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Marchantia thalli Transformation - Sulfadiazine Selection

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We use this protocol and it's working

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Abstract

Here we provide a simplified protocol for Agrobacterium-mediated stable transformation of regenerating thalli of the model liverwort Marchantia polymorpha that involves sulfadiazine herbicide selection. The protocol is adapted from Kubota et al. (2013; https://doi.org/10.1271/bbb.120700), which should be referenced for further detail.

Overview

1 This page includes a simplified protocol for Agrobacterium-mediated stable transformation of regenerating thalli of the model liverwort *Marchantia polymorpha.* The protocol is adapted from Kubota et al. (2013; https://doi.org/10.1271/bbb.120700), which should be referenced for further detail.

1.1 Basic order:
Grow gemmae
Cut thalli and promote regeneration
Co-culture with agrobacterium
Wash and plate on selection

Preparing Stock Solutions of Sulfadiazine, other Herbicides, and Transformation Media

2 Obtain the following herbicides/chemicals as needed: Sulfadiazine (Sigma Aldrich S6387), Hygromycin B (Duchefa H0192), Chlorsulfuron (Duchefa C0177), Cefotaxime (Duchefa C0111), Acetosyringone (Sigma D134406 or ThermoFisher 115540050).

2.1 Using the table below, generate the following stock solutions. Stocks dissolved in water must be sterilized through a 0.22 um syringe-driven filter under aseptic technique.

	A	B	C
	Reagent	**Stock Concentration**	**Solvent**
	Sulfadiazine	50 mg/ml	DMSO
	Hygromycin B	50 mg/ml	Water
	Chlorsulfuron	50 mg/ml	DMSO
	Cefotaxime	250 mg/ml	Water
	Acetosyringone	200 mM	DMSO

3 0M51C media is used when co-culturing regenerating thalli and agrobacterium. While the final co-culture media should be made fresh on day of use, a 10x 0M51C can be aliquoted and kept at -20 °C for
up to four months. Prepare 10x 0M51C media using the following recipes:

3.1 **10x 0M51C Stock Solution (1L)**

After combining ingredients, make up to 1 litre with dH_2O. DO NOT AUTOCLAVE. Media made from this stock will be filter sterilized before use and autoclaving will degrade components. Aliquot (recommend 50mL volumes) and freeze (-20 °C) as soon as possible. Stock will be yellow in colour.

A	B
10x 0M51C Stock Solution	For 1 L
KNO3	20 g
NH4NO3	4 g
MgSO4-7H2O	3.7 g
CaCl2-H2O	3 g
KH2PO4	2.75 g
EDTA-NaFe(III)	0.4 g
B5 Microcomponents (Recipe Below)	10 mL
B5 Vitamin (Recipe Below)	10 mL
0.75% KI	1 mL

3.2 B5 Microcomponents (100 mL)

Made in dH_2O

A	B
B5 Microcomponents	For 100 mL
NaMoO4-2H2O	25 mg
CuSO4-5H2O	2.5 mg
CoCl2-6H2O	2.5 mg

	A	B
	ZnSO4-7H2O	200 mg
	MnSO4-4H2O	800 mg
	H3BO	300 mg

3.3 **B5 Vitamin (100 mL)**

Made in dH_2O

	A	B
	B5 Vitamin	For 100 mL
	Inositol	10 g
	Nicotinic Acid	0.1 g
	Pyridoxine Hydrochloride	0.1 g
	Thiamine Hydrochloride	1 g

Generating pMpGWBs02-based Expression Vectors in Agrobacterium

4 Obtain pMpGWBs02 destination vector and transform into ccdB survival cells. Use lab stocks of DB3.1 or purchase commercially available competent cells like One Shot™ ccdB Survival ™ 2 T1R Competent Cells (ThermoFisher, A10460). Follow established or manufacturer protocols. All subsequent gateway cloning can be performed using standard DH5a or similar strains.

5 Follow manufacturer's protocol to perform BP reactions to generate pENTR clones of your gene of interest using BP Clonase II (ThermoFisher, 11789020). Verify by sequencing or PCR as needed.

6 Follow manufacturer's protocol to perform LR reactions using your pENTR clone and the pMpGWBs02 vector to generate a 35S::GOI (Gene of Interest) construct.

7 Consult the Gateway Cloning user guide for additional details (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/gatewayman.pdf).

8 After validating the final construct by sequencing or PCR, transform the final expression clone into *Agrobacterium tumefaciens* into GV3101 by electroporation or conjugation.

9 Validate successful transformation by PCR of your gene of interest and create glycerol stocks.

Making Marchantia Culture Media

10 **½ MS B5-Plant**: This standard growth media is used to grow Marchantia polymorpha for transformation.
pH with 1M KOH to 5.7 before filling to final volume of 1L with dH_2O. Autoclave.

	A	B
	½ MS B5 Plant	For 1 L
	MS Salts and Gamborg B5 Vitamins (Duchefa M0231)	2.2g
	Duchefa Plant Agar (Duchefa P1001)	12g

11 **½ MS B5-Plant + Sucrose**: This promotes thallus regeneration prior to transformation.

Melt desired amount of ½ MS B5-Plant media and add filter sterilized (or autoclaved) 40% sucrose solution for a final concentration of 1.5% sucrose. Eg. 18.5 mL 40% sucrose (w/v) to 500 mL media before pouring plates.

12 **0M51C co-culture media**: This liquid media promotes the growth of both the thalli and agrobacterium of the transformation.

	A	B	C
	Co-culture Media Recipe	500 mL	1 L
	Water	450 mL	900 mL
	10x 0M51C stock	50 mL	100 mL
	Sucrose	10 g	20 g
	L-Glutamine (Sigma-Aldrich G8540)	0.15 g	0.3 g
	Casamino acids (Merck 2240-500gm)	0.075 g	0.15 g
	pH5.5 with 0.1M KOH		

A	B	C
Filter sterilize with vacuum pump		
Acetosyringone stock, 200 mM	375 ul	750 ul

13 **Transformation Selection Media** (1/2 MS B5 Plant plus antibiotic):
N.B. Cefotaxime is used to kill any remaining agrobacterium after washing, and should be included along with whichever selection agent(s) are being used.

A	B	C
Selection	**Final Concentration**	500 mL Media
Sulfadiazine	5 ug/mL	50 uL stock
Hygromycin B	14 ug/mL	140 uL stock
Chlorsulfuron	5 ug/mL	50 uL stock
Cefotaxime	112.5 ug/mL	225 uL stock

14 **½ MS B5-Bacto**: This standard growth media is used to maintain Marchantia polymorpha stocks and is used when growing plants for experiments. In our conditions, *Marchantia polymorpha* grown on this media show less signs of stress and produce more gemmae cups.

A	B
½ MS B5 Bacto	For 1 L
MS Salts and Gamborg B5 Vitamins (Duchefa M0231)	2.2g
Difco Bacto agar (Becton & Dickinson 214030)	12g
pH with 1M KOH to 6.7 before filling to final volume of 1L with dH2O. Autoclave.	

Marchantia Thallus Transformation

15 Plate Marchantia gemmae onto ½ MS-Plant media. Take care not to overcrowd gemmae as this impacts thallus growth. We generally plate a 6x6 grid of gemmae into a 9 cm square culture dish.

In our conditions, we plate gemmae on a Thursday or Friday (week 0) and cut on the Tuesday of week 3, after 18-19 days of growth (16 hour light /8 hour dark cycle).

16 At 3 weeks of age, cut thalli into 4x fragments per plant as described in Kubota et al. (2013) under aseptic technique using a scalpel and transfer thalli to ½ MS-Plant media supplemented with sucrose. Apical notches must be removed in this step, and thalli should be placed on the media rhizoids down.

Allow thalli to regenerate on the sucrose plates for 3 days.

17 Two days after sectioning thalli, grow Agrobacteria in 10 mL of LB supplemented with appropriate antibiotics by shaking (180 RPM) at 28 °C overnight.

18 On thallus transformation day, prepare 0M51C co-culture media and then filter sterilize using a vacuum bottle-top filter (Sigma Aldrich S2GPT01RE, or equivalent). Add 750 uL Acetosyringone stock per 1L 0M51C co-culture media after filter sterilization in aseptic conditions. Prepare this media fresh for every transformation.

19 Add 50 mL of 0M51C co-culture media to a sterile flask (125-150mL wide-mouth) and add 40-50 pieces of thalli (at this stage, 1 plate of regenerating thalli per flask).

N.B. We find the easiest method of moving thalli from plates to flasks is to gently scrape the plate with a sterile spreader. The rhizoids of the thalli will stick lightly to the spreader, which can then be tapped off into the flask.

20 Prepare Agrobacteria by centrifuging the overnight cultures (10 minutes at 4000 rpm) and resuspending in 0M51C co-culture media.

21 Add 1 mL of Agrobacteria to each flask as needed.

Recommended to use a minimum of two flasks per construct, expect to use more flasks (or repeat multiple times) for constructs that may harm plant growth or development.

22 Shake thalli/flasks on an orbital shaker at 135 rpm in standard Marchantia growth conditions for 3 days.

23 To wash thalli, transfer the content of each flask into individual sterile 50 mL Falcon tubes. Washing must be performed in a Laminar flow hood using aseptic technique.

24 Slowly decant liquid into a large waste bottle while keeping thalli pieces in the tube, add 45 mL of sterile water, close the tube tightly, and shake to wash the thalli.

25 Repeat this washing step at least 5 times to clean thalli of Agrobacteria. Check solution is no longer cloudy, and rhizoids look clean.

26 Decant final wash and replace the liquid with a strong cefotaxime solution (1.5 mg/mL final concentration or 1.5 mL stock per 250mL water) and incubate for 40 minutes.

27 After the 40 minutes has elapsed, decant off as much of the cefotaxime solution as possible. Using sterile technique plate the pieces of thalli on Transformation Selection Media using the appropriate selectable agents. Seal the plate with micropore tape.

An empty sterile plates can be used to tip cleaned thalli onto to make separating thalli out easier.
We generally plate an 7x7 grid of thalli on a 9cm square culture plates. Try to arrange regenerating thalli such that the new growth sites are pointing away from each other.

28 Return the plates to standard growth conditions. Transformants should begin appearing 3-5 weeks after plating.

29 When transformants produce gemmae, transfer gemmae from an individual cup to fresh Transformation Selection Media. If cups are not apparent at early stages we typically move individual primary transformants to fresh selection media to promote gemmae development.

30 Validate transformants in the next gemmae generation.