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Preparation of Knockdown Transformants of Unicellular Charophycean Alga, *Closterium peracerosum-strigosum-littorale* Complex

Jun Abe[1], Naoko Hirano[2], Ayumi Komiya[2], Naho Kanda[2], Anri Fujiwara[2], Sachie Hori[2], Yuki Tsuchikane[1] and Hiroyuki Sekimoto[1, 2*]

[1]Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo, Japan; [2]Division of Material and Biological Sciences, Graduate School of Science, Japan Women's University, Bunkyo-ku, Tokyo, Japan

[*]For correspondence: sekimoto@fc.jwu.ac.jp

[Abstract] To prepare the knockdown transformants of the *Closterium peracerosum-strigosum-littorale* (*C. psl.*) complex, particle bombardment was applied with a newly constructed vector (pSA0104) with an endogenous constitutive promoter fused to a DNA fragment corresponding to an antisense strand of a target gene. Using a hygromycin resistance gene (*aph7"*), hygromycin-resistant colonies were selected. After the second screening, integration of the vector into the genome was checked by PCR and the knockdown effect was evaluated by Western blotting using a specific antibody against the target protein.

Materials and Reagents

1. 0.6 µm gold microcarriers (Bio-Rad Laboratories, catalog number: 165-2262) or 0.25 µm gold nanoparticle (BBI Solutions, catalog number: EMGC250)
2. Cell culture dishes, 90 mm x 20 mm polystyrene (AGC TECHNO GLASS CO., catalog number: SH90-20)
3. 50 ml culture tube (Thomas Scientific, Labcon, catalog number: 3181-345-008)
4. 1.5 ml microtube (BMbio, catalog number: BM-15)
5. Parafilm (VWR International, Bemis, catalog number: PM996)
6. Micropore surgical tape (3M, catalog number: 1530-0)
7. Qualitative filter paper No. 2 (Toyo Roshi Kaisha, Advantec, catalog number: 00021110)
8. Test tube for incubation of transformant (AGC TECHNO GLASS CO., catalog number: TST-SCR16-150)
9. pSA0104 Vector
10. Species: Heterothallic *C. psl.* complex strains [NIES-67 (mt^+) and NIES-68 (mt^-) (the National Institute for Environmental Studies)]
11. KOD-plus NEO DNA polymerase (TOYOBO CO., catalog number: KOD-401)

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12. KOD-FX DNA polymerase (TOYOBO CO., catalog number: KFX-101)
13. GENEART seamless cloning and assembly kit (Thermo Fisher Scientific, Invitrogen™, catalog number: A13288)
14. High Pure Plasmid Isolation kit (Roche Diagnostics, catalog number: 11754785001)
15. Spermidine (Wako Pure Chemical Industries, catalog number: 191-13831)
16. Agar powder (Nacalai tesque, catalog number: 01028-85)
17. Ethanol absolute (Wako Pure Chemical Industries, catalog number: 057-00451)
18. Glycerol (Wako Pure Chemical Industries, catalog number: 075-00616)
19. Quant-iT dsDNA Assay Kit, broad range (Thermo Fisher Scientific, Invitrogen™, catalog number: Q-33130)
20. Hygromycin B (Wako Pure Chemical Industries, catalog number: 085-06153)
21. QuickExtract Plant DNA Extraction Solution (Epicentre, catalog number: QEP80705)
22. Calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$] (Wako Pure Chemical Industries, catalog number: 039-00735)
23. Potassium nitrate (KNO_3) (Wako Pure Chemical Industries, catalog number: 160-04035)
24. Disodium β-glycerophosphate pentahydrate (Sigma-Aldrich, catalog number: 50020-1000 G)
25. Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) (Nacalai tesque, catalog number: 21003-75)
26. Vitamin B_{12} (Wako Pure Chemical Industries, catalog number: 226-00343)
27. Biotin (Wako Pure Chemical Industries, catalog number: 023-08711)
28. Thiamine HCl (Wako Pure Chemical Industries, catalog number: 201-00852)
29. 2-amino-2-hydroxymethyl-1, 3-propanediol (Wako Pure Chemical Industries, catalog number: 011-16381)
30. Hydrochloric acid (HCl) (Nacalai tesque, catalog number: 18321-05)
31. $Na_2EDTA{\cdot}2H_2O$ (Wako Pure Chemical Industries, catalog number: 345-01865)
32. Iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) (Wako Pure Chemical Industries, catalog number: 091-00872)
33. Manganese(II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$) (Nacalai tesque, catalog number: 21211-45)
34. Zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$) (Nacalai tesque, catalog number: 37011-62)
35. $CoCl_2{\cdot}6H_2O$ (Wako Pure Chemical Industries, catalog number: 003-00368)
36. $Na_2MoO_4{\cdot}2H_2O$ (Wako Pure Chemical Industries, catalog number: 019-00247)
37. Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) (Wako Pure Chemical Industries, catalog number: 039-00431)
38. C medium (see Recipes)

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39. PIV metals (see Recipes)
40. Conditioned C medium (see Recipes)
41. MI medium (see Recipes)

Equipment

1. 300 ml Erlenmeyer flask (AGC TECHNO GLASS CO., catalog number: 4980FK300)
2. Growth chamber (Nippon Medical & Chemical Instruments, model: KCLP-1400II CT), being discontinued
3. Handmade hemocytometer (1 x 1 mm, grid length x grid width) (not commercially available)
4. Thermal cycler (Thermo Fisher Scientific, Applied Biosystems™, model: veriti200)
5. Centrifuge (Hitachi Ltd., model: CF16RX)
6. Swing rotor (Hitachi Ltd., model: T5SS31)
7. Angle rotor (Hitachi Ltd., model: T15AP31)
8. Centrifuge (KUBOTA Corporation, model: 1920), being discontinued
9. Angle rotor (KUBOTA Corporation, model: RA-48J), being discontinued
10. Cute mixer (EYELA, model: CM-1000)
11. Qubit fluorometer (Thermo Fisher Scientific, Invitrogen™, catalog number: Q32857), being discontinued
12. Ultrasonic cleaner (Sigma-Aldrich, Branson®, model: 3510J-DTH)
13. Gene transfer system (Tanaka co., model: IDERA GIE-III)
14. Fluorescence stereomicroscope LEICA MZ16 F (Leica Microsystems)
15. Micro Pick and Place System (Nepa Gene Co., catalog number: MPP-300)
16. Light microscope (Olympus, model: CK-40), being discontinued

Procedure

A. Preparation of cells for transformation

1. Culture vegetative cells in 150 ml C medium (http://www.nies.go.jp/biology/mcc/home.htm) at 23 °C under a 16 h light (28 µmol/m^2/s) and 8 h dark cycle in 300 ml Erlenmeyer flasks.
2. Collect the C. psl. complex cells from the mid-logarithmic to early stationary phase (9-11 days of culture) in a 50 ml culture tube and concentrate by centrifugation (1,100 *x g* for 2 min at 23 °C) using a swing-type rotor (T5SS31). For the slowdown, deceleration should be set to the slowest speed.
3. Re-suspend the cells in a small aliquot of fresh C medium and count the cells under a light microscope (CK40) using a handmade hemocytometer. Adjust to a density of 5 x

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10^6 cells ml^{-1} by adding fresh C medium.

4. Spread the cell suspension (1 x 10^6 cells) using a glass bacterial spreader onto a 90-mm plate containing C medium with 1.5% agar (w/v), prepared one day previously and stored at room temperature.
5. Culture the cells at 23 °C for 2 days under continuous light at 10 µmol m^{-2} s^{-1} (Figure 1).

Figure 1. Photograph of gold particles sedimented by centrifugation

B. Preparation of constructs for transformation of the *C. psl.* complex (approximately 4-5 days)

1. To avoid off-target silencing, the DNA region used for the antisense expression should be carefully checked, using the BLASTN program, against unpublished RNAseq and a genome database of the *C. psl.* complex.
2. Amplify the required region of target DNA by PCR with KOD-plus-NEO DNA polymerase from plasmid clones (2 min at 94 °C, followed by 30 cycles of 10 sec at 98 °C and 1 min/kbp at 68 °C). In the case of *CpRLK1* gene, 2,180 bp fragment, encoding the extracellular domain of CpRLK1 protein, was amplified. The primer DNAs should include extra sequences corresponding to the vector sequences (ex. 5'-ccagcatgactagtctcgagTTCGGGCTGTTGCTTCGGCGTCA-3' and 5'-gcttcatcaaattactcgagTGGGTGCCGCCGTAGGTTAATAT-3'), which are required for cloning into the pSA0104 vector (Figure 2, Hirano *et al.*, 2015) using the GENEART kit. The enzyme mix provided with the kit recognizes and assembles the vector and PCR fragments sharing terminal end-homology. The pSA0104 vector contains a promoter region of *CpHSP70* (*5'CpHSP70*) for expression of the target gene and a hygromycin resistance gene (*aph7"*, Berthold *et al.*, 2002) for selection (Figure 2).

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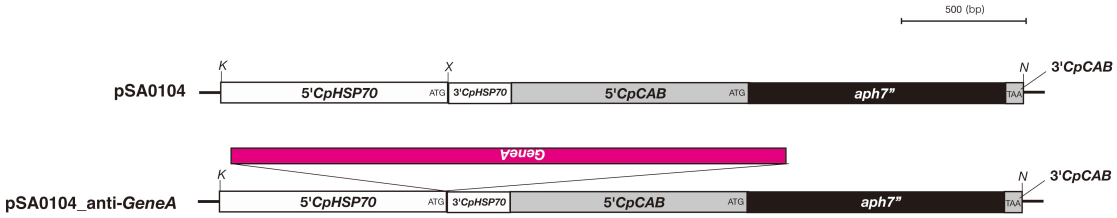
A



B

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      2080      2090      2100      2110      2120      2130      2140      2150      2160
ACGGTCTGAAATGATCTGTTAGGGTATTGaGCAATAAACGTACGCACGAGACACACCCTAAGTTCCCAAACCACTGTCGTTCAGCTAAAT

      2170      2180      2190      2200      2210      2220      2230      2240      2250
GTGTGCAACCCGTGTGTCACCATGTCTTGTGTAAATTTAATCAATCTATATttTTTcTTgaAAACGCTTGATGCtTTCATAGTtTTTACC

      2260      2270      2280      2290      2300      2310      2320      2330      2340
AACCCaAATTTGTAGCACCAAGAaGCTAATCTACAaGCCCAGTTCTAAAGAcCACaGTGTTTGATATGGGCTGaAATtCCcGGTATGTAA

      2350      2360      2370      2380      2390      2400      2410      2420      2430
CCAGTTAcCAAAGGCGTATATTCCATAGCAGTGTTGCTCAACAGCAGCACAAATGTCGTGACGTgGCaCGGGCATACAAGCTAGAATGGG

      2440      2450      2460      2470      2480      2490      2500      2510      2520
AATAAAGTGCGGCgCAAAAACCGgAAGACCATcACTCACCCAtGTACCtCTCGGTCCCTGTTCCCGCGAAGTtCcGcgatGCTCTGGTGT

      2530      2540      2550      2560      2570      2580      2590      2600      2610
CGGCCACGTCACTGAAATTCCTTGTGCTGTGAAATTCCCGTATCATCTGATTGGCGATCCCTGATTTTGAAATCTTCAGCTGAAGTTTTA

      2620      2630      2640      2650      2660      2670      2680      2690      2700
AACCTGTCTTCCGTTGCCACGTCGACAAACCCTCTCTAAGTTCCAACTCCTTGGGCACTCACGTATGCCCTCTCCCCCACAGCTTTTGCT

      2710      2720      2730      2740      2750      2760      2770      2780      2790
ATTTAGTCACGACTTTCCCCACTTGATAATTGTTAACAGGATTTCTGTGGTGCCAATTGTCAAGTCTTCCACCTCCGATTTCCTGTTCTC

      2800      2810      2820      2830      2840      2850      2860      2870      2880
CGTCACCATGggatccACACAAGAATCCCTGTTACTTCTCGACCGTATTGATTCGGATGATTCCTACGCGAGCCTGCGGAACGACCAGGA
     /
    NcoI
      2890      2900      2910      2920      2930      2940      2950      2960      2970
ATTCTGGGAGCCGCTGGCCCGCCGAGCCCTGGAGGAGCTCGGGCTGCCGGTGCCGCCGGTGCTGCGGGTGCCCGGCGAGAGCACCAACCC

      2980      2990      3000      3010      3020      3030      3040      3050      3060
CGTACTGGTCGGCGAGCCCGGCCCGGTGATCAAGCTGTTCGGCGAGCACTGGTGCGGTCCGGAGAGCCTCGCGTCGGAGTCGGAGGCGTA

      3070      3080      3090      3100      3110      3120      3130      3140      3150
CGCGGTCCTGGCGGACGCCCCGGTGCCGGTGCCCCGCCTCCTCGGCCGCGGCGAGCTGCGGCCCGGCACCGGAGCCTGGCCGTGGCCCTA

      3160      3170      3180      3190      3200      3210      3220      3230      3240
CCTGGTGATGAGCCGGATGACCGGCACCACCTGGCGGTCCGCGATGGACGGCACGACCGACCGGAACGCGCTGCTCGCCCTGGCCCGCGA

      3250      3260      3270      3280      3290      3300      3310      3320      3330
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      3340      3350      3360      3370      3380      3390      3400      3410      3420
GCGGGAACGCCGCGCGGCGACCGTCGAGGACCACCGCGGGTGGGGCTACCTCTCGCCCCGGCTGCTGGACCGCCTGGAGGACTGGCTGCC

      3430      3440      3450      3460      3470      3480      3490      3500      3510
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      3520      3530      3540      3550      3560      3570      3580      3590      3600
CGAGGTCACCGGGATCGTCGACTTCACCGACGTCTATGCGGGAGACTCCCGCTACAGCCTGGTGCAACTGCATCTCAACGCCTTCCGGGG

      3610      3620      3630      3640      3650      3660      3670      3680      3690
CGACCGCGAGATCCTGGCCGCGCGCTGCTCGACGGGGCGCAGTGGAAGCGGACCGAGGACTTCGGCCCGCGAACTGCTCGCCTTCACCTTCCT

      3700      3710      3720      3730      3740      3750      3760      3770      3780
GCACGACTTCGAGGTGTTCGAGGAGACCCCGCTGGATCTCTCCGGCTTCACCGATCCGGAGGAACTGGCGCAGTTCCTCTGGGGGCCGCC

      3790      3800      3810      3820      3830      3840      3850      3860      3870
GGACACCGCCCCCGGCGCCactagtggatccTAAATGCGTGAATTGTGTGCATAATTATATGTAGTGCAATTTTCGCTCCGGCggccgcc
                                                                          /
                                                                        NotI
      3880      3890      3900      3910      3920      3930      3940      3950      3960
accgcggtggagctccagcttttgttccctttagtgagggttaattgcgcgcttggcgtaatcatggtcatagctgtttcctgtgtgaaa

      3970      3980      3990      4000      4010      4020      4030      4040      4050
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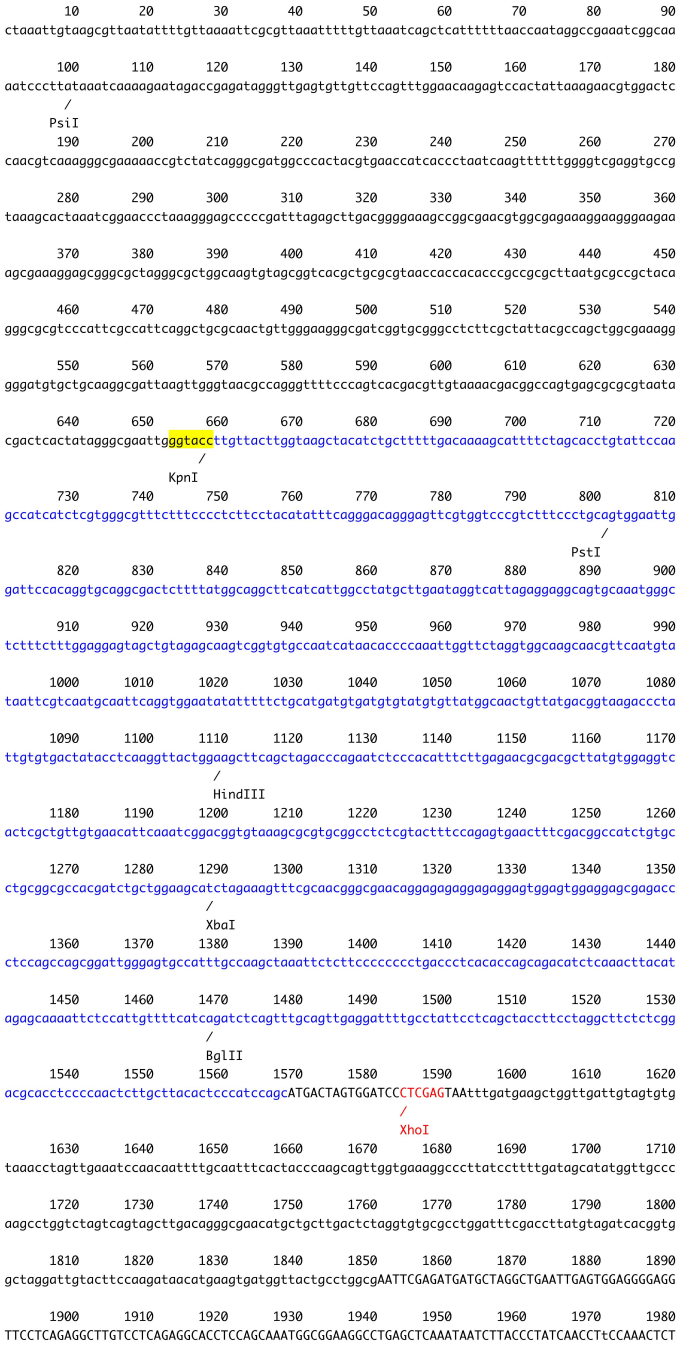

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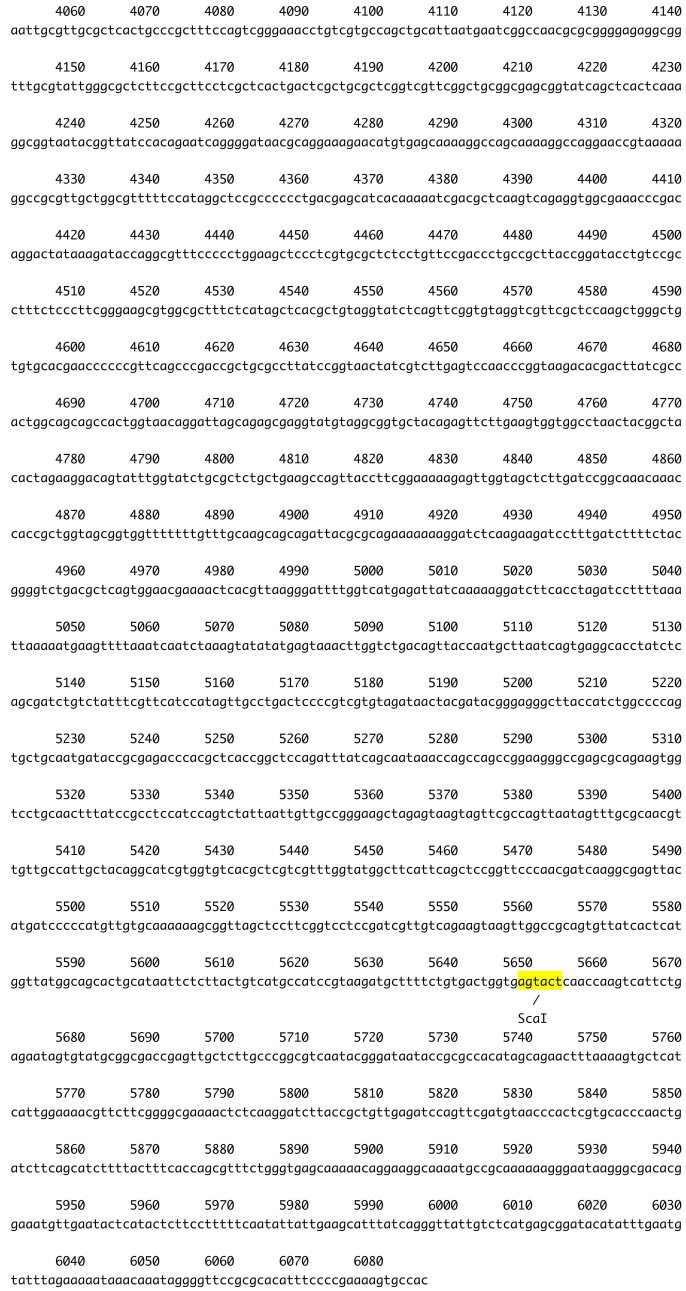

Figure 2. The plasmid vector for transformation of *C. psl.* complex. A. Constructs of pSA0104 and pSA0104_anti-*GeneA*. The promoters and untranslated regions of *CpCAB1* and *CpHSP70* genes are shown as light gray and white boxes, respectively. The *aph7"* gene (hygromycin resistance gene) is indicated by black box. Forward and reverse primers used for plasmid construction are shown as arrows under the boxes. Initiation codon (ATG) and stop codon (TAA) are indicated in their respective boxes. The plasmid backbone of the construct was pBluescript II SK+. *K*, *Kpn*I; *N*, *Not*I; *X*, *Xho*I. B. The sequence of pSA0104. The restriction sites used for cloning and for linearization have

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been highlighted. Blue character indicates the promoter and untranslated regions of *CpCAB1*. Pink character indicates *aph7"* gene.

3. Using the GENEART kit, insert the amplified DNA fragment corresponding to the antisense strand of a target gene into the *Xho*I site (immediately after *pCpHSP70*) of the pSA0104 vector.
4. After confirmation of the sequence, prepare a sufficient amount of plasmid DNA from an *E. coli* culture using a High Pure Plasmid Isolation kit, or the standard Alkaline SDS method, followed by phenol/chloroform purification (Birnboim and Doly, 1979).
5. Quantify the DNA concentration accurately using a Qubit fluorometer and the Quant-iT dsDNA Assay Kit.
6. To linearize the constructs, digest the vector arm region using suitable restriction enzymes, according to the manufacturer's instruction. In most cases, *Not*I, *Kpn*I, or *Sca*I (on pBluescript II SK+) can be used (Figure 2).

C. Microcarrier stock preparation
1. Add 60 mg of gold particles and 1 ml of 70% (w/v) ethanol solution to a 1.5 ml microtube.
   Note: 0.25 µm of gold particles would result in a higher transformation efficacy than 0.6 µm (Abe et al., 2008), however, this may be expensive. Normally, 0.6 µm gold particles are used.
2. Vortex at 2,000 rpm using a micromixer for 5 min.
3. Allow to sediment for 15 min at room temperature.
4. Further sediment by centrifugation using a T15AP31 rotor at 18,800 x *g* for 10 secfI.
5. Carefully remove the solution with a pipette.
6. Add 1 ml of sterile MilliQ water and vortex for 2 min. Sediment by centrifugation as in step B4. Discard the water. Repeat 3 times.
7. Add 1 ml of 50% sterile glycerol solution and vortex for 1 min.
8. Stored at -20 °C until needed.

D. Coating the microcarriers with the plasmid DNA
1. Thaw the microcarriers (step C8) and suspend by sonication for 5 min using an ultrasonic cleaner (set the mode "sonics").
2. Add the following reagents to the 82.5 µl (4.95 mg, for 20 shots) of microcarrier suspension in a 1.5 ml microtube. Mix by vortexing briefly after the addition of each reagent.
   a. 82.5 µl of linearized construct (for 400 ng/µl of empty pSA0104, which is 6,086 bp in length). Adjust the concentration to the equal mol value depending on the size of the construct.

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b. 250 µl of 2.5 M $CaCl_2$ (sterile filtered)
c. 100 µl of 0.1 M spermidine (sterile filtered)

3. Vortex for 2 min.
4. Incubate for 30 min at room temperature. Invert the tube gently every 10 min.
5. Sediment by centrifugation at 8,300 x *g* for 10 sec (RA-48J rotor) using a Kubota 1920 centrifuge.
6. Discard the supernatant without disturbing the microcarrier sediment.
7. Add 750 µl of 70% (v/v) ethanol and vortex for 2 min.
8. Sediment and discard the supernatant as in steps D5-6.
9. Add 750 µl of absolute ethanol and vortex for 2 min.
10. Sediment and discard the supernatant as in steps D5-6.
11. Add 250 µl of absolute ethanol. Seal the cap with parafilm to minimize ethanol evaporation until needed.

E. Particle bombardment

Note: This protocol is optimized for the IDERA GIE-III gene transfer system (Figure 3). Further optimization may be required if other particle delivery systems are used.

Figure 3. Photograph of IDERA GIE-III gene transfer system

1. Prepare an autoclaved top agar solution [0.4% (w/v) agar in C medium] and incubate the melted agar solution in a water bath at 42 °C.
2. Place a barotolerant chamber on a clean bench (Figure 4).

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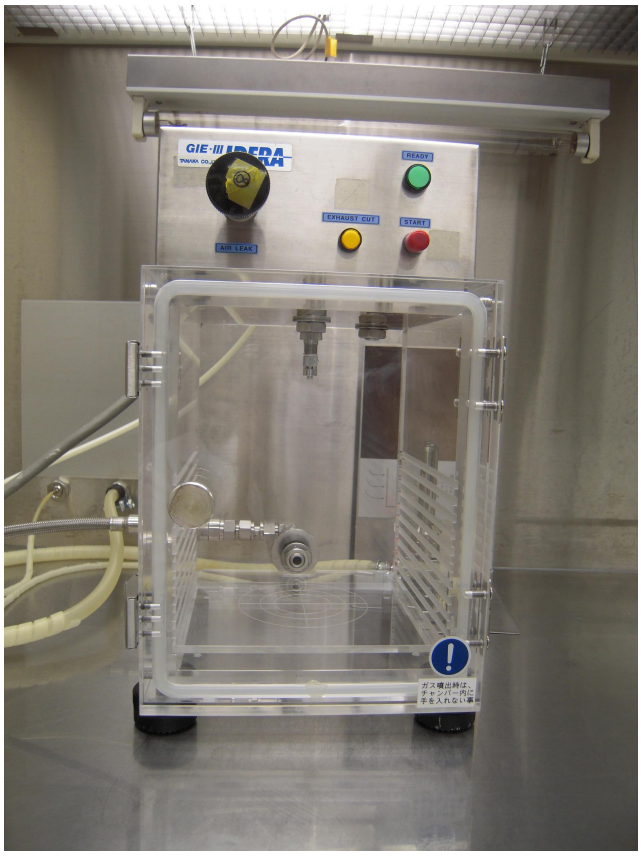

Figure 4. Photograph of barotolerant chamber on a clean bench

3. Suspend the microcarriers (step D11) completely by vortexing.
4. Add 3.3 µl of microcarrier suspension into each of three wells (Figure 5) of autoclaved DNA cartridges.

   Note: Before adding the suspension, we recommend bombarding the empty cartridges to empty the dishes. This step cleans and prevents clogging of the wells.

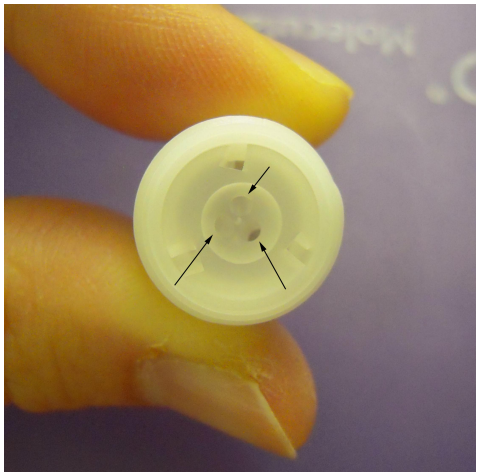

Figure 5. Photograph of DNA cartridge focusing on three wells. The arrows

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indicate the wells.

5. Place the DNA cartridge and precultured *C. psl.* complex on the culture dish (step A5, Figure 6) into the chamber (Figure 7).

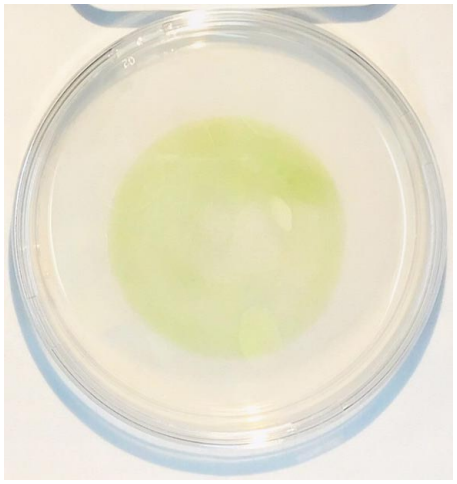

Figure 6. Photograph of *Closterium* cells cultured for 2 days on 90-mm plate containing C medium with 1.5% agar (w/v)

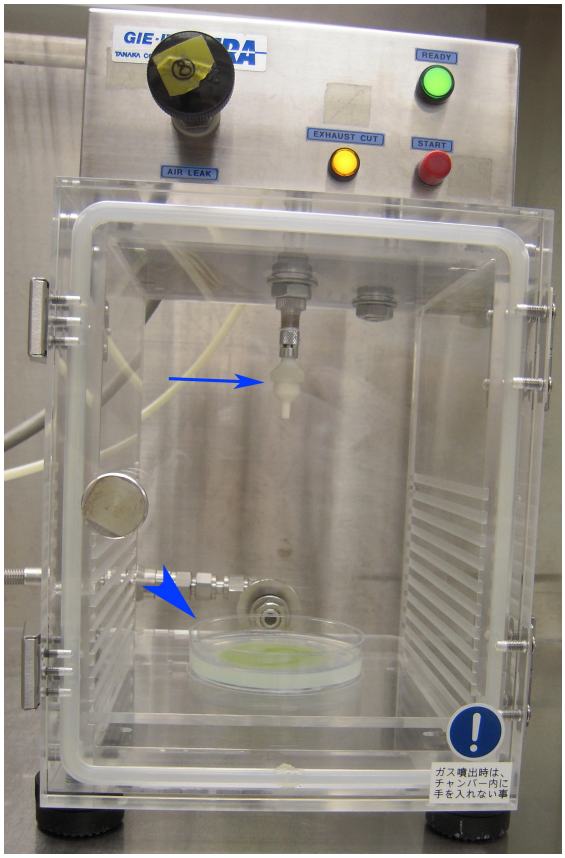

Figure 7. Photograph of barotoletant chamber just before the bombardment.

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Arrow indicates the DNA cartridge and arrowhead indicates the cultured plate.

6. Start bombardment using the following parameters (Figure 8):

Gun-to-target distance	13.0 cm
Helium exit pressure	5.5 kgf/cm
Vacuum in the barotolerant chamber	710-715 mmHg
Opening time of gas valve to allow rapid flow of helium gas	0.05 sec

Figure 8. Photograph of IDERA GIE-III focusing on the parameters

F. Selection of hygromycin resistant cells

1. Immediately after bombardment, add 5 ml of melted top agar (step E1) and allow to stand for 30 min.
2. Add 4 ml of C medium to the agar plate. Seal the plate using surgical tape and incubate for 2 d at 23 °C under continuous light (10 µmol m^{-2} s^{-1}) to enable accumulation of the aminoglycoside phosphotransferase (aph'' protein).
3. Carefully discard the C medium on the agar by decantation. Do not disturb the top surface of the agar.
4. Add 5 ml of C medium containing 50-100 µg ml^{-1} hygromycin B to the plate to select the hygromycin-resistant colonies.

   Note: The suitable concentration of hygromycin B for screening should be tested before transformation as this depends on the strains used. In our experience, a lower concentration (10 µg ml^{-1}) is suitable to select for certain C. psl. complex strains.
5. Incubate the cells for 3 to 5 weeks, with a weekly replacement of fresh. hygromycin-containing medium, under continuous light at 23 °C.
6. Pick the surviving colonies (Figure 9) and streak onto 0.8% (w/v) agar plates containing conditioned C medium (Abe *et al.*, 2011) and hygromycin. The unknown factor(s) for cell proliferation, which was secreted from growing cells into surrounded environment, would be included in the conditioned medium and would facilitate the cell division.

   Note: Use a stereomicroscope to check for contamination of the colonies with bacteria and/or fungi (Figure 10). If contamination occurs, wash the colony repeatedly (at least

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three times) by the capillary washing method (Andersen and Kawachi, 2005) or isolate a single cell using the Micro Pick and Place System.

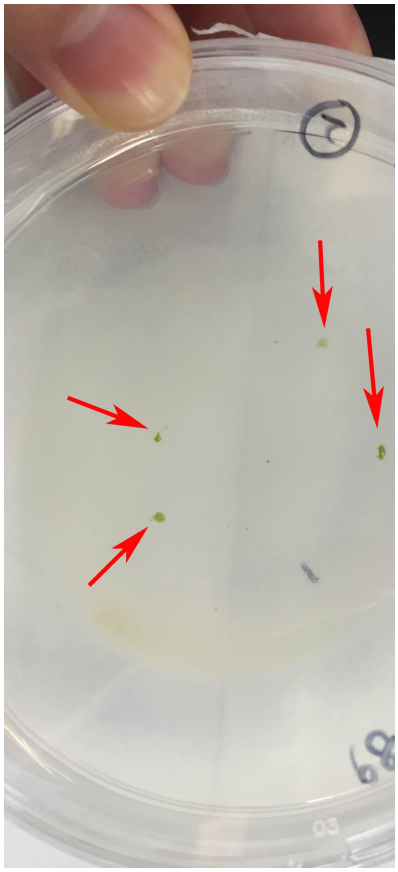

Figure 9. Photograph of surviving colonies after the selection. Arrows indicate the survived colonies, which have been incubated in C medium containing 50 µg ml^{-1} hygromycin B for 5 weeks.

Figure 10. Photograph of a contaminated colony. Scale bar = 200 µM

7. Pick the single colony on the plates (step F6) and transfer to 5 ml of conditioned

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medium in a test tube. Incubate at 23 °C under a 16 h light and 8 h dark cycle for 2-3 weeks.

Note: If contamination occurs, washing and isolation of a single cell is required as described in Note of step F6.

8. Transfer the proliferated cells from step F7 to fresh C medium and maintain under normal conditions (step A1).

G. Confirmation of successful integration of the constructs into the genome
   1. Isolate crude genomic DNA from cells using QuickExtract Plant Extraction Solution, according to the manufacturer's instruction.
   2. Amplify the integrated DNA from the genomic DNA by PCR with KOD FX DNA polymerase (2 min at 94 °C, followed by 40 cycles of 15 sec at 98 °C and 1 min/kbp at 68 °C).

H. Evaluation of knockdown effects
   1. If specific antibody against the target protein is being used, check the protein expression levels by Western blotting.
   2. Select those transformants showing a high reduction in protein expression levels for further characterization, because the knockdown effect will vary and some will not show a distinct reduction in protein expression.
   3. Evaluate the phenotype of the selected knockdown transformants. In our case, the sexual responses of transformants in MI medium was mostly evaluated.

Recipes

1. C medium

   Stock solution for 5 L of C media

10% (w/v) $Ca(NO_3)_2 \cdot 4H_2O$ solution	7.5 ml
5% (w/v) KNO_3 solution	10 ml
5% (w/v) disodium β-glycerophosphate pentahydrate solution	5 ml
4% (w/v) $MgSO_4 \cdot 7H_2O$ solution	5 ml
0.0001% (w/v) vitamin B_{12} solution	0.5 ml
0.0001% (w/v) biotin solution	0.5 ml
0.0112% (w/v) thiamine HCl solution	0.45 ml
PIV metals	15 ml
2-amino-2-hydroxymethyl-1, 3-propanediol	2.5 g

   Adjust pH to 7.5 with HCl

   a. PIV metals

   Component (for 500 ml)

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$Na_2EDTA{\cdot}2H_2O$	500 mg
$FeCl_3{\cdot}6H_2O$	98 mg
$MnCl_2{\cdot}4H_2O$	18 mg
$ZnSO_4{\cdot}7H_2O$	11 mg
$CoCl_2{\cdot}6H_2O$	2 mg
$Na_2MoO_4{\cdot}2H_2O$	1.25 mg

2. Conditioned C medium (Abe *et al.*, 2011)
   Incubate wild-type mt^+ or mt^- cells in fresh C medium for 14-20 days
   Collect the cultured medium by filtration using qualitative filter paper and sterilize the filtered medium by autoclaving (121 °C for 15 min)
3. MI medium
   Stock solution for 5 L of MI media

5% (w/v) $CaCl_2{\cdot}2H_2O$ solution	10 ml
5% (w/v) disodium β-glycerophosphate pentahydrate solution	5 ml
4% (w/v) $MgSO_4{\cdot}7H_2O$ solution	5 ml
0.0001% (w/v) vitamin B_{12} solution	0.5 ml
0.0001% (w/v) biotin solution	0.5 ml
0.0112% (w/v) thiamine HCl solution	0.45 ml
PIV metals	15 ml
2-amino-2-hydroxymethyl-1, 3-propanediol	2.5 g

   Adjust pH to 8.5 with HCl

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