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Agrobacterium-mediated transformation of *Zymoseptoria tritici*

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We use this protocol and it's working

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Abstract

This protocol describes the procedure for transforming plasmids into the plant pathogenic fungus *Zymoseptoria tritici* by *Agrobacterium tumefaciens*-mediated transformation. It is based on the protocol first described by Zwiers and De Waard (2001; doi: 10.1007/s002940100216) and utilises *A.tumefaciens* ternary vectors developed by Sidhu et al. (2015a; doi: 10.1016/j.fgb.2015.04.015), Sidhu et al. (2015b; doi: 10.1016/j.fgb.2015.03.021) and Kilaru et al. (2015; doi: 10.1016/j.fgb.2015.03.018).

Section 1 details the recipes for media used in the protocol

Section 2 describes how to make competent *A. tumefaciens* cells

Section 3 details the process of transforming *A. tumefaciens* competent cells with plasmid vectors

Section 4 describes the process of *A. tumefaciens*-mediated *Z. tritici* transformation

Attachments

PDF

Agrobacterium-mediat...

132KB

Materials

Chemicals for media preparation (see section 1 of protocol)
Selective antibiotics (see section 1 of protocol)
Zymoseptoria tritici strain for transformation
Agrobacterium tumefaciens strain EHA105
Plasmid DNA from *E. coli* miniprep
Liquid nitrogen
Nitrocellulose discs (Product number '325 P cellulose film' from AA packaging limited, Preston, UK used here)
Tweazers

1. Media Recipes

1 **Induction Media (IM)**

- Make stock solutions in deionised water (diH_20) according to Table 1, with K_2HPO_4/KH_2PO_4/NaCl combined to make 'Stock A', which can be autoclaved and used in a sterile environment to make IM for mutliple transformations.
- To prepare fresh for each transformation, dissolve the required amount of MES in 100 ml of deionised water before adding appropriate amount of each stock solution (Table 1).

Table 1 Induction medium components

	A	B	C	D	E
	Reagent	**Stock Solution**	**Concentration factor**	**Volume stock for 1l**	**Final conc.**
	K2HPO4	200mM (34.8 g/l)	20x (Stock A)	50 ml	10 mM
	KH2PO4	200mM (27.2 g/l)			10 mM
	NaCl	50mM (2.92 g/l)			2.5 mM
	MgSO4.7H20	1M (246.47 g/l)	x500	2 ml	2 mM
	CaCl2	100 mM (11.1 g/l)	x142.85	7 ml	0.7 mM
	FeSO4	10 mM (2.78 g/l)	x1000	1 ml	10 µM
	(NH4)2SO4	1M (132.1 g/l)	x250	4 ml	4 mM
	Glucose	1M (180.16 g/l)	x100	10 ml	10 mM
	Glycerol	20% (v/v)	x40	25 ml	0.5%
	MES	-	-	7.81 g/l (solid)	40 mM

- Add diH_20 to give the final volume
- Adjust the pH to 5.6 using 1M NaOH
- Decant 1/5 of the prepared IM for liquid medium (IM broth)
- Add 2% Agar (w/v) for solid media (IM agar)
- Autoclave both and place IM broth in fridge until use
- Once cooled, amend IM agar with kanamycin (100 µg/ml) and acetosyringone (40 µg/ml) and pour plates. Store in the fridge once plates are set.

2 **Aspergillus Minimal Medium (AMM) agar**

- Prepare 1l stock of 20x Salts solution (120 g/l $NaNO_3$, 10.4 g/l KCl, 10.4 g/l $MgSO_4.7H_2O$, 30.4 g/l KH_2PO_4), autoclave and store at 4ºC
- To prepare 1000x Trace elements stock solution, add reagents in Table 2 in order to 80 ml diH_2O, allowing each to dissolve before adding the next.

Table 2 Trace elements components

	A	B
	Reagent	**Mass for 100ml**
	ZnSO4.7H2O	2.2g
	H3BO3	1.1g
	MnCl2.4H2O	0.5g
	FeSO4.7H2O	0.5g
	CoCl2.5H2O	0.16g
	CuSO4.5H2O	0.16g
	(NH4)6Mo7O24.4H2O	0.11g
	Na4EDTA	5g

- Heat Trace elements stock solution to boiling, cool to 60º, adjust pH to 6.5 – 6.8 with 5M KOH and adjust the volume to 100 ml.
- Autoclave Trace elements stock solution, then store in the dark at 4º. (Note: stored in the fridge, the $FeSO_4$ may preciptitate out of solution but solution will still work)
- Prepare 1l of Aspergillus MM agar by combining the following:
  - 10 ml of 20x Salts
  - 1 ml of 1000x Trace elements
  - 10g Glucose
- Add to 800ml diH_2O and adjust the pH to 6.5 with 5M KOH, then adjust volume to 1l.
- For solid medium add 10 g/l Agar No. 1 and autoclave.
- Once cool add antibiotics to remove *A. tumefaciens* and select for transformant *Z. tritici* colonies

3 **Basal Medium (BM) agar**

- Prepare basal medium (1.7 g/l Yeast nitrogen base w/o amino acids or NH_4SO_4, 2 g/l Asparagine, 1 g/l NH_4NO_3, 10 g/l Glucose) in diH_2O
- Adjust the pH to 6 with 1M Na_2HPO_4
- Add 2 g/l agar and autoclave
- Once cool add antibiotics to remove *A. tumefaciens* and select for transformant *Z. tritici* colonies

4 **YPD Agar Media**

- Prepare YPD (10 g/l Yeast extract, 20 g/l Peptone, 20 g/l Glucose, 20 g/l Agar) in diH_2O and autoclave.

5 Prepared the following **antibiotics stock solutions**, before being filter sterilised and stored at –20°C. These are added to cooled media when required.

- **Rifampicin:** 100 mg/ml in DMSO
- **Kanamycin:** 100 mg/ml in H_2O.

- **Cefotaxime:** 250 mg/ml in H_2O.
- **Streptomycin:** 100 mg/ml in H_2O.
- **Ampicillin:** 100 mg/ml in H_2O.
- **Hygromycin B:** 50 mg/ml in H_2O.
- **Sulfonylurea**: 10 mg/ml in DMF (Dimethylformamide)
- **Glufosinate Ammonium** (BAR selection): 100 mg/ml in H_2O
- **Carboxin**: 40 mg/ml in Ethanol
- **Geneticin** (G418 selection): 200 mg/ml in H_2O
- **Acetosyringone:** 40 mg/ml in DMSO

2. Making competent *Agrobacterium tumefaciens* cells

6 Inoculate 5 ml LB broth amended with Rifamipicin (100 µg/ml) with a colony of *A. tumefaciens* or 15 µl of *A. tumefaciens* glycerol stock.

7 Incubate overnight at 28°C with shaking at 250 rpm.

8 The next day, inoculate 50 ml LB broth amended with Rifamipicin (100 µg/ml) in a sterile 250 ml conical flask with 2 ml of the above overnight culture.

9 Incubate flask at 28°C with shaking at 250 rpm for ~8 hours until it reaches OD_{600} of 0.6.

10 Chill the flask on ice for 5 minutes.

11 Pellet cells in a 50 ml Falcon tube at 3000 rpm at 4°C for 5 min.

12 Resuspend cells in 1 ml of 20 mM $CaCl_2$.

13 Make aliquots and store at −80°C (50 µl of cells used per transformation).

3. Transformation of *A. tumefaciens*

14 Thaw *A. tumefaciens* competent cells on ice (takes **1-2 hours**).

15 Add 10 µl of plasmid miniprep DNA to 50 µl of *A. tumefaciens* competent cells in a 1.5 ml Eppendorf tube. Mix gently by flicking tube.

16 Freeze tubes in liquid nitrogen. Wait until bubbling subsides then remove the tube.

17 Thaw frozen tubes in 37°C water bath for 5 minutes.

18 Add 0.5 ml LB Broth without antibiotics.

19 Incubate cells at 28°C with 200 rpm shaking for 2-3 hours.

20 Pellet cells and resuspend with 150 µl LB broth before plating onto LB agar plates amended with Kanamycin (100 µg/ml) and Rifampicin (100 µg/ml).

21 Incubate plates at 28°C for 2 days.

22 Pick one transformant colony and inoculate 5 ml LB Broth amended with Kanamycin (100 µg/ml) and Rifampicin (100 µg/ml).

23 Incubate overnight 28°C with 250 rpm shaking.

24 Make glycerol stock of transformed *A. tumefaciens* cells (850 µl of cells + 150 µl sterile glycerol).

4. *Agrobacterium*-mediated transformation of *Z. tritici*

25 **Before transformation day:**
Five days before the transformation, inoculate a YPD agar plate with the *Z. tritici* strain to be used in transformation and incubate at 19^{O}C.

26 Four days before transformation, streak LB agar amended with Kanamycin (100 µg/ml) and Rifampicin (100 µg/ml) with *A. tumefaciens* glycerol stock and incubate at 28°C for 2-3 days before use for fungal transformation.

27 The day before transformation, inoculate a single colony of *A. tumefaciencs* cells into 4 ml of LB media in a 50 ml falcon tube, amended with Kanamycin (100 µg/ml) and Rifampicin (100 µg/ml) and grow at 28°C with 250 rpm shaking overnight.

28 The day before transformation, prepare IMbroth and IM agarplates amended with Kanamycin (100 µg/ml) and acetosyringone (40 µg/ml).

29 **Transformation day:**
Measure the OD_{600} of the overnight culture.It can be used when it has reached an OD_{600} of at least 0.75 to around 1. (Ensure the blank for spectrophotometer readings is LB + Rifampicin).

30 Dilute *A. tumefaciens* overnight culture to an OD_{600} of 0.09 in IM (amended with Kanamycin 100 µg/ml and Acetosyringone 40 µg/ml) in a volume of 10 ml in a 50 ml sterile flask.

31 Grow cultures at 28°C/250 rpm. Measure OD_{600} every hour, until it reaches an OD_{600} of 0.21-0.24 (maximum 0.28). This usually takes 1.5-3 hours. (Ensure the blank for spectrophotometer readings is IM containing a similar dilution of LB + Rifampicin to *A. tumefaciens* cultures).

32 While *A. tumefaciens* cultures are incubating, place a single sterile nitrocellulose disc dipped in sterile water on the centre of each induction plate using sterile tweazers. Make sure to drain as much water as possible off the disc before laying it on the plate. Leave plates open to dry.

33 (Optional: Pour 5-10 sterile glass beads into each IM agar plate if plating out too many transformant plates for spreading with L-shaped spreader).

34 Harvest the *Z. tritici* yeast-like cells by adding 2 ml sterile H_2O to the *Z. tritici* plate and spreading thoroughly using an L-shaped spreader and transfer to a 15 ml falcon tube.

35 Pass *Z. tritici* cell suspension through a 100 µm sterile cell strainer/miracloth to remove clumped cells.

36 Measure the concentration of yeast-like cells on a haemocytometer, diluting where necessary.

37 Dilute *Z. tritici* yeast-like cells to $5x10^6$ cells/ml in sterile H_2O.

38 Equal volumes of the *A. tumefaciens* culture and *Z. tritici* cells are mixed together.

39 Prepare the following controls
- 'No *Agrobacterium*' control (equal amounts of induction media and *Z. tritici* cells) as a control for background growth of *Z. tritici* on selective plates.

- 'No selection' control (equal amounts of *Z. tritici* and *A. tumefaciens*) to assess *Z. tritici* growth without selection.

40 Plate 150 µl aliquots of the above mixtures onto IM agar plates with nitrocellulose discs and spread with L-shaped spreader (or glass beads).

41 Allow plates to dry before placing in a plastic box containing silica gel beads to avoid build up of moisture in plates. (Note: no need to parafilm plates).

42 Incubate the plates for 48 h at 19 °C.

43 **Two days after transformation:**
Prepare one of the following media for selection depending on selective marker used. Remember to pour a plate for the 'No selection' control plate before adding the *Z. tritici* selective antibiotic.

- AMM agar with Hygromycin B (200 µg/ml) – HYG^Rselection
- AMM agar with glufosinate ammonium (200 µg/ml) – BAR^R selection
- AMM agar with Geneticin (200 µg/ml) - $G418^R$selection
- BM agar with Sulfonylurea (10 µg/ml) – SUR^Rselection
- Czapek Dox agar with Carboxin (40ug/ml) – $sdi1^R$ selection

44 Add the following antibiotics to kill *A. tumefaciens* (including 'No selection' control plate): Cefotaxime 250 µg/ml **OR** Timentin 100µg/ml, **AND** Streptomycin 100 µg/ml **AND** Ampicillin 100 µg/ml.

45 When the plates are set, transfer the nitrocellulose discs using sterile tweazers from IM plates onto selective plates.

46 Incubate the plates at 19 °C. Colonies should be visible after around 10 days but can take up to 3 weeks to be ready for subculturing.

47 **Putative Transformants:**
After 2-3 weeks, any colonies which appear are patch subcultured onto the following media depending on selective marker used:

- $HYG/G418/sdi1^R$: YPD plates amended with Hygromycin OR Geneticin (200 µg/ml) OR Carboxin (40 µg/ml) and antibiotics
- BAR: AMM plates with Glufosinate ammonium (200 µg/ml) and antibiotics
- SUR: BM plates with sulfonylurea (10 µg/ml) + antibiotics

48 Plates are incubated at 19 °C for 5-7 days and any growing colonies are then verified by PCR screening.

49 Confirmed transformants are subcultured on selection media a second time before being stored in 50% glycerol at -80^{o}C.