General Information:

Protocol from: Pierre-François Perroud

Host laboratory: Stuart McDaniel laboratory, University of Florida

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Protocol title: *Ceratodon purpureus* transformation

Protocol use: This technique aims to establish transgenic lines of the moss *C. purpureus*. It can be used to remove or modify a specific locus (e.g. gene deletion or gene tagging) or to introduce gene expression of any sequence of interest. The goal of the present project is to express fluorescent proteins from loci situated on male and female sex chromosome.

Introductory notes:

Ceratodon purpureus transformation procedure is adapted from the well-established protocols for the moss *Physcomitrella patens* described in Cove *et al*. 2009. The media used to grow tissue before the transformation as well as during the transformation procedure are identical to those in use with *P. patens* methods. The full set of recipes is repeated at the end of this protocol.

The procedures described here lead to isolation of transgenic moss specific to the used vector. Necessary molecular characterization (locus genotyping and copy number evaluation) of such transgenic plants is not part of the present protocol, but follow standard techniques in common use in plant molecular biology field. Similarly, vector cloning (specific to each project) as well DNA amplification methods and restriction cut needed prior any moss specific procedure are described here. But, maps and sequences of both vectors used in this particular project are available in Appendix I.

For ease of comprehension, the procedure is described in three different protocols: protoplast production, protoplast transformation and finally protoplast regeneration and plant selection. Finally, note that this procedure seems to work similarly with both strains used in the study Gg-1 (female) and R40 (male).

Protocols described hereafter need to be performed at room temperature (<22°C) if not mentioned otherwise and in a sterile environment, e.g. vertical or horizontal laminar flow hood. Standard *in vitro* plant precautions concerning sterility (e.g. surface cleaning, reagent filtering and autoclaving) are required.

Cove DJ, Perroud P-F, Charron AJ, McDaniel SF, Khandelwal A, Quatrano RS (2009) The Moss *Physcomitrella patens*. A Novel Model System for Plant Development and Genomic Studies. In 'Emerging Model Organisms, a Laboratory Manual'. (Eds C D.A. and G A.) pp. 69-104 Cold Spring Harbor Laboratory Press: New York

A. *C. purpureus* protoplasts production

1) Harvest and transfer rapidly tissue from four Petri dishes of six-days old moss protonemata grown on BCDA medium into 15 ml of 8.5% mannitol. Do not let the tissue dry.

2) Add 5 ml of driselase 2% (for a final driselase concentration of 0.5%) and incubate 45 minutes at room temperature with occasional gentle plate swirling. The suspension should be green after 45 minutes with a rapidly sedimenting colored component, protoplasts.

3) Filter the suspension through 100 μm sieve and let incubate at room temperature for 15 minutes.

4) Filter the suspension to 30-35 μm sieve and pour the flow-though containing isolated protoplasts into a tube that can withstand low speed centrifugation (e.g. 50 ml conical "Falcon" tube).

5) Centrifuge 5 minutes at 250 g to sediment the protoplast.

6) At this stage, a dark green pellet should be visible and the supernatant should be brownish (driselase color), not green. Discard the supernatant and resuspend the pellet unto 20 ml of mannitol 8.5% supplemented with 10 mM $CaCl_2$ (wash #1)

7) Centrifuge 5 minutes at 250 g to sediment the protoplast.

8) At this stage, a dark green pellet should be visible and the supernatant should be mostly clear. Repeat step 6 and 7 (wash #2)

9) Resuspend the pellet unto 20 ml of mannitol 8.5% supplemented with 10 mM $CaCl_2$ and evaluate the protoplast number in suspension using a hemacytometer (Fisher Scientific, USA, Cat. # 0267110).

You have now a suspension with a known number of protoplast that can be used for transformation (see protocol B). Alternatively, they can be plated on PRMB for any regeneration test specific to your interest. Typical protoplast yield is one million protoplasts per Petri dish plate. One single protoplast transformation requires between 3.5×10^5 to 5×10^5 protoplasts.

C. purpureus protoplast transformation

1) In a 15 ml sterile tube, put 15 µg of vector DNA resuspended in sterile TE with a maximum of 30 µl volume. This DNA can be produced by standard PCR amplification or plasmid prep amplification. With plasmid amplified DNA, however, the vector must be cut on each side of the transformation vector producing a linear, open-ended DNA fragment for transfection. This fragment type improves DNA integration and reduces the number of episomal transient transformants.

2) Add to the TE plus DNA 300 µl of protoplast resuspended in MMM buffer. Protoplast concentration in the MMM buffer can be anywhere between $1.2x10^6$ to $1.6x10^6$ without affecting transformation efficiency, with the volume ratio for each component presented in this protocol.

3) Add 300 µl of PEG transformation solution and mix gently (do not vortex or pipet up and down the suspension) but thoroughly, producing visually uniform solution.

4) Incubate the tube for 5 minutes in a 45°C water bath.

5) Let the tube stand at room temperature for 10 minutes.

6) The PEG transformation solution is toxic to the protoplasts. Dilute the transformation mix by adding, at one minute interval, five times 300 µl, then five times 1 ml of mannitol 8.5% supplemented with 10 mM $CaCl_2$ (6.5 ml total). Mix gently by swirling the tube after each dilution step.

7) Let the tube stand at room temperature for 30 minutes. At this stage protoplasts have been transformed and are ready to be plated.

8) To concentrate the protoplast suspension, centrifuge 5 minutes at 250 g to sediment the protoplast. Discard the supernatant and add up to 2 ml of fresh mannitol 8.5% supplemented with 10 mM $CaCl_2$.

9) Add 3 ml of 45°C PRMT (previously melted in the microwave and cooled down to 45°C in a water bath), mix well by pipetting and quickly pour 1.25 ml of the mix per PRMB plate overlaid with sterile cellophane (4 plates per transformation tube).

10) Let the plates stand for 30 minutes to one hour in the laminar flow hood (covered and sealed) before transferring the plate to the an incubator set at 25°C and with a long light cycle (16 hours light/8 hours dark). The manipulations for the day are done. See the protocol for regeneration and selection.

B. Plant regeneration and selection.

1) *Week 1: protoplast regeneration*:
Let the protoplasts grow for 6 to 7 days on the transformation plates. Observe the plates for: 1) the number of dividing protoplasts, since this is the actually number that count to evaluate transformation efficiency (it should be as high as possible), and 2) bacterial or fungal contamination. You can potentially contain a weak contamination with vancomycin, but dispose of plates with any strong bacterial contamination or fungal contamination. After a week of growth, most of what can regenerate will have and you should see between 2 to 15 cells per plant. This is numerically the most variable step of the procedure, and this number can vary depending upon the starting moss tissue, minor variation in the reagents or timing of steps during the earlier procedures (A and B).

2) *Week 2: First selection*
Transfer the cellophane containing the regenerating protoplasts onto a BDCA plate supplemented with the appropriate antibiotic (for which resistance is present in the transformation vector) - in this project Hygromicin B at 25 µg/L. Incubate plates for a week in standard growth conditions. Do not extent this step since antibiotics in general are light sensitive and your selection pressure is decreasing with time and hence you may be maintaining transient episomal transformants. During this step most of the plants should die, leaving 50 to 250 growing plants

3) *Week 3-4: release from first selection*
Transfer cellophane containing living transformants and dead non-transformants onto a BDCA plate. Most of your growing moss plants are still transient transformants. These two weeks of selection release will allow strong growth of the plants leading to dilution of the episomal element so such plant will end up to be antibiotic sensitive and die in the next selection step.

4) *Week 5: Second selection*
Transfer cellophane onto BDCA plate supplemented with the appropriate antibiotic (for which resistance is present in the transformation vector) – again, in this project Hygromicin B at 25 µg/l. This step will kill up to 90% of the remaining growing plants. You should be left with stable (or chromosome integrated) transformant.

5) *Picking transformants*
At the end of step 5 (after 6-8 days of second selection), pick using a stereoscope any plant still growing and transfer them unto BCDA medium for tissue amplification. Look carefully at the filament growing at the edge of your plants, since they should grow well in stably transformed plants, but will the most sensitive cell of any transient transformants.

C. Recipes.

If not mentioned otherwise, sterilization is performed by autoclaving at 121ºC for 20 min for volumes up to 500ml, 40 min for volumes between 500 and 1000ml. By default all stock solutions are autoclaved and assumed sterile after wise. In doubt, resterilize! Analytical grade chemicals should be used where possible.

I. Stock solution:

8.5% (w/v) D-mannitol

D-mannitol	85 g
ddH_2O	up to 1 l

$CaCl_2$ 1M

$CaCl_2.6H_2O$	219 g
ddH_2O	up to 1 l

Driselase 2%

Driselase powder (Sigma)	2 g
8.5% (w/v) D-mannitol	up to 100 ml

Proceed as follow: add both components and stir the solution for 1 hour at room temperature. Transfer unto 50 ml tube and centrifuge 20 minutes at 4500 g. Filter sterilize the supernatant that should brown but clear. Make 5 ml aliquot in sterile tubes and store at -20ºC. Thaw 1 hour before use.

TES (Hoagland's A-Z trace element solution):

H_3BO_3	614 mg	$MnCl_2.4H_2O$	389 mg
$Al_2(SO_4)_3.K_2SO_4.24H_2O$	55 mg	$CoCl_2.6H_2O$	55 mg
$CuSO_4.5H_20$	55 mg	$ZnSO_4.7H_2O$	55 mg
KBr	28 mg	KI	28 mg
LiCl	28 mg	$SnCl_2.2H_2O$	28 mg
		ddH_2O	to 1 l

MMM buffer

Mes	2 g (1%)
Mannitol	17 g (8.5%)
$MgCl_2.6H_2O$	610 mg (15 mM)
ddH_2O	up to 150 ml

pH 5.6 with KOH and up to 200 ml with ddH_2O

PEG transformation solution

Mannitol	7 g (0.38 M)
Calcium nitrate	2.36g (0.1M)
PEG 4000 (Serva)	40 g (40 % w/v)

Tris pH 8	1ml of 1M Tris pH 8.0
ddH_2O	up to 100 ml

!!FILTER STERELIZE and make of 3 ml aliquot before store at -20ºC!!

Solution B

$MgSO_4.7H_2O$	25 g
ddH_2O	up to 1 l

Solution C

KH_2PO_4	25 g
ddH_2O	500ml

Adjust pH to 6.5 with minimal volume of 4 M KOH and make up to 1 l with additional distilled H_20.

Solution D

KNO3	101g
ddH_2O	up to 1 l

II. Media

BCDA medium

solution B	10 ml	
solution C	10 ml	
solution D	10 ml	
TES	1 ml	
$FeSO_4.7H_2O$	12.5 mg	
di-ammonium(+)tartrate	920 mg	(=5mM)
agar	7 g	
ddH_2O	to 1 l	

Sterilize by autoclaving and let cool to 50ºC.
Add 1 ml of $CaCl_2$ 1M for a final concentration of 1 mM
Any supplemental antibiotic is added from stock at this stage.

PRMB (Protoplast regeneration medium, bottom layer)

solution B	10 ml	
solution C	10 ml	
solution D	10 ml	
TES	1 ml	
$FeSO_4.7H_2O$	12.5 mg	
di-ammonium(+)tartrate	920 mg	(=5mM)
D-mannitol	60 g	
agar	7 g	
ddH_2O	to 1 l	

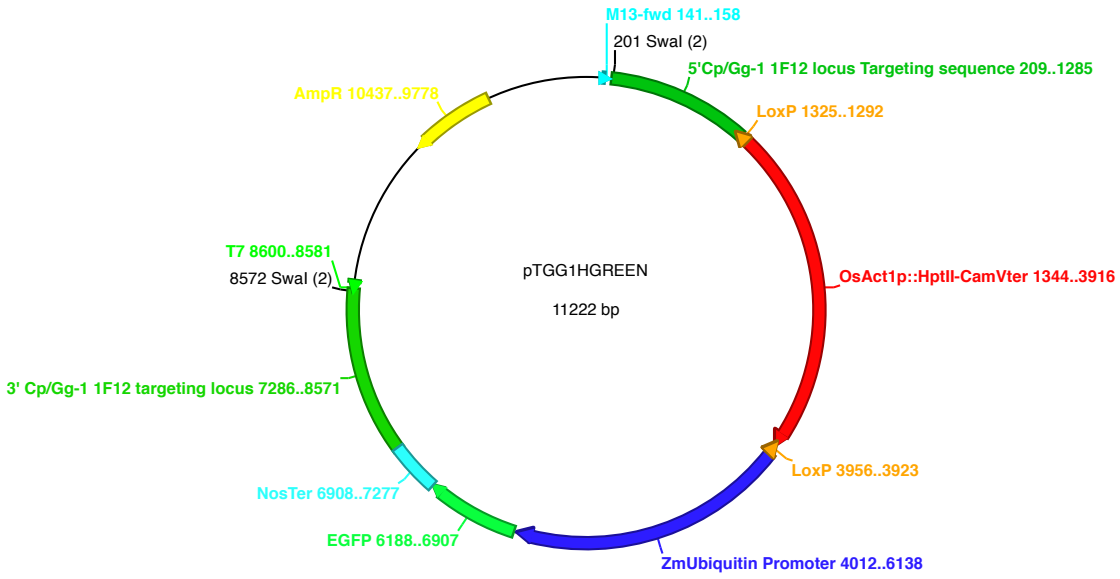
Sterilize by autoclaving and let cool to 50ºC.
Add 10 ml of $CaCl_2$ 1M for a final concentration of 10 mM

PRMT (Protoplast regeneration medium, top layer)

solution B	10 ml	
solution C	10 ml	
solution D	10 ml	
TES	1 ml	
$FeSO_4.7H_2O$	12.5 mg	
di-ammonium(+)tartrate	920 mg	(=5mM)
D-mannitol	80 g	
agar	4 g	
ddH_2O	to 1 l	

Sterilize by autoclaving and let cool to 50ºC.
Add 10 ml of $CaCl_2$ 1M for a final concentration of 10 mM

pTGG1HGREEN is a vector targeting a GFP expression cassette and a hygomycin resistance cassette to the female sex chromosome of *Ceratodon purpureus*.



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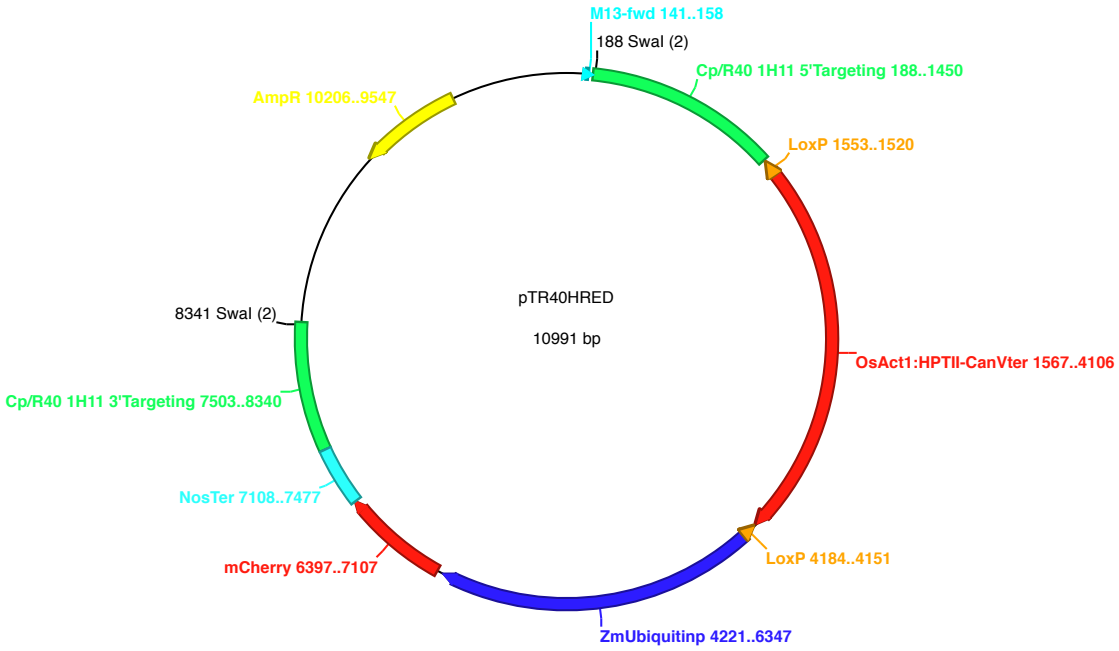
pTR40HRED is a vector targeting mCherry expression cassette and a hygomycin resistance cassette to the male sex chromosome of *Ceratodon purpureus*.



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