Sugar beet (*Beta vulgaris* L.)

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Summary

Creating transgenic plants is invaluable for the genetic analysis of sugar beet and will be increasingly important as sugar beet genomic technologies progress. A protocol for *Agrobacterium*-mediated transformation of sugar beet is described in this chapter. Our protocol is optimized for a sugar beet genotype that performs exceptionally well in tissue culture, including the steps of dedifferentiation, callus proliferation, and regeneration. Because of the infrequent occurrence of such a genotype in sugar beet populations, our protocol includes an *in vitro* propagation method for germplasm preservation. The starting materials for transgenic experiments are aseptic shoots grown from surface-sterilized seed balls. Callus is induced from leaf explants and subsequently infected with *Agrobacterium*. Plantlets are regenerated from transgenic callus and vernalized for flowering, if necessary. The efficiency of transformation was quite high; in our laboratory, the culture of only 10 leaf explants, on average, generated one transgenic plant.

Key Words: *Agrobacterium tumefaciens*; *Beta vulgaris*; binary vector; callus; genetic transformation; regeneration; sugar beet

1. Introduction

Sugar beet is one of the two major sugar crops in the world (*1*). Sugar beet breeding has been continuously conducted since the 18th century (*2*) to promote increased yields and to overcome problems arising in sugar beet cultivation. The breeding traits have been analyzed by genetic approach to investigate the mechanism how the phenotypes are expressed. Recently, several genes responsible for some breeding traits have been identified via positional cloning, including *Hs*$^{pro-1}$ (nematode resistance), *B* (annual habitat), and *X* (the restorer-of-fertility gene for cytoplasmic male sterility) (*3*-*5*). As exemplified by these reports, obtaining the nucleotide sequence that corresponds to a gene locus of interest has now become a practical objective for sugar beet researchers due to recent advances in molecular biological techniques. Moreover, the pace of sugar beet genetics is expected to accelerate with the release of the (nearly) complete nucleotide sequence of the sugar beet genome (*6*). This advance will allow gene sequences involved in processes that are currently difficult to tackle at the molecular genetics level: for example, genes associated with yield or root quality will be identified by combining quantitative trait locus analysis and fine-scale chromosome-segment-substitution-line analysis.

To investigate the function of genes, transgenic experiments play a pivotal role. Therefore, methods for making transgenic plants are very important. As for sugar beet, there

are several reported instances of generating transgenic plants (*7*). Transgenic sugar beets have been produced through *Agrobacterium*-mediated transformation, microprojectile-mediated DNA delivery, and DNA uptake by-protoplasts using polyethylene glycol or electroporation (reviewed in [*8*]). However, sugar beet researchers have observed that a transgenic method developed in one laboratory does not always become routine in another laboratory, and sometimes the method is not reproducible in another laboratory. The reasons for these problems include: the method is genotype dependent; the method requires an extremely large number of explants; and details of the protocol are not publically available (*7, 8*). Therefore, creating transgenic sugar beets is a significant obstacle for the genetic analysis of this crop.

In this chapter, we describe in detail our latest protocol for producing transgenic sugar beets, in which the number of explants required is manageable; one transgenic plant is obtained from c.a. 10 explants. However, we note that our method is also genotype dependent, which is consistent with the general observation that most sugar beets are recalcitrant against dedifferentiation and regeneration in tissue culture (*7, 9*). In fact, sugar beet lines showing efficient dedifferentiation and regeneration in response to tissue culture are limited (*7, 10*). This observation suggests that the frequency of genotypes suitable for transgenic experiments is quite low in sugar beet populations, making such germplasm invaluable. In this context, our

protocol includes a method for the *in vitro* clonal propagation of sugar beet plants.

The framework of our method was originally developed by one of us in 1999 (H. Tamagake, unpublished data). Since then, we have continuously improved the protocol that starts with obtaining aseptic shoots from which leaves are excised as primary explants. Callus is induced from the leaf explants and is subsequently infected with *A. tumefaciens*. Plantlets are regenerated from transgenic callus. We have observed very few genetic chimeras consisting of cell types both having and lacking transgenes in our transgenic sugar beets since our protocol first worked. Transmission of transgenes to the next generation via sexual reproduction has been confirmed (*5*).

2. Materials

2.1. Agrobacterium tumefaciens *strain and binary vector*

We used *A. tumefaciens* strain LBA4404 (see [*11*] for selectable marker information) and pMDC123 as the binary vector, which is equipped for the Gateway cloning system (*12*) (*see* **Note 1**). The backbone of the binary vector is derived from one of the pCambia series (http://www.cambia.org/) (*12*), and *A. tumefaciens* transformed with pMDC123 is kanamycin resistant. pMDC123 has the *Basta*^{r} (bialaphos resistance) selectable marker in its T-DNA

region.

2.2 Plant materials

Sugar beet line NK-219mm-CMS was used (*see* **Notes 2 and 3**). This line was developed at HARC/NARO (*13*) as a cytoplasmic male-sterile (CMS) line whose male sterility is conferred by a mitochondrial type designated as Owen, after a sugar beet geneticist who first described sugar beet CMS (*14*).

2.3 Stock solutions

2.3.1. Phytohormones and growth regulator

1. 6-Benzylaminopurine (BA): add 50 mg BA to ~10 mL water. Add drops of 1 M NaOH until the BA is dissolved (the amount of 1 M NaOH is within the range of 500 µL to 1 mL) and adjust the final volume to 15 mL with water. Store at 4˚C.
2. Abscisic acid (ABA): add 15 mg ABA to ~10 mL water. Add drops of 1 M NaOH until the ABA is dissolved (the amount of dropped 1 M NaOH is within the range of 500 µL to 1 mL) and adjust the final volume to 15 mL with water. Store at 4˚C.
3. 2, 3, 5-Triiodobenzoic acid (TIBA): add 15 mg TIBA to 15 mL of dimethyl sulfoxide (DMSO). Store at 4˚C.
4. Indole-3-butyric acid (IBA): add 15 mg IBA to 0.5 mL of ethanol and adjust the final

volume to 15 mL with ~90˚C water. Store at 4˚C.

2.3.2. Antibiotics and selective agents

1. Meropenem (Dainippon Sumitomo Pharma, Osaka, Japan): 50 mg/mL stock in ddH_2O. Filter-sterilize using sterile 0.2 µm, hydrophilic filters and store at -20˚C.
2. Kanamycin: 50 mg/mL stock in ddH_2O. Store at -20˚C (*see* **Note 4**).
3. Streptomycin: 50 mg/mL stock in ddH_2O. Store at -20˚C (*see* **Note 4**).
4. Rifampicin: 50 mg/mL stock in DMSO. Store at -20˚C.
5. Hygromycin B: 100 mg/ mL stock in ddH_2O. Filter-sterilize and store at -20˚C.
6. Bialaphos (Wako Pure Chemical Industries, Osaka, Japan): 5 mg/ mL stock in ddH_2O. Filter-sterilize and store at -20˚C.

2.3.3. Other solutions

1. Acetosyringone (1- [4-Hydroxy-3, 5-dimethoxyphenyl] ethanone): 100 mg/mL stock in DMSO. Store at -20˚C.
2. Sterilizing Solution containing 1% (v/v) sodium hypochloride (use commercial bleach) and ~0.5% (v/v) Tween 20.

2.4. Culture media

2.4.1. For Agrobacterium

1. LB Medium: 10 g/L Bacto Tryptone, 5 g/L Yeast Extract, and 10 g/L NaCl. When used in solid form, add 15 g/L purified agar. For growing transformed LBA4404, the final antibiotic concentrations in LB are: 50 mg/L streptomycin; 50 mg/L kanamycin; and 50 mg/L rifampicin.

2.4.2. For sugar beet

1. Modified Murashige-and-Skoog (mMS) Medium (based on [*15*]; *see* **Note 5**): 1900 mg/L KNO_3, 825 mg/L NH_4NO_3, 440 mg/L $CaCl_2 \cdot 2H_2O$, 370 mg/L $MgSO_4 \cdot 7H_2O$, 170 mg/L KH_2PO_4, 6.2 mg/L H_3BO_3, 16.9 mg/L $MnSO_4 \cdot 4H_2O$, 8.6 mg/L $ZnSO_4 \cdot 7H_2O$, 0.83 mg/L KI, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L $Na_2 \cdot$EDTA, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, 2.0 mg/L Glycine, 100 mg/L myo-inositol, 30 g/L sucrose and 250 mg/L 2-morpholinoethanesulfonic acid (MES). The pH is adjusted to 5.8 with 1M KOH. When necessary, agar (8.0 g/L) or gellan gum (2.5 g/L) are added to solidify the medium, indicated by '+gellan gum' or '+agar', respectively, in this chapter.
2. Propagation Medium: mMS supplemented with 0.10 mg/L final-concentration BA. Solidify with agar.

3. Root Induction Medium: mMS supplemented with 1.00 mg/L final-concentration IBA. Solidify with agar.
4. Callus Induction Medium: mMS supplemented with 0.25 mg/L final-concentration BA. Solidify with gellan gum, when necessary (indicated by '+gellan gum').
5. Washing Medium: mMS supplemented with: 0.25 mg/L final-concentration BA; 100 mg/L final-concentration meropenem; and 100 mg/L final-concentration hygromycin B or 5 mg/L final-concentration bialaphos, depending on the selectable marker in the T-DNA region of the binary vector used for the transgenic experiment.
6. Selection Medium: mMS supplemented with: 0.25 mg/L final-concentration BA; 50 mg/L final-concentration meropenem; 100 mg/L final-concentration hygromycin B or 5 mg/L final-concentration bialaphos, depending on the selectable marker of the binary vector. Solidify with agar. The selective agent is added after autoclaving.
7. Regeneration Medium: mMS supplemented with: 1.00 mg/L final-concentration BA; 1.00 mg/L final-concentration ABA; 1.00 mg/L final-concentration TIBA; and 100 mg/L final-concentration hygromycin B or 5 mg/L final-concentration bialaphos, depending on the selectable marker of the binary vector. Solidify with agar.

2.5. Other materials

1. Sterile plastic petri dishes: we use 90 x 15 mm and 90 x 20 mm, for *Agrobacterium* and sugar beet, respectively.
2. Vacuum oven: LHV-112 type (Espec, Osaka, Japan) (*see* **Note 6**).
3. Parafilm (4-inch).
4. New Medel film (25 mm) (Aglis, Yame, Japan), a grafting tape.
5. Glass test tubes (flat bottom) (25 x 100 mm), and cognate plastic caps with a drilled 7 to 8 mm diameter hole. The hole is sealed with Milli-Seal Membrane Seal (Merck Millipore, Billerica, MA) (*see* **Note 7**).
6. Glass tissue culture bottles (62 x 109.2 mm), and cognate plastic caps with a drilled 2- to 3-mm diameter hole that is sealed with Milli-Seal Membrane Seal (*see* **Note 7**).
7. Glass Erlenmeyer flasks (100-mL type with 28-mm diameter neck) (*see* **Note 8**) capped with two layers of aluminum foil.
8. Stainless-steel mesh (1 mm sieve-opening) (e.g. code 91-03 29-3; Sansyo, Tokyo, Japan; Fig. 1A). Sterilize by autoclaving.
9. Stainless-steel laboratory spatula (e.g. code 91-1249-4, Sansyo; Fig. 1B). Sterilize by autoclaving.
10. Sterile plastic 10 mL pipettes.

11. Glass Pasteur pipettes whose tips are cut to expose a wider bore (~□4 mm) (e.g. komagome pipette, code K-PIPET-LT2; Asahi Techno Glass, Tokyo, Japan; Fig. 1C). Sterilize by autoclaving.
12. Sterile Pipetman (P1000) and cognate tips.
13. Culture pots: unglazed pottery. Various sizes are necessary to adapt to the growing plants. Sterilize by autoclaving. Plastic pots are also good.
14. Soil: Kentakun, organic fertilizer-premixed soil (~pH 7) (Ecogreen Okoppe, Okoppe, Hokkaido, Japan) (*see* **Note 9**). Sterilize by autoclaving.

3. Methods

3.1. Growing aseptic seedlings

1. Soak seed balls in concentrated sulfuric acid for 30 min to 1 hr. Rinse in running water and manually remove the pericarps. Enclose the seed balls in a bag made of single-layer gauze and soak the bag in running water overnight.
2. Soak the gauze bag containing the seed ball in 70% v/v ethanol for 30 sec, then transfer to Sterilizing Solution for 15 min under vacuum (~760 mm Hg) (room temperature) (*see* **Note 6**).

3. In a laminar flow hood, rinse the gauze bag containing the surface-sterilized seed balls in sterile water (30 sec x 3).

4. After the final rinse, transfer the bag to a sterile petri dish and open with a sterile surgical knife (scalpel). Embed the seed balls in fresh mMS Medium+gellan gum (30 mL/plastic petri dish) (no more than 20 seed balls/petri dish). Seal the petri dishes with two layers of New Medel film (*see* **Note 10**). Incubate at 30˚C in the dark.

5. After germination of the seed balls, transplant the aseptic seedlings to fresh mMS Medium+gellan gum (15 mL/glass test tube) (1 seedling/glass test tube). Grow the aseptic seedlings at 25˚C under a 16 hr light-8 hr dark photoperiod (30 to 40 µmol/m^2/s).

3.2. Propagating seedlings in vitro *(see Note 11)*

1. For each aseptic seedling, excise the apical meristem with its surrounding tissue. Place the small explants on fresh Propagation Medium (40 mL/glass tissue culture bottle). Grow the explants at 25˚C under a 16hr light-8hr dark photoperiod. Multiple shoots should appear within two weeks (*see* **Note 12**).

2. Select single shoots from shoot bundles so as to contain a meristem at the base, and place each shoot on fresh mMS Medium+agar (40 mL/glass tissue culture bottle). Grow

the shoots at 25˚C under a 16 hr light-8 hr dark photoperiod (Fig. 2A).

3. If roots have not emerged after two weeks, prepare Root Induction Medium (40 mL/glass tissue culture bottle). Place unrooted shoots on the fresh Root Induction Medium to induce root formation. Transplant rooted shoots to fresh mMS Medium+agar (40 mL/glass tissue culture bottle).

3.3. Inducing callus and establishing suspension cells

1. Grow the aseptic seedlings or shoots in bottles until ~4 to 8 leaves have formed.
2. Cut the leaves of the aseptic seedlings or shoots into pieces of ~1.5 cm^2 (*see* **Notes 13 and 14**). Place the explants on fresh Callus Induction Medium+gellan gum (30 mL/plastic petri dish) (Fig. 2B). Grow the explants at 25˚C in the dark for 6-7 weeks.
3. Select friable calli that are not brown (Fig. 2C) and place 0.5 g fresh weight (FW) of callus into each Erlenmeyer flask containing 30 mL of liquid Callus Induction Medium. Rotate the flasks at 120 rpm, 25˚C, in the dark to start suspension cultures. Continue culturing for 10 days.

3.4. Preparing the **Agrobacterium** *culture for infection*

1. Introduce the binary vector into *A. tumefaciens* strain LBA4404 by electroporation (*16*). Inoculate a single colony of the established transformant into 5 mL of liquid LB

Medium with appropriate antibiotics. Rotate 120 rpm for 2-3 days at 25˚C in the dark. Store aliquots of the culture as 40% glycerol stocks at -80˚C.

2. Inoculate 200 µL of the glycerol stock into 5 mL of liquid LB Medium with appropriate antibiotics. Rotate 120 rpm for two to three days at 25˚C in the dark to prepare the *A. tumefaciens* suspension for inoculation.

3.5. **Agrobacterium** *infection and cocultivation*

1. From the sugar beet suspension cells, remove cells that are browned, have formed adventitious roots, or exhibit any other anomaly.
2. Using a stainless-steel sieve and a laboratory spatula, strain the suspension cells and transfer 0.5 g fresh weight of cells to the Erlenmeyer flask containing fresh liquid Callus Induction Medium. Rotate the flasks at 120 rpm at 25˚C in the dark for three to four days to prepare the sugar beet suspension for *Agrobacterium* infection (Fig. 2D).
3. Add 100 µL of the *A. tumefaciens* suspension and 30 µL of acetosyringone to the sugar beet suspension. Continue co-cultivation for three to four days (rotating 120 rpm, 25˚C, in the dark).

3.6. Washing sugar beet suspension cells

1. After co-cultivation, remove the medium with a sterile plastic pipette. Add 15 mL of

fresh Washing Medium.

2. Using a glass Pasteur pipette, gently wash the inside surface of the Erlenmeyer flask and the sugar beet suspension cells with the added Washing Medium. Remove the old Washing Medium and add 15 mL of fresh Washing Medium and repeat washing.
3. Remove the old Washing Medium, add 30 mL of fresh Washing Medium and allow the Erlenmeyer flasks to sit for 1hr without shaking in a laminar flow hood.
4. Remove the old Washing Medium and resuspend the sugar beet cells in fresh Washing Medium (15 mL). Using a glass Pasteur pipette, place 2- to 3-mL aliquots of the suspension on fresh Selection Medium with selective agent (30 mL/plastic petri dish) and swirl the plate to evenly distribute the sugar beet cells on the plate. Do not move the plate until the cells are firmly attached (for approximately 30min to 1hr). Remove excess liquid medium by P1000 pipetman. Grow the cells at 25˚C in the dark for four to six weeks (Fig. 2E).

3.7. Selection of transgenic callus and shoot regeneration

1. Find and keep colonies growing until they reach 5-7-mm diameter on the Selection Medium (Fig. 2F).
2. Select callus clumps from the colonies (Fig. 2F) and place on fresh Regeneration

Medium (30 mL/plastic petri dish) (5 clumps/plate). Grow the clumps at 25˚C under a 16hr light-8hr dark photoperiod for two weeks, until green tissue that looks like adventitious embryos, buds or leaves reaches 5 to 20 mm in size (Fig. 2G).

3. Embed the regenerated organs in fresh mMS Medium+agar (40 mL/glass tissue culture bottle) in such a way that leaf-like parts face upward (Fig. 2H). Grow at 25˚C under a 16hr light-8hr dark photoperiod.
4. When shoots are established, remove browned or extremely vitrified portions and transfer the shoots (or base of the shoots with meristems) onto fresh mMS Medium+agar in glass tissue culture bottle.
5. Transfer the shoot onto fresh mMS Medium+agar in glass tissue culture bottle every three to six weeks. Repeat two to three times (*see* **Note 15**).

3.8. Transplanting and acclimation

1. Grow the regenerated plantlet until it has two to three leaves of ~5 cm in size and multiple roots of 1 to 3 cm in length (*see* **Note 16**).
2. Open the cap of the glass tissue culture bottle (*see* **Note 17**). Grow the plantlet at 25˚C under a 16hr light-8hr dark photoperiod for three to four days.
3. Remove medium from the roots of regenerated plantlets by gently washing with water.

Transplant the plantlet into the pot. Grow the plantlet at 25˚C under a 16 hr light-8 hr dark photoperiod (*see* **Note 18**).

3.9. Vernalization

1. Transfer acclimated plants to a 5˚C growth chamber with a 24 hr light photoperiod (*see* **Note 19**). Continue the low temperature treatment for four months (*see* **Note 20**).
2. After the low temperature treatment, transfer the plant to a greenhouse at 25˚C with a 24 hr light photoperiod (*see* **Note 21**). As plants get larger, transplant to new pots of the appropriate size. Expect to see inflorescences after two- to three months.

4. Notes

1. We have also used pMDC32, which has *Hyg*^{r} (hygromycin resistance) as a selectable marker, and successfully obtained transgenic sugar beets (H. Kagami, T. Mikami and T. Kubo, unpublished data). Except for the selective agent, the protocol is identical to that used with pMDC123. In this chapter, the concentration of hygromycin is given for the convenience of the readers.
2. The recurrent parent (i.e. the so-called maintainer line) of NK-219mm-CMS is NK-219mm-O, a male fertile line that was developed at HARC/NARO (*13*).

Consequently, the nuclear genomes of NK-219mm-O and NK-219mm-CMS are nearly identical. We saw no apparent difference between the two lines in terms of their tissue culture responses, suggesting that any cytoplasmic effect on tissue culture, if any, is below a detectable level (H. Kagami, T. Mikami, and T. Kubo, unpublished data).

3. In our experience, genotype is the principal factor that influences the efficiency of callus formation, callus proliferation, and plant regeneration in sugar beet (H. Tamagake, unpublished data; H. Kagami, T. Mikami, and T. Kubo, unpublished data). Therefore, screening for genotypes associated with high performance in tissue-culture experiments was a prerequisite for our protocol. Very few Japanese sugar beet lines perform comparably to NK-219mm-O and NK-219mm-CMS (H. Tamagake, unpublished data). In addition, although NK-219mm-O and NK-219mm-CMS are superior lines on average, there is some intralineage variation of the tissue-culture performance in NK-219mm-O (and NK-219mm-CMS), perhaps, due to genetic heterogeneity of these lines. Because of this observation, we selected the best individual with the highest efficiency of callus formation, callus proliferation, and plant regeneration before starting transgenic experiments. We obtained one to three such individuals from ~30 plants of NK-219mm-O or NK-219mm-CMS. The selected host

plants are being maintained and propagated as genetic clones (*see* ***3.2.***).

4. We do not sterilize antibiotics for the selection of transformed *E. coli* and transformed *A. tumefaciens*.
5. This is the basal medium for our protocol. Modifications include: reduced NH_4NO_3 concentration (1/2 compared to the original MS) and the addition of MES buffer, which is absent from the original MS formulation.
6. An aspirator may also work.
7. The tubes and the bottles with medium must be capped loosely when they are sterilized by autoclaving.
8. We observed poor proliferation of callus when Erlenmeyer flasks with thinner necks were used. This may be due to inefficient aeration that may also influence other steps of our protocol.
9. We have used other brands of commercial soil with a neutral pH.
10. New Medel is used to seal plates containing sugar beet tissues, organs, calli, or plantlets in all the steps of this protocol. For *Agrobacterium*, we use Parafilm.
11. This step is useful for preserving the germplasm of interest (*see* **Note 3**).
12. Propagation Medium is replaced by mMS+agar when shoot multiplication is

unnecessary.

13. Both leaf blades and petioles are suitable for callus induction. The type of explant does not influence the efficiency of callus formation, callus proliferation, or plant regeneration, as far as we experienced.

14. If a genetic clone of the seedling is necessary, keep the remaining part with a meristem and go to step ***3.2.***

15. If rooting is not observed, see step ***3.2.***3 for a procedure to induce root formation.

16. Viable plantlets are less vitrified. Plants originating from the same colony (***3.7.***1.) are considered as the same line.

17. At the beginning, the opening should be small. Gradually enlarge the opening until the cover can be removed. This is a crucial step for acclimation.

18. Shoots start to grow following the completion of the acclimation period. Because the plantlets are fairly well acclimated by this time, we do not cover (e.g. transparent plastic hood) the plantlets. However, in the undesirable conditions such as low humidity or continuous wind flow, plantlets should be covered. Lightly moist soil is sufficient as too much water is bad for root elongation.

19. We use light emitting diodes (LED) as a light source because they generate very little

heat.

20. The length of the low-temperature-treatment period may depend on the plant genotype. Because NK-219mm-CMS and NK-219mm-O are known to be highly resistant to bolting (i.e. requiring a long period of low temperature for flowering), a four-month low-temperature treatment was necessary. A shorter period may be sufficient for plants that are less resistant to bolting.

21. Our greenhouse takes sunlight in the day-time, which is beneficial to the growth of sugar beet (H. Kagami, T. Mikami, and T. Kubo, unpublished data). However, perhaps because sugar beet is a long-day crop domesticated in the high latitude area (i.e. Europe), natural day-length is occasionally insufficient to induce flowering in Japan. Therefore, we use supplemental light in our greenhouse to extend the day-length. The type of light used at night is important. We use incandescent light (~10 μmol/m^2/s) and suggest that fluorescent lights should not be used as the sole light source. Constant illumination by fluorescent lights failed to induce flowering of sugar beet (R. Ohgami, T. Mikami, and T. Kubo, unpublished data). On the other hand, we saw no inhibitory effect of fluorescent light on the onset of flowering (Y. Ishibashi, T. Mikami, and T. Kubo, unpublished data). Taken together, it is likely that fluorescent lights lack the

specific wavelength of light that can induce sugar beet flowering.

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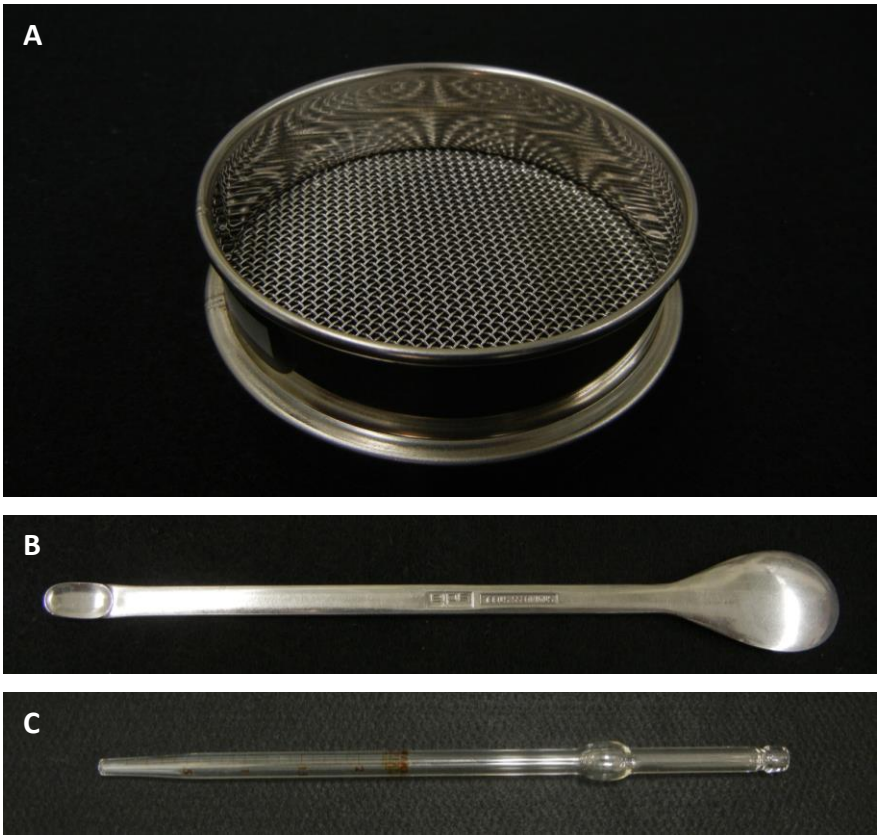
Figure legends

Figure 1 Photographs of utensils used in our laboratory. **(A)** Stainless-steel mesh (80-mm diameter). **(B)** Stainless-steel laboratory spatula (180 mm in length). The smaller spoon is used for straining the suspension callus. **(C)** Glass Pasteur pipette (245 mm in length). The rubber bulb is not shown.

Figure 2 Photographs of selected stages in our protocol for generating transgenic sugar beets. **(A)** Propagated shoots in a tissue culture bottle. **(B)** Excised leaf blades and petioles on Callus Induction Medium. **(C)** Callus proliferation from explants. The arrow indicates white and friable callus suitable for subsequent steps. **(D)** Sugar beet suspension cultures ready for *Agrobacterium* infection. **(E)** Distribution of *Agrobacterium*-infected cells on a Selection Medium plate. **(F)** Growth of resistant callus on a Selection Medium plate (indicated by the arrow). **(G)** Green tissue that is about to differentiate into a leaf-like organ. Such tissue forms a shoot. The scale bar is 2 mm. **(H)** Regenerated organ is embedded into solid mMS Medium. Leaf-like structures face upward. The scale bar is 10 mm.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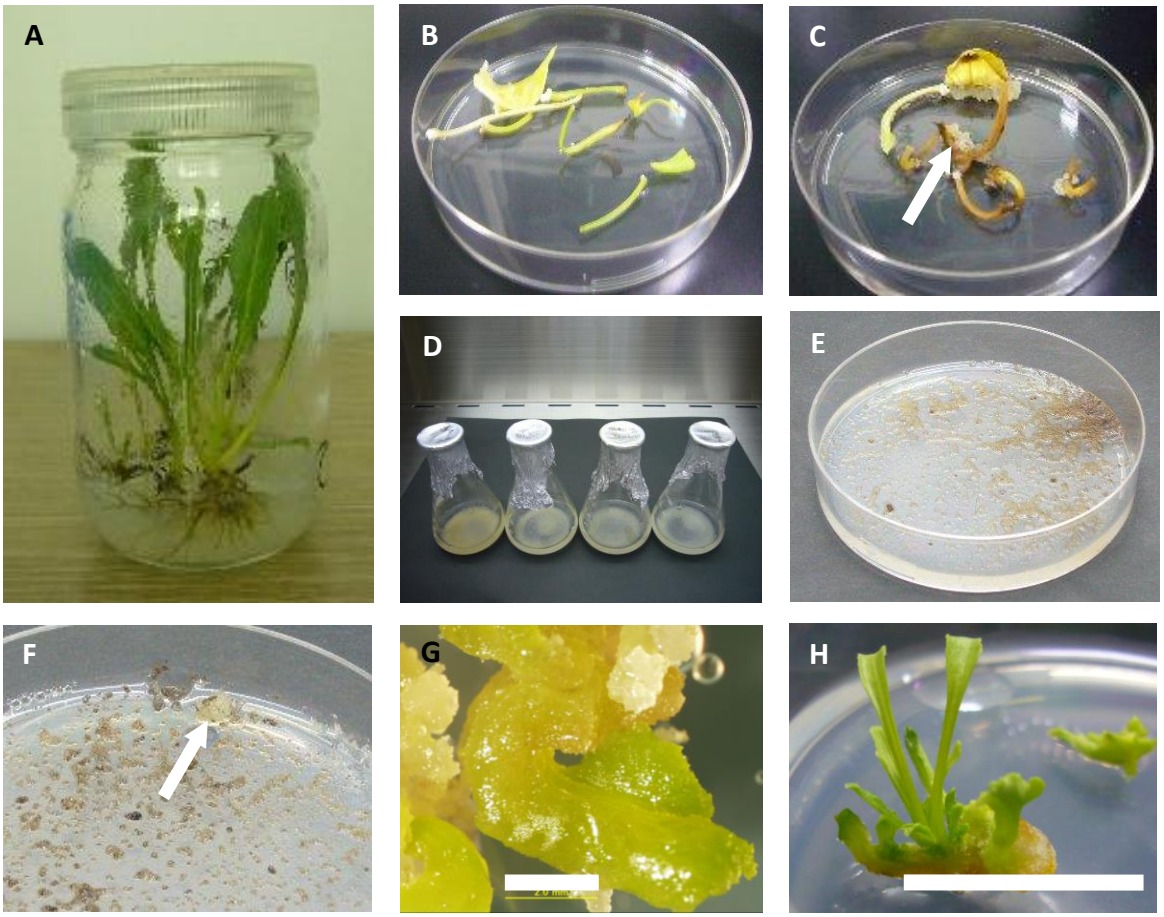
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