BAP 3.0
MS	1.8 ± 1.2	2.6 ± 1.8	2.8 ± 1.9	4.3 ± 2.2	1.9 ± 1.2	1.0 ± 1.0
B5	1.3 ± 1.0	2.0 ± 1.8	1.8 ± 1.6	2.8 ± 1.9	2.0 ± 1.3	1.1 ± 1.1

Figure 4

Table 3 The number of regenerated shoots per cotyledon under the combined effect of MS and B5 base media with ABA 1.0 mg/l and different BAP concentrations using the Chinese long 461 genotype after 20 days of shoot regeneration culturing.

Table 4 | Combined effect of *Agrobacterium* infection time and *Agrobacterium* concentration (OD_{600}) on cucumber shoot regeneration using an ABA/BAP concentration of 1.0/1.5 mg/l in the shooting medium and the Xintaimici genotype.

	Regeneration efficiency (%)		
Infection time	OD_{600}= 0.05	OD_{600}= 0.25	OD_{600}= 0.5
5 min	59.0	78.7	54.1
15 min	68.1	86.0	38.0
30 min	68.6	78.0	34.4

Figure 5

Table 4 Combined effect of Agrobacterium infection time and Agrobacterium concentration (OD600) on cucumber shoot regeneration using an ABA/BAP concentration of 1.0/1.5 mg/l in the shooting medium and the Xintaimici genotype.

Steps	Problem	Possible reasons	Solution
6	Poor germination	Seeds not of good quality	Use fresh, plump seeds and do a seeds germination rate test before use.
		Seeds surface sterilization	Sterilize seeds in ethanol no more than 1 min and in sodium hypochlorite no more than 20 min, and then rinse with excess amount of water.
		Germination medium	Use B5 medium instead of MS medium.
11	The two cotyledons of the seeds are difficult to detach	The seeds germination or sprouting time is too short	Leave the seeds under germination condition for another half day to one day.
	The two cotyledons are curled	The seeds germination time is too long or sprouting conditions are not suitable	Reduce the seeds germination time and make sure the seeds under good conditions for germination (28° C,dark).
15	Bacterium growth on or around the explants such as Red bacterium or other bacterium	Contamination from the endopleura or seed endophyte	Use Cl_2 (add 2 ml HCl to 15 ml NaClO in a seal container, please be careful) sterile the seeds for 1-2 hours, not for long, before the step 1.
			Cut the radicles off first and always change the filter paper to avoid the endophytes on radicles.
		Contamination from other microorganisms	Use sterile implements and be careful of operate under laminar flow.
		Contamination from Agrobacterium bacterium	Change one new Agrobacterium bacterium.
16	No shoot regeneration	Inappropriate ABA or BAP concentration	Check and regulate the amount and ratio of ABA and BAP.
		Growing point did not touch the medium	Always check and insert the growing point into medium as the cotyledon growing.
		The explants has been dead	Discard these explants.
18	No root regeneration	The differentiated part did not remove clearly or the hormones of ABA/BAP used for differentiation in the shoots affect the rooting	Cut off the differentiated part at the end part of the shoots clearly and recut the end part of the shoots every 1-2 weeks three times.
		The end of shoot did not inserted into the medium	Cut off the big leaves and always check the end of shoot is inserted into the medium.
		Inappropriate selective agent composition	Check the agent type and the selective agent concentration.
		The negative transgenic plants	Discard these plants.
23	Death of plants	Roots not developed well	Use roots well developed plants.
25	No specific insertion fragments bands	The genome DNA concentration is not proper	Try to use different DNA concentrations.
		The negative transgenic plants	Discard these plants.
27	Loss of plants or fruits	The inappropriate growth condition	Control the temperature, light, water and soil condition for the plants growth flowering and fruiting.
	No seeds or no ripe seeds	Unsuccessful pollination	Use 2-3 male flowers from the same plants to pollinate to female flowers and make sure the pollen of male flowers is normal development.
		Transgenic effect	Check whether if the insert gene or fragment insertion sites affect the seeds development.

Figure 6

Table 5 Troubleshooting advice

Supplementary Files

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- supplement0.jpg