Conjugation of Thalassiosira pseudonana Version 3

Jernej Turnsek

Abstract

This protocol was used to express a nourseothricin (*nou*/*nat*) resistance gene and a gene encoding a silaffin precursor TpSil3p-APEX2 fusion protein in *Thalassiosira pseudonana* (*Tp*) strain CCMP1335 using conjugation by largely following Karas et al. (2015). In addition to the protocol, I am listing some of my observations working with this diatom species. Please check the 'Before start' and 'Guidelines' sections for more information including a list of *Tp* episomes I've constructed and which are available upon request. For additional background on my overacrching experimental aim, please refer to this document.

1. E. V. Armbrust, The Genome of the Diatom Thalassiosira Pseudonana: Ecology, Evolution, and Metabolism. *Science*. **306**, 79–86 (2004).

2. B. J. Karas *et al.*, Designer diatom episomes delivered by bacterial conjugation. *Nat. Commun.* **6**, 6925 (2015).

Keeping in mind that diatom episomes contain a yeast centromere, it would be very interesting to see if conjugation could be adapted to organisms beyond diatoms.

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Guidelines

[1] 'Crashing phenomenon' in *Tp* cultures

In my experience *Tp* CCMP1335 has to be subcultured frequently - every ~2 weeks - to avoid 'crashing'. I usually dilute my cultures 1:10 in fresh L1 medium. By 'crashing' I mean sudden take over of what I believe is a naturally associated bacterium in the culture. This usually happens in the stationary phase over the course of 1 or 2 days during which brown diatom culture becomes milky due to bacteria. It could be a standard contamination issue, but I've pretty consistently observed this phenomenon even after ordering completely fresh stocks from the NCMA. I analyzed the bacterium via 16S sequencing and some simple growth experiments and confirmed it was a *Pseudomonas* species. You can read more about how I've done this here and find the paper associated with my 16S and *rpoD* (*Pseudomonas*-specific) analysis here.

Interestingly, such *Tp-Pseudomonas* interplay was already observed in the late 1970's.

[2] Episomes for *Tp* conjugation available upon request

All episomes used in my conjugation experiments including their maps are available upon request. Send an email to turnsek@fas.harvard.edu, turnsek@ucsd.edu or jturnsek@jcvi.org and we'll go from there/I can provide more information. Here is a list of available episomes:

pTpPuc3 - empty nourseothricin resistance cassette-containing episome for *T. pseudonana* conjugation

eGFP - 2 different enhanced GFP constructs

DreidelTeal - blue chromogenic protein

TpSil3p-eGFP - frustule-associated silaffin precursor protein TpSil3p tagged with eGFP

TpSil3p-APEX2-FLAG - frustule-associated silaffin precursor protein TpSil3p tagged with APEX2 and FLAG

TpSil3p-APEX2-6xHis - frustule-associated silaffin precursor protein TpSil3p tagged with APEX2 and 6xHis

NOTE:

All the genes above were cloned into pTpPuc3 Kan^R backbone via Gibson Assembly starting 36 nucleotides downstream of the HIS3 gene. They are flanked by a constitutive fcp promoter and an associated terminator. Here are their sequences in FASTA format:

>Tp_fcp_promoter

GCGCTTTTTCCGAGAACTCCCCATAAGTCAACGGCTCCAATCAAGAATGTATCCGACAACGGCGAGCATAGCAA
CACGTCCGTCTTTGGAGTAGAATCATCATGTTGTGGATGAATACACAGA

TGAATGACATTAAAAGCATGAACATGTTAGAGAGTAGGAGGTAGAGATTGATATGGTAGCATTGCGATGTTTGT
TTTTGGTCAGCATATGATGAGTGGATACCAATATGATGAAAGTTGAATCT

CGCGTTTGAGCTCAGCGGTACGTTATTGATCGAAAGTAGCCTGATCAAAATCCTTGGAGAGTACAAGAGGATCA

AAGAATCCAGTGGGGGCGATAACTCCAAGCTCGTTCTCAAAGAGGCAA

TGGAGGTAGAAACTCATCCCAGTTGAGAAGAAGTGAAGGCAGTGGCGGTGGCGAAAGCAGAGGCAACGAGGA
CAGACTTCCTGTGGGTTGATGCAACGAATATTTCCAGAAGGAGAAG

TTTAGAGAGTTGAACCGCTACCTACAATGACAAAGTATCGTATCGATTTTGATGTTGGTTGGTTATGAATTCAAA
CTGTAAGTTGGATTGTGAGAAGATCAGAAGTTGAACGAACACATCTTTC

CGATCATTCACCTCCACACTGCAACAACACGGTACTTCTTCCGCGGCAGGTCTCTGTCGCCATTCTCTTGTCCTG
TTGTTGGCTGTGAGACGAGGAAAGCAACGACAAGTTTCACAAAAGGG

AGTTCCTTTAACGAGATATGTTTTTTATAAAGAGTCCCAATAGAAAGACAAATTGATTCCTCCGTGCAAACGCGC
AAATAAACACCACGTCCATTATATCCATATCTTTCAGAGTATCCAACAAGT

GTTGAAGGACAGGTAGTTGAAGTAACGTATCTTCCCCCTCGACTGGATCCATCAACAAGGCGAACAAATCCATT
CAACCTCTCATAAATTATCTGATTTACCAAACC

>Tp_fcp_terminator

ATACTGGATTGGTGAATCAATGAGCCGTAGCACAATGGTTACATTCGGCTAGCTAAGATCCAATGGCAAGGACC
AAGTGCTGGAACTTGTTTTGCTTTAGCAGATCTTAGCGTGAGAGGTATT

TGTCCTCTGTCAGGAGTAGATAGTAGATGTTCTTTTTAAACTAAAATGCTAACTGTTCCGAATTCCTCATCGCAGC
TAATCCGTACATCAAAAGACAAAATGCTAGGTATGTGTACTACATCTCC

TGTTGCTAGATAAGACATATGATAGGAAACACACCATCAATAGTCATTGTAGCTTTACTTATACTACGCATTTGCA
CTTTCCCCTGAGTGGCAGAGGCGCATTGAGAAAATCGATCTCAACATAG

TTTATGTAGCATCCCCTAGATCCATTACTTTAAGTCTCCTTCGTCTTTGGTGTAGGCATGTTGGACACAACGAGG
TAAAACACAACACAAACAATGTGTCCAGCAAAGTAGTAGCTGCTCCAGT

TCT

Before start

[1] Preparation of donor bacteria

1.1. Any *E. coli* strain will probably work just fine, but I stuck with the one from the original publication: EPI300 (Epicentre, catalog # EC300110).

1.2. Electroporate pTA-Mob into EPI300 cells and select on LB agar plates w/ 10 or 20 µg/mL gentamycin.

NOTE:

Please refer to the following publication to learn more about the mobilization plasmid pTA-Mob: T. A. Strand, R. Lale, K. F. Degnes, M. Lando, S. Valla, A new and improved host-independent plasmid system for RK2-based conjugal transfer. *PLoS One*. **9**, e90372 (2014).

1.3. Make pTA-Mob-containing strain chemically competent.

1.4. Tranform your engineered pTpPuc3 cargo plasmid into pTA-Mob-containing cells.

NOTE:

If you already have a working *Tp* plasmid that you successfully transformed via biolistics or some other method, you can easily convert it into a conjugation-compatible vector. Refer to Karas et al. (2015) for details on how to perform this conversion.

1.5. Select double transformants on LB agar plates with 10 or 20 µg/mL gentamycin and 50 or 100 µg/mL kanamycin.

1.6. Store plates at 4°C.

[2] Preparation of L1 medium and L1 1% agar plates

I was using L1 Medium Kit and Gulf of Maine Seawater, both from NCMA, to make my liquid medium and plates.

NOTE:

I also had success with ESAW, f/2 and Daigo's IMK media, but never tested them for the full conjugation protocol. Daigo's IMK can be ordered on this website by using a serch term "IMK" (select catolog # 392-01331).

2.1 L1 medium

I would usually make batches of 1L by combining seawater, N, P and Si sources from the kit followed by autoclaving, cooling down to ~50°C and adding vitamins and antibiotics. The resulting medium

had pH ~8.5. Before starting a diatom culture I would adjust the pH with HCl to ~8.0. Starting pH anywhere between 7.8 and 8.2 should be fine. All my liquid cultures were grown in a batch mode at constant light, ~18^{o}C and moderate shaking (~100 rpm). I never buffered the medium and/or aerate with air or CO_2.

NOTE:

The resulting medium contain some precipitates due to Si and seawater-derived compunds. You can use a combination of microwaving and filter sterilization instead which should prevent precipitation.

2.2 1/2xL1 1% agar plates w/ 5% LB

I would always prepare 0.5L and make ~12 plates 40 mL each. For 0.5L combine and mix seawater, N, P and Si sources from the kit, add agar and 5% LB. Autoclave, cool down to ~50 ^{0}C and add vitamins.

NOTES:

- 5% LB is **v/v not w/v**. I had success preparing plates w/v, but **v/v is what you should use**. In other words: 1.25 g LB powder per 1 L.

- 1/2xL1: cut all N, P, Si and vitamin volumes from the kit in half.

- I had success with Bacto Agar and Sigma's high purity agar.

2.3.1/2xL1 1% agar plates w/ 100 µg/mL nourseothricin

Same as above except the LB part; add nourseothricin when cooled down to ~50 ^{0}C. My nourseothricin stocks are 200 mg/mL.

NOTE:

I observed a lot of background - false positive - colonies on plates with only 50 µg/mL nourseothricin so I switched to 100 µg/mL. You can read more about nourseothricin on the following website which I found very useful.

Protocol

Growth and preparation of E. coli donor

Step 1.

Pick bacterial colonies from your Gent+Kan plates and inoculate 10 mL LB medium. Grow overnight.

NOTES

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Start growing colonies early in the afternoon the day before conjugation to get enough biomass. ~16-20 hrs should suffice. Expect OD_{600} ~2 next morning.

Growth and preparation of E. coli donor

Step 2.

Measure OD_{600} and start a 150 mL LB subculture (recommended starting OD_{600} either 0.05 or 0.1).

Growth and preparation of E. coli donor

Step 3.

Grow at 37°C until OD_{600} reaches 0.3-0.4.

Growth and preparation of E. coli donor

Step 4.

Centrifuge at 4,000 rpm, 10°C, for 10 min.

DURATION

00:10:00

Growth and preparation of E. coli donor

Step 5.

Decant supernatant and resuspend in 800 µL SOC.

Growth and preparation of Thalassiosira pseudonana cells

Step 6.

Tp was cultured in L1 medium as described in the 'Before start' section.

NOTES

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- I've never tested if cell density before spinning cells down matters with respect to final conjugation outcome same as the Alverson Lab did for P. tricornutum. They observe ~8 x 10^6 cells/mL to be a sweet spot. In my hands spinning cells down at ~4-8 x 10^6 cells/mL seemed to work fine. According to my information harvesting T. pseudonana at ~0.8 x 10^5 cell/mL works best for biolistic experiments.

- I counted cells with BioRad's TC20 automatic cell counter.

Growth and preparation of T. pseudonana cells

Step 7.

Spin down 2 x 10^8 cells at 4000 rpm, 10°C, for 10 min.

DURATION
00:10:00

Growth and preparation of T. pseudonana cells

Step 8.

Decant supernatant and resuspend pellet in 1 mL L1 medium.

NOTES
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I usually saved some supernatant and used it for resuspension and later on for scraping cells before selection (see Steps 14 and 15).

Conjugation

Step 9.

Mix 200 µL *T. pseudonana* cells and 200 µL *E. coli* cells in a 1.5 mL tube.

Conjugation

Step 10.

Pipette up and down a few times.

Conjugation

Step 11.

Plate on 1/2xL1 1% agar plates w/ 5% LB.

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Make sure the plates are dry. Leave them open at 37 ^{0}C for an hour or so before plating diatom-bacteria co-cultures.

Conjugation

Step 12.

Incubate in dark at 30°C for 90 minutes.

DURATION
01:30:00

Conjugation

Step 13.

Move plates to your standard *Tp* growth conditions - in my case 18°C and constant light - for 4 hours.

DURATION
04:00:00

Conjugation

Step 14.

Add 1 mL L1 medium and scrape with a cell scraper or L spreader.

Selection

Step 15.

Expect to recover 500 µL co-culture suspension after scraping.

Selection

Step 16.

Plate 200 µL of the resulting suspension on pre-dried 1/2xL1 1% agar plates w/ 100 µg/mL nourseothricin.

Selection

Step 17.

Leave at 18°C and constant light until colonies appear - 2 weeks.

Screening

Step 18.

Here are a few ways to confirm the presence and expression of your heterologous gene in resulting colonies:

1. Growth under selection pressure.

Make sure colonies are able to grow in liquid L1 medium with 100 µg/mL nourseothricin (Nou100). Pick colonies with a small tip or better a toothpick and inoculate 0.5 mL L1 medium. Once you observe growth subculture in larger volume.

2. PCR

- Use 1 µL of diatom culture as a template to amplify your expression cassette.

- Confirm the absence of donor DNA by amplifying *E. coli*-specific genes.

NOTE:

I used primers to amplify the *corC* gene. Make sure you always run *E. coli* EPI300 posititve control.

- Confirm the absence of live donor cells by plating some diatom culture on LB plates w/o antibiotics.

NOTE:

Any remaining donors cells and donor DNA are gone after a few liquid subcultures. You can also patch colonies on fresh 1/2xL1 Nou100 plates.

3. RT-PCR

- Purify total RNA from Nou100 diatom culture, convert it to cDNA and use it to run a PCR with heterologous gene-specific primers.

- An example of my result with controls can be found here.

4. Episome recovery

- Perform a diatom miniprep as described by Karas et al. (2015).

- Transform *E. coli* with diatom-derived DNA.

- Select on LB agar plates with 50 µg/mL kanamycin.

- Miniprep, digest and analyze on a gel.

NOTE:

You will learn more about the state of your episome from this analysis.

5. Western blot

6. Protein-specific assays

- enzymatic assay, microscopy etc.

I've had success with 1, 2 and 3; tried 4, 5 and 6 without success.