

Transformation Protocol for Rice – Abbreviated

Seed plating on 2N6 – dark

↓ 4 weeks

Subculture onto 2N6 – dark

↓ 4 – 10 days

Co cultivation onto 2N6-AS – dark

↓ 3-7 days

Selection on 2N6-TCH – dark

↓ 4 weeks, subculture onto 2N6-TCH every 2 weeks

Transfer proliferating calli onto 2N6-TCH-dark

↓ 2 weeks

Regeneration onto RGH6-dark

↓ 7 days

Transfer to light

↓ 4-6 weeks

Plantlets onto ½ MSH - light

↓

Transfer plants to the glasshouse

Transformation Protocol for Rice

Seed Material: *Oryza sativa* L. ssp japonica cvs. Millin or Nipponbare.

Callus Induction:

- Dehusk seeds; usually 20-30 grams are required for an experiment.
- Treat husked seeds with 70% ethanol for one minute.
- Wash seeds thoroughly with distilled water.
- Sterilise seeds with a solution of 80% commercial bleach (4.0% hyper chlorite m/v) and one drop Tween-20 rotate, gently for 30 minutes at room temperature on wheel.
- Wash the bleached seeds in the laminar flow 3 times with sterile water.
- Blot dry on sterile filter paper.
- Plate seeds on **2N6 media**, 12-15 seeds per plate.
- Allow callus induction and proliferation to continue for 3-4 weeks, depending on the vigour of the seed. Millin takes 3 weeks, Nipponbare takes 4 weeks.

Callus Subculture:

- After callus induction the proliferated embryogenic calli are subcultured into pieces around 2.5mm in size and placed on fresh 2N6 media for a further 7-10 days.

Bacteria Preparation:

- Two to three days before the transformation, streak out bacteria containing plasmid of interest on **YM solid media** containing antibiotic. The antibiotic will depend on the plasmid being used.
- Grow the bacteria at 28°C for 2-3 days.

Transformation:

- The bacteria are resuspended in **AAM media** containing 100µm acetosyringone (19.62 mg/L). The bacteria is scraped or washed off culture plates using AAM media and shaken to resuspend. Resuspend to OD₆₀₀ of about 1.0.
- The resuspended bacteria should be left to sit 2-3 hours at room temperature.
- Transfer the suspension mix to a larger container and add the calli. Swirl and leave calli to sit for approximately 40 minutes at room temperature.
- Blot dry for 20 minutes on sterile filter paper and plate onto **2N6-AS** plates.
- Co-cultivate with the calli for 2-3 days (*A. tumefaciens*) or 7 days (*S. meliloti*) in the dark in growth room (26°C).

Transformation Protocol for Rice- cont.

Callus washing:

After co-cultivation the calli need to be washed to remove bacteria.

- Prepare a solution of Claforan (250mg/L) and Timentin (200mg/L) in sterile water.
- Transfer the calli to the solution, swirl and leave for 20-30 minutes. (During this time most of the bacteria are released from the calli.)
- Pour off the solution and wash calli again a few more times until the solution is clear.
- Blot dry calli on sterile filter paper.
- Transfer calli to **2N6-TCH** plates. These contain Claforan and Timentin to kill any remaining bacteria. The hygromycin is for selection.
- Incubate in the dark, 26°C.

Selection:

- Transfer the calli to fresh 2N6-TCH plates every two weeks. Due to selection pressure, the calli will become light to dark brown during this period.
- Small transgenic hygromycin resistant calli will start proliferate (grow) after two to four weeks on selection. Proliferating calli are a creamy-white color.
- Transfer proliferating to 2N6-TCH media for another 2 weeks.
- Calli can be assayed for GUS activity at this stage.

Regeneration:

- After two weeks or when proliferating calli have increased in size, transfer to regenerating media, **RGH6**. The calli should be a creamy-white colour.
- Leave the calli on regenerating media for one week in the dark, 26°C.
- Transfer to the light, 26°C.

Plantlet Formation:

In about 1 to 2 weeks you will notice calli turning green and then shoots will start differentiating.

- The shoots can then be transferred to rooting media **1/2MSH**.
- Once plants have formed they can be transferred to the glasshouse.

Media and Solutions for Rice Transformation

2N6 (Seed Plating)

(modified from Chu et al, 1975)

	<u>1L</u>
Sucrose	30g
Casamino acids	1.0g
Proline	0.5g
Glutamine	0.5g
Sigma N6 salts	4.0g
N6 Vitamins 100x	10ml
2,4-D (1mg/ml)	2ml

- pH 5.8
- Phytigel 2.5g/L
- Autoclave
- Or, if not using Sigma N6 (Chu's) salts add:
 - N6 Macro 20x 50ml/L
 - N6 Micro 1000x 1ml/L
 - Fe₂EDTA (Iron) 100x 10ml/L

N6 Macro 20x

	<u>20x (g/L)</u>	<u>Final concentration</u>
		<u>1 x mM</u>
KNO ₃	56.6	28.0
(NH ₄) ₂ SO ₄	9.26	3.5
KH ₂ PO ₄	8.00	2.9
MgSO ₄ ·7H ₂ O	3.70	0.75
CaCl ₂ ·2H ₂ O	3.32	1.13

- Store 4°C
- Substituting chemicals:
 - Ca Cl₂ 2.5g/L
 - MgSO₄ 1.8g/L

N6 Micro 1000x

	<u>1000x (g/L)</u>	<u>Final concentration</u>
		<u>1 x mM</u>
MnSO ₄ ·4H ₂ O	4.40	19.7
H ₃ BO ₃	1.60	25.9
ZnSO ₄ ·7H ₂ O	1.50	5.2
KI	0.80	4.8

- Store 4°C

FeSO₄EDTA Iron 100x

	<u>g/1L</u>	<u>1 x mM</u>
FeSO ₄ .7H ₂ O	2.78	0.1
Na ₂ EDTA	3.72	0.1

- Store 4°C in dark bottle

N6 Vitamins 100x

	<u>100x (mg/L)</u>	<u>Final concentration</u> <u>1 x μM</u>
Myo-inositol	10g	555
Glycine	200	26.6
Thiamine-HCl	100	3.0
Nicotinic acid	50	4.0
Pyridoxine-HCl	50	2.4

- Store 4°C
- Substituting chemicals:
 - Pyridoxine 41mg/L

2,4-D (1mg/mL) (2,4-Dichlorophenoxy acetic acid)

Dissolve 400mg 2,4-D in 400mL 70% methanol

- Store 4°C

2N6-AS (co cultivation media)

	<u>1L</u>
Sucrose	30g
Casamino acids	1.0g
Glucose	10g
Sigma N6 salts	4.0g
N6 Vitamins 100x	10ml
2,4-D (1mg/ml)	2ml

- pH 5.2
- Phytigel 3.5g/L
- Autoclave
- When ready to pour add:
 - 1ml Acetosyringone (100mM stock)
- Or, if not using Sigma N6 (Chu's) salts add:
 - N6 Macro 20x 50ml/L
 - N6 Micro 1000x 1ml/L
 - Fe₂EDTA (Iron) 100x 10ml/L

Acetosyringone (100mM) (AS)

Dissolve 392mg acetosyringone in 20mL Dimethylsulfoxide (DMSO)

- No need to sterilize
- Store -20°C in 1mL aliquots

Claforan (250mg/ml)

Add 8ml sterile Milli-Q H₂O to 2g Claforan

- Store 4°C in dark

Timentin (200mg/ml)

Add 15ml sterile Milli-Q H₂O to 3g Timentin

- Store 4°C

2N6-TCH (Selection media)

	<u>1L</u>
Sucrose	30g
Casamino acids	1.0g
Sigma N6 salts	4.0g
N6 Vitamins 100x	10ml
2,4-D (1mg/ml)	2ml

- pH 5.2
- Phytigel 3.5g/L
- Autoclave
- When ready to pour add:
 - 1ml Hygromycin (50mg/ml)
 - 1ml Claforan (250mg/ml)
 - 0.5ml Timentin (200mg/ml)
- Or, if not using Sigma N6 (Chu's) salts add:
 - N6 Macro 20x 50ml/L
 - N6 Micro 1000x 1ml/L
 - Fe₂EDTA (Iron) 100x 10ml/L

RGH6 (Regeneration medium)

	<u>1L</u>
Sucrose	30g
Glutamine	0.5g
Proline	0.5g
Casein enzymatic hydrolysate	0.3g
N6 Macro 20x	50ml
N6 Micro 1000x	1mL
Fe ₂ EDTA (Iron) 100x	10mL
N6 Vitamins 100x	10ml
BAP (1mg/mL)	3ml
NAA (1mg/mL)	500µL

- pH 5.8
- Phytigel 6g/L
- Autoclave
- When ready to pour add:
 - 1ml Hygromycin (50mg/ml)

BAP (1mg/ml) (6-Benzylaminopurine)

Add 1N KOH drop wise to 100mg BAP until dissolved. Make up to 100mL with Milli-Q H₂O

- Store 4°C
- Benzyl adenine (BA) is chemically the same as 6-Benzylaminopurine (BA or BAP)

NAA (1mg/ml) (Naphthalene acetic acid)

Dissolve 100mg NAA in 1mL absolute ethanol. Add 3mL 1N KOH. Make up to 80mL with Milli-Q H₂O. Adjust pH to 6.0 with 1N HCl. Make up to 100mL with Milli-Q H₂O

- Store 4°C

½ MS-H medium (Rooting)

(Svab, et al, 1975)

	<u>1L</u>	<u>Final concentration</u>
Sucrose	10g	10%
MS Macro 10x	50mL	1/2x
MS Micro 1000x	0.5mL	1/2x
Fe ₂ EDTA Iron 100x	5mL	1/2x
N6 Vitamins 100x	5mL	1/2x

- pH 5.8
- Phytigel 2.5g/L
- autoclave
- when ready to pour add:
 - Hygromycin (50mg/mL stock) 1mL

MS Macro 10x

(Murashige and Skoog, 1962)

	<u>10x (g/L)</u>	<u>Final concentration</u> <u>1 x mM</u>
KNO ₃	19.0	18.8
NH ₄ NO ₃	16.5	20.6
CaCl ₂ .2H ₂ O	4.4	3.0
MgSO ₄ .7H ₂ O	3.7	1.5
KH ₂ PO ₄	1.7	1.25

- Store 4°C
- Substituting chemicals:
 - CaCl₂ 3.3g/L
 - MgSO₄ 1.8g/L

MS Micro 1000x

(Murashige and Skoog, 1962)

	<u>1000x (g/L)</u>	<u>Final concentration</u> <u>1 x µM</u>
MnSO ₄ .4H ₂ O	22.3	100
ZnSO ₄ .7H ₂ O	8.6	30
H ₃ BO ₃	6.2	100
KI	0.83	5.0
Na ₂ MoO ₄ .2H ₂ O	0.25	1.0
CuSO ₄ .5H ₂ O	25mg	0.1
CoCl ₂ .6H ₂ O	25mg	0.1

- Store 4°C
- Substituting chemicals:
 - MnSO₄.H₂O 16.9g/L

YM Media

	<u>1L</u>
Mannitol	10g
Yeast extract	0.4g
K ₂ HPO ₄ (10% w/v stock)	1 ml
KH ₂ PO ₄ (10% w/v stock)	4 ml
NaCl (10% w/v stock)	1 ml
MgSO ₄ .7H ₂ O (10% w/v stock)	2 ml

- pH 6.8
- Agar 15g/L
- Autoclave
- When ready to pour add:
 - Antibiotic selection if required

Keep poured plates for 2 days at room temperature to visualise any contamination, then store at 4°C.

AAM Liquid Medium (Hiei, et al, 1994)AAM- Hiei *et al.*, 1994 = modified AAAA - Toriyama *et al.*, 1985 = modified B5B5 - Gamborg *et al.*, 1968

	<u>1L</u>
Sucrose	68.5g
Glucose	36.0g
Casamino acids	500mg
AA Macro 10x	100ml
AA Micro 1000x	1ml
Fe ₂ EDTA (Iron) 100x	10ml
AA Amino Acids 100x	10mL
MS Vitamins 100x	10ml

- pH 5.2
- Autoclave in 250mL aliquots
- when ready to pour add:
 - Acetosyringone (100mM stock) 250µL/250mL

AA Macro 10x

	<u>10x (g/L)</u>	<u>Final concentration</u>
		<u>1 x mM</u>
KCl	29.50	39.5
MgSO ₄ .7H ₂ O	2.50	1.0
CaCl ₂ .2H ₂ O	1.50	1.0
NaH ₂ PO ₄ .H ₂ O	1.5	1.1

- Store 4°C
- Substituting chemicals:
 - Ca Cl₂ 1.13/L
 - MgSO₄ 1.2g/L

AA Micro 1000x

	<u>1000x (mg/100mL)</u>	<u>Final concentration</u>
		<u>1 x µM</u>
MnSO ₄ .4H ₂ O	1000	44.8
H ₃ BO ₃	300	48.5
ZnSO ₄ .7H ₂ O	200	7.0
KI	75	4.5
Na ₂ MoO ₄ .2H ₂ O	25	1.0
CuSO ₄ .5H ₂ O	2.5	0.1
CoCl ₂ .6H ₂ O	2.5	0.1

- Store 4°C

AA Amino Acids 100x

	<u>100x (g/100mL)</u>	<u>Final concentration</u>
		<u>1 x mM</u>
L-glutamine	8.76	6.0
L-aspartic acid	2.66	2.0
L-arginine	1.74	1.0
Glycine	0.075	0.1

- Store 4°C
- MS Vitamins 100x**

(Murashige and Skoog, 1962)

	<u>100x (mg/L)</u>	<u>Final concentration</u>
		<u>1 x (mg/L)</u>
Myo-inositol	10g	100
Nicotinic acid	50	0.5
Pyridoxine-HCl	50	0.5
Thiamine-HCl (10mg/mL)	1mL	0.1
Glycine (100mg/mL)	20µL	0.02

- Store 4°C
- Substituting chemicals:
 - Pyridoxine 41mg/L

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