Agrobacterium-mediated transformation of *Brassica napus* and *Brassica oleracea*

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***Agrobacterium*-mediated transformation is widely used for gene delivery in plants. However, commercial cultivars of crop plants are often recalcitrant to transformation because the protocols established for model varieties are not directly applicable to them. The genus *Brassica* includes the oil seed crop, canola (*B. napus*), and vegetable crop varieties of *Brassica oleracea*, including cauliflower, broccoli and cabbage. Here, we describe an efficient protocol for *Agrobacterium*-mediated transformation using seedling explants that is applicable to various *Brassica* varieties; this protocol has been used to genetically engineer commercial cultivars of canola and cauliflower in our laboratory. Young seedling explants are inoculated with *Agrobacterium* on the day of explant preparation. Explants are grown for 1 week in the absence of a selective agent before being transferred to a selective medium to recover transgenic shoots. Transgenic shoots are subjected to an additional round of selection on medium containing higher levels of the selective agent and a low-carbohydrate source; this helps to eliminate false-positive plants. Use of seedling explants offers flexible experiment planning and a convenient explant source. Using this protocol, transgenic plants can be obtained in 2.5 to 3.5 months.**

INTRODUCTION

Brassicas are closely related to the model plant *Arabidopsis*, which belongs to the *Brassicaceae* family. Developing tools that facilitate the transfer of knowledge from model plants, such as *Arabidopsis*, to agriculturally important plants, such as Brassicas, would aid the breeding of crops with novel, desirable characteristics[1]. Genetic transformation technology is vital to gene discovery, functional analyses and variety improvement. It has allowed the development of novel Brassica varieties, such as those producing nutriceutical, biodegradable plastic or biopharmaceuticals, by introducing novel genes from unrelated sources—something that cannot be achieved by conventional breeding methods. Conventional breeding of Brassica is labor and resource intensive and time consuming; it takes eight to ten generations to develop a new variety[2]. In contrast, genetic transformation provides direct means to introduce a specific gene or trait into a selected genotype without adversely affecting their desirable genetic background. Moreover, most traits introduced by gene-transfer method are dominant. Thus, a gene-delivery system suitable for commercial *Brassica* varieties would eliminate lengthy downstream breeding programs required to develop a commercial product when an agronomically inferior model variety is used. Additionally, a gene-transfer system that is routinely applicable to commercial cultivars of different genetic backgrounds would help in realizing the full potential of the genomics era in *Brassica* breeding.

Direct DNA-transfer methods for *Brassica* transformation

A number of direct DNA-transfer methods have been explored for *Brassica* transformation. These include PEG-mediated DNA uptake, electroporation, microinjection and microprojectile bombardment[3,4]. PEG-mediated direct DNA uptake of protoplasts has been attempted in *B. oleracea*[5,6] and *B. napus*[7–9] using protoplasts prepared from either mesophyll or hypocotyl. Electroporation and PEG-mediated DNA uptake relies on the introduction of DNA into plant protoplasts followed by plant regeneration. In general, protoplast isolation and regeneration of viable plants have been difficult to achieve with crop plants and are not readily reproducible. Moreover, regeneration of plants from the protoplast of Brassicas is genotype dependent. In addition, the use of protoplasts requires a longer tissue-culture period, leading to an increased risk of undesirable somaclonal variations (which are mostly due to stress imposed by *in vitro* culture). Thus, the usefulness of these direct DNA-transfer methods[4,7] in regenerating transgenic plants is limited.

Microspores of *Brassica* have been used for gene delivery using microinjection[10] and microprojectile bombardment has been tried for *B. napus* using microspores as explants[11,12]. This latter method involves propelling DNA-coated gold or tungsten particles into intact plant tissues or cells followed by regeneration of transgenic plants. However, the microprojectile method is not favored, as it leads to fragmentation of DNA during bombardment, insertion of backbone vector DNA and insertion of multiple gene copies. Multiple copies of the transgene can lead to transgene silencing and integration of vector DNA is undesirable especially in the present regulatory and consumer environment.

Agrobacterium-mediated transformation of *Brassica*

Agrobacterium-mediated transformation has become the most common method for Brassica transformation. Previous studies have used both *A. tumefaciens* and *A. rhizogenes* to obtain transgenic plants[3,4,13]. *A. rhizogenes*-mediated transformation involves production of hairy (transgenic) roots followed by plant regeneration from these roots. Studies of *A. rhizogenes*-mediated transformation in *B. oleracea*[14,15] found that the resulting transgenic plants had undesirable abnormal phenotypes such as reduced apical dominance, wrinkled leaves, altered flower morphology and reduced fertility[16]. Recovering phenotypically normal transgenic plants using *A. rhizogenes*-mediated transformation requires that the genotype to be transformed not only has the ability to produce adventitious roots[17] but also has the ability to regenerate normal transgenic plants from transgenic roots.

A. tumefaciens-mediated transformation depends on the susceptibility of plants to *Agrobacterium* infection and delivery of T-DNA from the binary plasmid into plant cells. The ability to regenerate transgenic plants from these transformed cells is also vital for successful transformation. Studies on the genetic factors controlling the susceptibility of *B. oleracea* to *A. tumefaciens* during *in vitro* shoot regeneration confirm that both the ability to regenerate shoots and the degree of susceptibility to *A. tumefaciens* are good indicators of the transformation potential of *B. oleracea* genotypes[18,19].

Advantages and limitations of *Agrobacterium*-mediated transformation

Compared to direct DNA-transfer methods, the *Agrobacterium*-mediated transformation method is considered to be simpler and less expensive, as it exploits the unique ability of *Agrobacterium* to introduce the transgene into plant cells. Unlike microprojectile bombardment method, the most commonly used direct DNA-transfer method, *Agrobacterium*-mediated transformation, does not require specialized equipment (e.g., gene gun) or expensive consumables such as gold particles. The feasibility of recovering low-copy-number transgenic *Brassica* plants has been demonstrated for various *Agrobacterium* strains. Thus, gene silencing, associated with multiple transgene copies, is less likely to occur with *Agrobacterium*-mediated transformation. In addition, *Agrobacterium*-mediated transformation delivers only the modified T-DNA of the plasmid, thereby eliminating the random integration of vector sequences often seen with microprojectile bombardment; this makes analysis of the transformed DNA (e.g., required by regulatory authorities) more straightforward. Finally, the *Agrobacterium* method offers the possibility of using plant transfer DNA (P-DNA), which resembles and functions like T-DNA, for plant transformation studies[20]; this would reduce the amount of intergenic (i.e., foreign) DNA in the transgenic plant, which is important for allaying public concerns[21].

Varieties amenable to *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation has allowed the generation of viable transgenic plants from many *Brassica* species[22–26]. Transformation has been tested in a variety of explant types such as hypocotyl, leaf, seed and seedling stems, and the efficiency of transformation reported varies with the cultivar, age of the donor plant and explant type[3]. Hypocotyl explants have been used to obtain transgenic *B. napus* and *B. oleracea* plants[22] while use of cotyledon explants in *B. napus* transformation has also been demonstrated[23]. However, commercial cultivars of Brassicas of economic importance, including canola, are generally considered to be recalcitrant to genetic transformation and regeneration[27,28]. In the case of canola, most of the transformation protocols reported are relatively specific to the model spring Westar cultivar, which is not agronomically desirable, and are not directly applicable to other spring and winter cultivars[27,29]. Moreover, there have been few reports on transgenic commercial cultivars of winter-type canola[27,30]. Our laboratory has been successful in developing a transformation protocol for commercial lines of *Brassica*[31,32]. The protocol is not only modified from de Block *et al.*[22] and Moloney *et al.*[23], but also includes a strategy to recover transgenic cells (i.e., the shoot) and involves an additional round of selection for effectively identifying transgenic shoots. The protocol described here has been successfully used to transform commercial cultivars (Oscar, Rainbow, RK7 (Taparoo), R125 (Charlton)) and model variety, Westar, of *B. napus*[31] and commercial lines (WG-11-1, B-4) of *B. oleracea* var *botrytis*[32] (see **Table 1**). It should be emphasized that successful genetic transformation relies on regeneration of viable shoots from the cultured tissues. Therefore, the potential for shoot regeneration of a given cultivar should be checked and optimized first before embarking on transformation.

TABLE 1 | *Agrobacterium*-mediated transformation of commercial cultivars of *B. napus* and *B. oleracea* using cotyledon as explants.

Genotype	Transformation efficiency(%)	Reference
B. napus, Westar	33.1	31
B. napus, Oscar	68.1	31
B. napus, RK7[a] (Taparoo)	67.6	31
B. napus, R125[a] (Charlton)	7.7	31
B. napus, Rainbow	11.9	31
B. oleracea var *botrytis*, B-4	9.5	32
B. oleracea var *botrytis*, WG-11-1	~7.0	32

Transformation efficiency (%) = number of transgenic shoots divided by total number of explants used multiplied by 100. B-4 and WG-11-1 are parent lines of commercial F_1 hybrid seeds of autumn cauliflower. [a]Commercial variety names are given in brackets.

Key factors for successful transformation

Our previous studies[31,32] on cauliflower (*B. oleracea*) and canola (*B. napus*) have identified several key factors for successful transformation.

The use of young seedling explants. Using seedling explants offers flexible experiment planning, a convenient explant source, reduced labor and maintenance cost of explant sources, and reduced contamination during *in vitro* culture. Explants from younger seedlings (~4 d) of *Brassica* seem to respond better in tissue culture than older seedlings.

***Agrobacterium* concentration and length of exposure during infection.** The *Agrobacterium* concentration (as determined by measuring OD at 650 nm) used for infection was found to be among the critical factors for explants turning necrotic, especially when octopine strains, such as LBA4404, were used. The OD (at 650 nm) of *Agrobacterium* strain LBA4404 should be adjusted to 0.05 to avoid explant necrosis. Similarly, longer infection time could also lead to necrotic explants resulting in failure of transformation.

Cocultivation time. During cocultivation of *Brassica* explants with *Agrobacterium*, growth of *Agrobacterium* on the explants should be avoided to prevent the explants turning necrotic or regenerating few or no transgenic plants.

Delayed exposure to the selective agent. The delay in exposing the explants to the selective agent such as kanamycin or hygromycin (depending upon the selectable marker used; note: *Agrobacterium*-specific antibiotic such as carbenicillin is included in the medium) is critical in promoting proliferation and recovery of transgenic cells that give rise to transgenic plants.

Inclusion of a selective agent during shoot initiation and shoot outgrowth. Selection during shoot initiation and shoot outgrowth encourages regeneration of putative transgenic shoots. However, the amount of selective agent used in the medium during these steps should not be too high and regenerating explants should be transferred to fresh medium every 2 weeks to maintain the selection pressure.

Additional selection steps, that is, transformant selection. This step is necessary to eliminate false positives and to select transgenic shoots. Transformant selection involves exposing the individual shoots to an increased amount of selective agent while lowering the carbohydrate source in the medium.

Explant type. *Agrobacterium tumefaciens*-mediated transformation of *Brassica* species using seedling (cotyledon) explants is described here. Hypocotyl explants have been used for *B. napus* and *B. oleracea* transformation[2,22,29,31]. Hypocotyl explants usually exhibit callus and shoot development during transformation from the top cells, away from the selective agent, leading to high numbers of escapees or false positives. Therefore, it is desirable to employ direct shoot regeneration and avoid callus development, possibly by omitting auxin from the shoot initiation medium. In addition, hypocotyl explants are more likely to turn black if the *Agrobacterium* concentration is too high during infection, or the cocultivation step is too long, or ingredients such as silver nitrate are omitted from the medium or gas exchange (e.g., use of micropore tape) during incubation is not appropriate. The protocol described here has also been used to produce viable transgenic plants from hypocotyl explants of several commercial varieties of *Brassica* in our laboratory, but the efficiency of transformation has generally been lower than that of cotyledon explants, and, usually, shoot initiation and shoot growth have taken longer time. In addition, root explants have also been evaluated for their regeneration[33,34] and transformation[31] capacities in our laboratory, but their transformation efficiency has been found to be low[31].

MATERIALS

REAGENTS

- *Brassica* seeds (see **Box 1**) ▲ **CRITICAL** The regeneration capacity of the target *Brassica* cultivar is vital and should be determined before applying this protocol. Parallel transformation of Westar cultivar (*B. napus*) with the chosen cultivar is recommended for initial experiments as a positive control.
- *Agrobacterium* strain, for example, LBA4404, carrying a binary vector harboring the gene of interest and a selectable marker such as kanamycin. Other strains of *Agrobacterium* such as AGL1 or EH101 could also be used. We routinely use LBA4404 (Invitrogen), which is a common strain, for *Brassica* transformation in our laboratory. ▲ **CRITICAL** We have successfully obtained transgenic *Brassica* plants using pBI101, pBIN plus and pCAMBIA binary vectors in our laboratory. However, we generally find that pBI101 results in fewer transgenic plants than either pCAMBIA or pBIN plus. The nopaline synthase promoter and terminator sequences were used to regulate expression of the selectable marker NPT-II.
- Sodium hypochlorite, 12.5% (wt/vol) (Chem-Supply, cat. no. ST044) ▲ **CRITICAL** Store in light-proof container in a cool place (below 25 °C). Sodium hypochlorite should not be stored for more than 4 months, as it degrades over time; it is recommended to buy a fresh stock every 2–3 months.
- Agar (Sigma, cat. no. A-1296) for LB medium
- Tryptone (Oxoid, cat. no. B246018)
- Yeast extract (Merck, cat. no. 1.03753.0500)
- Sodium chloride (NaCl; Merck, cat. no. 1.06404.5000)
- Sodium hydroxide (NaOH; Merck, cat. no. B871798624); dissolve 4 g in 100 ml water to prepare a 1 N stock ! **CAUTION** Causes severe burns. Avoid contact with skin and eyes. Wear suitable protective clothing, gloves and eye protection.
- Sterile distilled (glass distilled) water
- Ethanol (Chem-Supply, cat. no. EA043) ! **CAUTION** Flammable liquid, store in a cool place.
- Ethanol 70% (vol/vol) in distilled water
- Ethanol 50% (vol/vol) in distilled water
- Ammonium nitrate (NH_4NO_3; Chem-Supply, cat. no. AA003) ! **CAUTION** Harmful if swallowed. Irritant to eyes, skin and respiratory tract. Wear suitable protective gear. Hazardous oxidizing agent that can react violently with other incompatible materials.
- Potassium nitrate (KNO_3; Chem-Supply, cat. no. PA001) ! **CAUTION** Harmful if swallowed. Irritant to eyes, skin and respiratory tract. Wear suitable protective gear. Hazardous oxidizing agent that can react violently with other incompatible materials.
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$; Merck, cat. no. 10151.4Y)
- Potassium dihydrogen orthophosphate (KH_2PO_4; Ajax Finechem, cat. no. 391)
- EDTA (ethylenediaminetetraacetic acid disodium salt, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$; Chem-Supply, cat. no. EA023)
- Boric acid (H_3BO_3; Ajax Finechem, cat. no. A102)
- Manganese (II) sulfate (monohydrate) ($MnSO_4 \cdot H_2O$; Ajax Finechem, cat. no. A309)
- Zinc sulfate ($ZnSO_4 \cdot 7H_2O$; BDH, cat. no. 102994R) ! **CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$; Mallinckrodt, cat. no. 7782)
- Copper (II) sulfate (cupric sulfate, $CuSO_4 \cdot 5H_2O$; Ajax Finechem, cat. no. 171) ! **CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Cobalt (II) chloride hexahydrate ($CoCl_2$ $6H_2O$; Sigma, cat. no. C-8861)
- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$; Ajax Finechem, cat. no. 127) ! **CAUTION** Do not breathe dust, avoid contact with skin or eyes.
- Potassium iodide (KI; Merck, cat. no. 10212) ! **CAUTION** Do not breathe dust, avoid contact with skin or eyes. Wear suitable protective gear.
- Nicotinic acid ($C_6H_5NO_2$; Sigma, cat. no. N-0761) ! **CAUTION** Irritant to eyes, respiratory system, skin and eyes. Wear suitable protective gear.

BOX 1 | EVALUATION OF REGENERATION POTENTIAL OF THE EXPLANTS

The protocol described here relies on the regeneration of viable plants from cotyledon explants isolated from germinated seedlings; it is therefore necessary to evaluate the regeneration capacity of these explants from a given *Brassica* variety before attempting transformation. To optimize shoot regeneration from a given cultivar, test different levels of plant growth regulators, BAP (1–5 mg liter^{-1} is a good starting range). In addition, consider different levels of NAA in combination with BAP in shoot initiation medium.

(i) Surface sterilize and germinate seeds as described in Steps 1–6 of the main Procedure.

(ii) Prepare seedling explants as described in Steps 12 and 13 of the main Procedure.

▲ **CRITICAL STEP** As no *Agrobacterium* infection is required, omit Steps 7–11 and 14–16 of the main Procedure.

(iii) Follow Steps 17–19 of the main Procedure for shoot initiation and shoot outgrowth.

▲ **CRITICAL STEP** Do not include selective agents (antibiotics) in the medium during shoot initiation and shoot outgrowth.

(iv) Count the number of explants producing shoots and the number of shoots regenerated per explant. Determine the efficiency of regeneration and the average number of shoots (green) regenerated per explant. Usually the time frame of shoot and plant regeneration is shorter when this process is used than that indicated in the main Procedure.

- Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$; Sigma, cat. no. T-4625) ! CAUTION Do not breathe dust, avoid contact with skin and eyes and do not inhale. Wear suitable protective gear.
- Pyridoxine hydrochloride (vitamin B6, $C_8H_{11}NO_3 \cdot HCl$; Sigma, cat. no. P-9755) ! CAUTION Irritant to eyes, respiratory system and skin. Wear suitable protective gear.
- myo-Inositol ($C_6H_{12}O_6$; Sigma, cat. no. 13011)
- Sucrose ($C_{12}H_{22}O_{11}$; Chem-Supply, cat. no. SA030)
- Sodium chloride (NaCl; Merck, cat. no. 10241.AP)
- Phytagel (plant-cell-culture tested, powder; Sigma, cat. no. P-8169)
- Silver nitrate ($AgNO_3$; Merck, cat. no. 1.01512.0025) ! CAUTION Avoid contact and inhalation; it is highly toxic, a strong oxidizer, corrosive and light sensitive. Wear suitable protective gear.
- 1-Naphthalene acetic acid (NAA, $C_{12}H_9O_2K$; Sigma, cat. no. N-1145; see REAGENT SETUP) ! CAUTION Toxic, irritant to eyes, respiratory system and skin. Wear suitable protective gear. ▲ CRITICAL Prepare fresh.
- 6-Benzylaminopurine (BAP, $C_{12}H_{11}N_5$; Sigma, cat. no. B-3408) (see REAGENT SETUP) ! CAUTION Toxic, irritant to eyes, respiratory system and skin. Wear suitable protective gear. ▲ CRITICAL Prepare fresh.
- Gibberellic acid (GA_3, $C_{19}H_{22}O_6$; Sigma, cat. no. 48870) (see REAGENT SETUP) ! CAUTION Toxic, irritant to eyes, respiratory system and skin. Wear suitable protective gear.
- Indole-3-butyric acid (IBA, $C_{12}H_{13}NO_2$; Sigma, cat. no. I-5386) (see REAGENT SETUP) ! CAUTION Toxic if swallowed. Irritant to eyes, respiratory system and skin. Wear suitable protective gear.
- Indole-3-acetic acid (IAA, $C_{10}H_9NO_2$; ICN Biomedicals, cat. no. 102037) (see REAGENT SETUP) ! CAUTION Toxic if swallow. Irritant to eyes, respiratory system and skin. Wear suitable protective gear. ▲ CRITICAL Prepare fresh.
- Carbenicillin disodium salt ($C_{17}H_{16}N_2O_6SNa_2$; MP Biomedicals, cat. no. 195092)
- Kanamycin monosulfate ($C_{18}H_{36}N_4O_{11} \cdot H_2SO_4$; Sigma, cat. no. K-1377); prepare 50 mg ml^{-1} stock in water ! CAUTION Toxic, wear suitable protective clothes and gloves, face and eye protection. Do not breathe dust.
- Rifamycin (Sigma, cat. no. R-3501); prepare 100 mg ml^{-1} stock in DMSO ! CAUTION Toxic, wear suitable protective clothes and gloves, face and eye protection. Do not breathe dust.
- Adenine hemisulfate salt ($C_5H_5N_5$ $1/2H_2SO_4$, cat. no. A-9126)
- Ethylenediaminetetraacetic acid ferric sodium salt iron (III) sodium salt (ferric-EDTA; $C_{10}H_{12}N_2NaFeO_8$; Sigma, cat. no. E-6760)
- Polyvinylpyrrolidone (PVP 40,000; Sigma, cat. no. PVP40) ! CAUTION Do not breathe dust, avoid contact with skin or eyes. Wear suitable protective gear.
- LB liquid medium (see REAGENT SETUP)
- LB plates (see REAGENT SETUP)
- Murashige and Skoog (MS) medium (ref. 35; see REAGENT SETUP)
- Seed germination medium (see REAGENT SETUP)
- Infection and *Agrobacterium* suspension medium (see REAGENT SETUP)
- Cocultivation medium (see REAGENT SETUP)
- Callus induction medium (see REAGENT SETUP)
- Shoot initiation medium (see REAGENT SETUP)
- Shoot outgrowth medium (see REAGENT SETUP)
- Transformant selection medium (see REAGENT SETUP)
- Root initiation medium (see REAGENT SETUP)

EQUIPMENT

- Sterile 50 ml plastic tubes (Greiner Bio-One, cat. no. 227261)
- Sterile 10 ml plastic tubes (Greiner Bio-One, cat. no. 188261)
- Autoclave (Tuttnauer autoclave steam sterilizer, cat. no. 2540 EKA)
- Laminar flow hood for plant culture work (LAF Technologies, model DFM44)
- Biohazard hood for *Agrobacterium* plating and culture (LAF Technologies, model BSC1200)
- Steri 350 for sterilizing forceps and needles (Simon Keller AG, cat. no. CH-3400); flame could be used instead of specialized equipment
- Shaker (Ratek Instruments, model EOM5)
- Incubator for *Agrobacterium* plates (Thermoline, TS-400)
- Shaker incubator (temperature controlled) for *Agrobacterium* culture (Thermoline, orbital shaking incubator TLM)
- Growth room set at 25 °C with 16 h day-length (~3,500 lux at Petri-dish level); alternatively, growth chamber with similar light and temperature conditions could be used
- Sterile plastic Petri plates for microbiology (Greiner Bio-One, cat. no. 663180)
- Sterile plastic Petri plates for plant tissue culture (Greiner Bio-One, cat. no. 664160)
- Sterile, large plastic Petri plates (Greiner Bio-One, cat. no. 639160)
- Forceps, needles and scalpel (Terumo) ▲ CRITICAL Sterilization is required.
- Surgical blades (Livingstone International)
- Disposable drinking cups with lids (270 ml, sterile, gamma radiated; Dalton Packaging: cups, cat. no. 500102 and lids: cat. no. 500105; Steritech, gamma sterilization service)
- Plant growth culture vessels (6 cm × 6 cm, height 10 cm; Sarstedt Australia.) ▲ CRITICAL Sterilization required (alternative to drinking cups for shoot growth selection and root regeneration).
- Disposable sterile syringe filter, 0.2 mm (Minisart, cat. no. 16534)
- Surgical tape (3M Micropore, cat. no. 1530-1)
- Pipettes: P1000, P200, P20 (Gilson)
- Sterile pipette tips (Axygen Scientific: 1–200 µl, cat. no. T-200Y; 1–1,000 µl, cat. no. T-1000B)

REAGENT SETUP

LB medium (liquid) Dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in water to make up a total volume of 1 liter; pH 7.0; autoclave.

LB (solid) medium for *Agrobacterium* plates Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in water to make up a total volume of 1 liter; pH 7.0; autoclave. Add 50 µl of 100 mg ml^{-1} rifampicin stock and 50 µl of 50 mg ml^{-1} kanamycin stock to 50 ml LB solid medium.

LB liquid medium for *Agrobacterium* suspension Add 15 µl of 100 mg ml^{-1} rifampicin stock and 15 µl of 50 mg ml^{-1} kanamycin stock to 15 ml LB liquid medium.

MS major salts Dissolve 1,650 mg NH_4NO_3, 1,900 mg KNO_3, 370 mg $MgSO_4 \cdot 7H_2O$, 170 mg KH_2PO_4 and 43 mg ferric-EDTA in 1 liter of water.

MS minor salt stock Dissolve 620 mg boric acid, 2,230 mg $MnSO_4$, 860 mg $ZnSO_4 \cdot 7H_2O$, 25 mg $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg $CuSO_4 \cdot 5H_2O$ and 2.5 mg $CoCl_2 \cdot 6H_2O$ in 100 ml of distilled water. Store at −20 °C. ▲ CRITICAL Always thaw stock solution completely before taking an aliquot.

Vitamin stock Dissolve 100 mg nicotinic acid, 1 g thiamine hydrochloride, 100 mg pyridoxine hydrochloride and 10 g myo-inositol in 100 ml distilled water. Can be stored at −20 °C up to 3–4 months.

$CaCl_2$ stock Dissolve 15 g of $CaCl_2 \cdot 2H_2O$ in 100 ml distilled water. Can be stored at 4 °C up to 3–4 months.

KI stock Dissolve 75 mg of KI in 100 ml distilled water. Store in a dark glass bottle at 4 °C. Prepare fresh every 1–2 months.

NAA (10 mg ml^{-1}) Dissolve 10 mg NAA in 100 µl of 1 N NaOH. Add 900 µl sterile distilled water.

BAP (stock 1; 10 mg ml^{-1}) Dissolve 10 mg NAA in 100 µl of 1 N NaOH. Add 900 µl sterile distilled water.

BAP (stock 2; 2.5 mg ml^{-1}) Dissolve 10 mg NAA in 100 µl of 1 N NaOH. Add 900 µl sterile distilled water.

$AgNO_3$ (50 mg ml^{-1}) Dissolve 50 mg $AgNO_3$ in 1 ml of sterile distilled water.

GA_3 (2 mg ml^{-1}) Dissolve 2 mg GA_3 in 1 ml of 50% ethanol.

IBA (10 mg ml^{-1}) Dissolve 10 mg IBA in 100 µl of 1 N NaOH. Add 900 µl sterile distilled water.

IAA (10 mg ml^{-1}) Dissolve 10 mg IAA in 100 µl of 1 N NaOH. Add 900 µl sterile distilled water. ▲ CRITICAL Always prepare NAA, BAP, $AgNO_3$, GA_3, IAA, IBA stock fresh. ▲ CRITICAL Always prepare NAA, BAP, $AgNO_3$, GA, IAA, IBA stock fresh.

Seed germination medium Mix 0.5 strength of MS major salts (i.e., dissolve 825 mg NH_4NO_3, 950 mg KNO_3, 185 mg $MgSO_4 \cdot 7H_2O$, 85 mg of KH_2PO_4 and 21.5 mg ferric-EDTA in 1 liter of water), 0.5 ml of MS minor salt stock, 0.5 ml $CaCl_2$ stock, 0.5 ml KI stock, sucrose 10 g and phytagel 4 g $liter^{-1}$. Adjust the pH to 5.8 before addition of phytagel.

Infection and *Agrobacterium* suspension medium MS minimal organic liquid medium (1× major salts, 1 ml minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock and sucrose 20 g; pH 5.8; autoclave).

Cocultivation medium 1× MS major salts, 1 ml MS minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 100 µl $AgNO_3$, 75 µl BAP (stock 1, 10 mg ml^{-1}), 20 µl NAA and 5µl GA_3.

Callus induction medium 1× major salts, 1 ml minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 100 µl $AgNO_3$, 75 µl BAP (stock 1, 10 mg ml^{-1}), 20 µl NAA, 5 µl GA_3 and 500 mg carbenicillin.

Shoot initiation medium 1× major salts, 1 ml minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock and 20 g sucrose; pH 5.8. Add 4 g

phytagel, autoclave and add filter-sterilized 100 µl $AgNO_3$, 300 µl BAP (stock 1, 10 mg ml^{-1}), 20 µl NAA, 5 µl GA_3, 500 mg carbenicillin and 25 mg kanamycin.

Shoot outgrowth medium 1× major salts, 1 ml minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock, 20 g sucrose, 40 mg adenine hemisulfate and 500 mg PVP 40,000; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 5 µl BAP (stock 2, 0.25 mg ml^{-1}), 500 mg carbenicillin and 25 mg kanamycin.

Transformant selection medium 1× major salts, 1 ml minor salts, 2.9 ml $CaCl_2$ stock, 1 ml KI stock, 1 ml vitamin stock, 10 g sucrose, 40 mg adenine hemisulfate and 500 mg PVP 40,000; pH 5.8. Add 4 g phytagel, autoclave and add filter-sterilized 5 µl BAP (stock 2, 0.25 mg ml^{-1}), carbenicillin 500 mg and kanamycin 50 mg.

Root initiation medium 0.5× major salts, 0.5 ml minor salts, 1.95 ml $CaCl_2$ stock, 0.5 ml KI stock and 10 g sucrose; pH 5.8. Add 4 g phytagel, autoclave and add 100 µl filter-sterilized IBA stock solution.

PROCEDURE

Surface sterilization of seeds ● TIMING 30 min

1| Place seeds in sterile 50 ml plastic tubes and add approximately 35–40 ml sodium hypochlorite.

▲ CRITICAL STEP Do not exceed approximately 4–5 ml seeds per tube. Larger amounts of seeds per tube results in incomplete surface sterilization.

▲ CRITICAL STEP Seeds should be surface sterilized completely to avoid contamination by microorganisms and fungi.

2| Close the tube tightly and place it on a shaker. Shake vigorously at room temperature (RT, 22–25 °C) for 20 min.

3| Discard sodium hypochlorite and rinse the seeds five times by shaking for approximately 30 s in 40–45 ml sterile distilled water.

▲ CRITICAL STEP This and all subsequent steps should be performed in a laminar flow cabinet.

4| Decant the seeds into a sterile Petri plate for ease of handling the seeds in subsequent steps.

Seed germination ● TIMING 4 d

5| Transfer the surface-sterilized seeds with forceps onto Petri plates containing seed germination medium, allowing 10–12 seeds per plate.

▲ CRITICAL STEP Do not place too many seeds on a plate, as this makes picking up germinated seeds difficult. If using a large number of seeds, large Petri plates containing germination medium can be used. Up to 25 seeds could be placed in a large Petri dish.

6| Germinate the seeds in the dark at RT (22–25 °C) for 4 d.

▲ CRITICAL STEP Seeds germinated in dark or dim light (~ 650 lux) are more likely to be successfully transformed by *Agrobacterium* than light-germinated seedling explants.

? TROUBLESHOOTING

Agrobacterium preparation ● TIMING 4 d

7| Start *Agrobacterium* preparation on the same day as seed germination**.** Streak LBA4404 harboring binary vector on 2–3 LB plates containing 100 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin. Incubate the plates for 2 d at 28 °C.

! CAUTION Handle *Agrobacterium* in biohazard cabinet.

? TROUBLESHOOTING

8| Inoculate a single colony of *Agrobacterium* from one of the plates into 10 ml LB liquid medium containing 100 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin. Culture at 28 °C with shaking (250 r.p.m) for 36 h.

▲ CRITICAL STEP Shaking, temperature and duration (at least 36 h) are important for good bacterial growth.

9| Measure the OD at 650 nm using a spectrophotometer.

10| Spin the *Agrobacterium* culture down (4,300–5,500*g* is fine) for 10 min at RT. Remove the supernatant and rinse the pellet in liquid MS minimal organic medium (without antibiotics). If *Agrobacterium* pellet is disturbed while rinsing then spin (4,300*g* for 10 min at RT) again.

11| Resuspend the pellet in liquid MS minimal organic medium (without antibiotics) and adjust the OD (650 nm) to 0.05 with MS minimal organic medium.

Agrobacterium infection and cocultivation ● TIMING 2 d

12| Pull the seedlings (from Step 6) out of the germination medium and place in an empty Petri plate.

13| Cut the cotyledons, including approximately 2 mm stalk (petiole), from the seedling using a scalpel blade (**Fig. 1**).

▲ CRITICAL STEP Avoid including meristem when excising explants, as meristem does not get infected with *Agrobacterium* or transform easily and thus generates false-positive plants. Explants should be infected as soon as possible. If using a large number of

explants, perform the infection step in batches. Do not let explants dry out, as susceptibility to *Agrobacterium* infection decreases as the wound turns brown.

14| Dip the cut ends of cotyledon explants (i.e., petiole only) in *Agrobacterium* (harboring binary vector) suspension (OD 0.05, from Step 11) for 30 s (**Fig. 1**).

15| Place the infected explants onto cocultivation medium using forceps. Place the cotyledon explants upright with the cut ends embedded in the medium (**Fig. 1**), allowing roughly ten explants per Petri plate. Seal the Petri plates using 3M surgical tape.
▲ CRITICAL STEP Use of 3M surgical tape is necessary, as this allows gas exchange during incubation. Avoid transferring liquid with explants while placing them on cocultivation plates, as this might lead to overgrowth of the *Agrobacterium* resulting in inhibition of transgenic shoot regeneration.

16| Incubate under dim light (~660 lux) or in the dark at 25 °C for 2 d.
▲ CRITICAL STEP Dim light (~660 lux) or dark conditions are required for this step.
? TROUBLESHOOTING

Shoot initiation ● TIMING 4–5 weeks
17| Transfer explants into callus induction medium containing 500 mg liter^{-1} carbenicillin (to select against the *Agrobacterium*) using sterile forceps. Seal Petri dishes with 3M surgical tape. Incubate under dim light at 25 °C for 1 week.
▲ CRITICAL STEP Do not use the transgene selective agent (kanamycin) at this point; the aim is to kill the *Agrobacterium* while still allowing plant cell proliferation.
? TROUBLESHOOTING

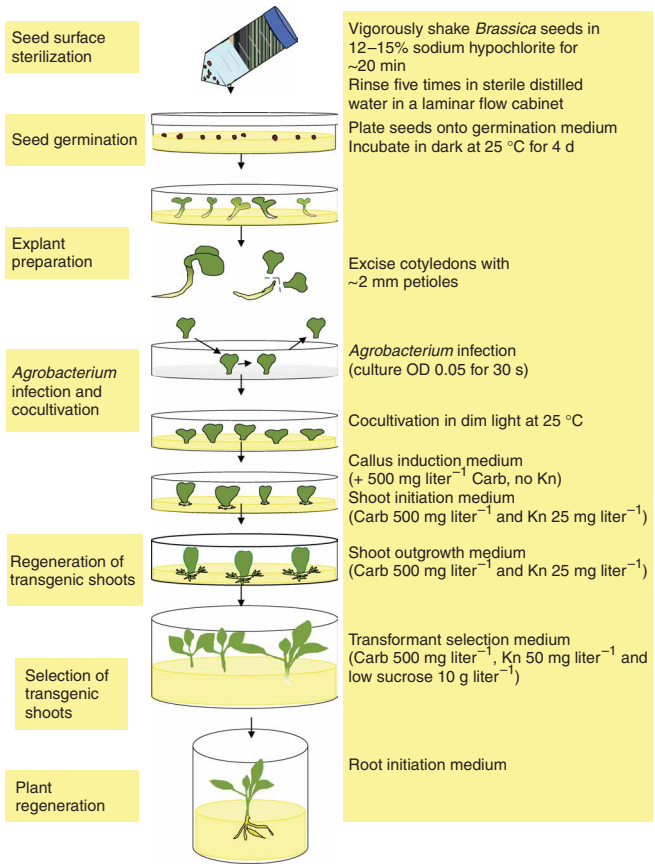

Figure 1 | Overview of the steps involved in *Agrobacterium*-mediated transformation of *Brassica*. Carb, carbenicillin; Kn, kanamycin.

18| Transfer the explants to shoot initiation medium containing 500 mg liter^{-1} carbenicillin and 25 mg liter^{-1} kanamycin using sterile forceps. Incubate in light (16 h; ~3,300 lux at Petri dish level) at 25 °C for 4 weeks.
▲ CRITICAL STEP Culturing in fresh shoot initiation medium after 2 weeks stimulates shoot production. Use freshly prepared kanamycin plates for selection. Depending upon the cultivar used, this step could take less time, that is, 2–3 weeks.
? TROUBLESHOOTING

Shoot outgrowth ● TIMING 2–4 weeks
19| Transfer the explants with shoot initials to shoot outgrowth medium containing 500 mg liter^{-1} carbenicillin and 25 mg liter^{-1} kanamycin using sterile forceps. Incubate under light (16 h) at 25 °C for 2–4 weeks.
▲ CRITICAL STEP Use 3M tape to seal the Petri plates. Harvest/remove fully grown shoots (2–4 cm long) to encourage further shoot outgrowth.
? TROUBLESHOOTING

Transformant selection ● TIMING 2–3 weeks
20| Transfer shoots, using sterile forceps, to plastic drinking cups or tissue culture vessels containing transformant selection medium with reduced sucrose (10 g liter^{-1}), high kanamycin (50 mg liter^{-1}) and 500 mg liter^{-1} carbenicillin. Ensure that the base of the shoots is well embedded in the medium. Place five to six shoots per cup (**Fig. 2a**). Incubate in dim light at 25 °C for 2–3 weeks. This step is effective in eliminating nontransgenic or false-positive shoots and effectively selects for transgenic shoots.
▲ CRITICAL STEP Do not overcrowd the cup with shoots. Ensure that the ends of shoots are placed well into the medium. Each shoot should be intact, well separated from callus cells or other shoot initials. The presence of callus cells or shoot initials could result in the formation of (potentially nontransgenic) shoots in subsequent steps. This is an effective way to eliminate escapees or false positives (**Fig. 2a**).
? TROUBLESHOOTING

Root initiation ● TIMING 2 weeks

21| Transfer green shoots to cups containing root initiation medium with 500 mg liter^{-1} carbenicillin, using sterile forceps making sure base/ends of the shoots are placed well into the medium. Incubate in light at 25 °C for 2 weeks.
▲ CRITICAL STEP Roots should start appearing after a week.
? TROUBLESHOOTING

Establishment under glasshouse conditions ● TIMING 1 week

22| Remove the plants from cups with forceps. Wash briefly with lukewarm running tap water to remove traces of phytagel.
▲ CRITICAL STEP Transfer only shoots with well-developed roots. Do not let shoots wilt during the transfer.

23| Fill pots with the potting mix and water the pots so that the soil is moist.
▲ CRITICAL STEP Use new or well-cleaned pots; previously used pots should be washed and disinfected. Potting mix should be autoclaved to kill bacteria and fungi.

24| Place the regenerated shoot into the potting mix by making a hole (depth depending upon the root length) in the soil. Compact the soil around the shoot and water slightly.

25| Place the pots containing shoots in a mist chamber for 1 week before transferring to normal glasshouse conditions (**Fig. 2b**).
? TROUBLESHOOTING

● TIMING
It takes approximately 10–14 weeks to complete the above procedure.
Steps 1–6, seed sterilization and germination: 4 d
Steps 7–11, *Agrobacterium* preparation: 4 d (this should be carried out in parallel to Steps 1–6)
Steps 12–16, infection and cocultivation with *Agrobacterium*: 2 d
Steps 17 and 18, shoot initiation: 4–5 weeks
Step 19, shoot outgrowth: 2–4 weeks
Step 20, transformant selection: 2–3 weeks
Step 21, root initiation: 2 weeks
Steps 22–25, establishment under glasshouse: 1 week

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2.**

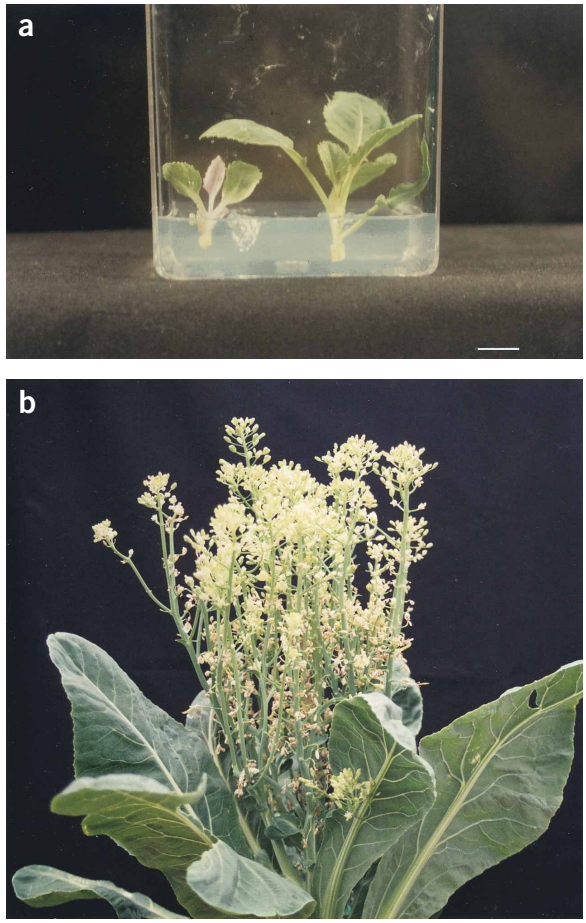

Figure 2 | Transgenic *Brassica oleracea* plants. (**a**) Transformant selection: green transgenic shoot and white leaf nontransgenic shoot. (**b**) Transgenic plant at flowering stage. Figure reprinted with permission of Springer Science and Business Media from ref. 32.

TABLE 2 | Troubleshooting table.

Steps	Problem	Possible reasons	Solution
6	Poor or variable seed germination	Seeds not of good quality or old	Use fresh seeds
		Seeds are non-viable due to suboptimal storage conditions	Store seeds in air-tight container in dry conditions at ≤4 °C
		Seed surface sterilization	Too long in sodium hypochlorite—watch the duration in sodium hypochlorite carefully. Rinse with excess amount of water at lease five times to remove traces of sodium hypochlorite

TABLE 2 | Troubleshooting table (continued).

Steps	Problem	Possible reasons	Solution
	Seeds or seedling contaminated	Inadequate seed surface sterilization	Increase duration in sodium hypochlorite to 30 min followed by rinsing five times using excess amount of sterile water
		Inadequate operation of washing and seed-plating steps	Carry out in a laminar hood. Use sterilized water and forceps
7	*Agrobacterium* fails to grow	*Agrobacterium* does not contain binary vector	Check for the presence of binary vector Introduce binary vector into *Agrobacterium*
16	Explants turning black	*Agrobacterium* concentration too high Silver nitrate lacking in the medium Use of other sealing tapes (Parafilm, Nescofilm)	Adjust final Agro suspension OD to 0.05 Check for silver nitrate in the medium Use micropore tape to seal the plates
	Bacterium growth on or around the explants	Contamination from other microorganisms	Use sterile implements and operate under laminar flow
	Agrobacterium growth on explants	Lack of carbenicillin in the medium	Add carbenicillin to the medium
17 and 18	No shoot regeneration Too many shoots regenerated	Inappropriate selective agent/medium composition Lack of selective agent	Check the type and amount of selective agent Check the amount of selective agent
19	No shoot outgrowth from shoot initials	Inappropriate shoot outgrowth medium	Check the composition of shoot outgrowth medium
20	All shoots surviving on TSM	Lack or inappropriate concentration of selective agent and sucrose	Check concentration of kanamycin and sucrose in the medium
	All shoot turning white on TSM	Too high concentration of selective agent	Check the amount of selective agent
		Regeneration of nontransformed plants	Check *Agrobacterium*-contained desired plasmid
21 and 25	Loss of plants	Roots not properly developed Desiccation or wilting during transfer	Check for well-developed roots Provide humid environment during and after the transfer to pots

TSM, transformant selection medium.

ANTICIPATED RESULTS

This protocol has been used to generate transgenic plants from commercial cultivars of different Brassicas such as canola (*B. napus*)[31] and cauliflower (*B. oleracea* var *botrytis*)[32]. The transformation efficiency from some commercial cultivars (Oscar and Taparoo) of canola was over 67% (see ref. 31), whereas that from the commonly used model Westar variety produced transgenic plants with 33% efficiency (**Table 1**). Of course, the transformation efficiency varies with the regeneration potential of the *Brassica* crop variety used, but, nevertheless, viable transgenic plants have been regenerated with reasonable efficiency from most of the varieties tested in our laboratory.

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