Protocal for Hybrid poplar NM6 transformation

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***Agobacterium* strain**: *Agrobacterium* C58 w/pBI121 construct

Plant material:

GH tissue: 3-5 month, 100-200cm in height, young leaf from greenhouse.

In vito plant tissue: propagate in box 1-2 month leaf.

Protocol:

Transformation using GH leaves:

1. Streak *Agrobacterium* single colony on a LB plate containing appropriate antibiotics. Incubate at 28°C for 2 days.

2. Pick up one isolated *Agrobacterium* colony to inoculate 3mL of LB medium with the appropriate antibiotics and grow for 20 hours in a shaker at 28°C, 200rpm.

3. Subculture the *Agrobacteria* 10uL into 20mL of LB containing suitable antibiotics and 20uM Acetosyringone. Grow overnight in a shaker at 28°C, 200rpm

4. Excise young leaves from green house grown plants.
   Notes: Keep in mind, must pick up young leaf (from trees top 4-7) and immediately follow next step.

5. Surface sterilize plant material collected in greenhouse in 10% Clorox solution for 20 minutes, followed rinse at least three times in sterile dd water.

6. Using sharp blade, leaves were excised into 10mm squares along the midrib with five wounds on the rib. Dip the lead discs into the overnight-grown *Agrobacteria* and inoculated for 5 minutes.
   Notes: A new razor blade should be used for each construct.

7. Gently blot explants dry to remove excess *Agrobacteria.* Place upside down on callus induction medium.

8. The inoculated explants were co-cultivation on CIM1 at 25°C in darkness for 2 days.

9. Explants were rinsed several times in sterile dd water to remove most of the agrobacteria., Briefly blot dry on a sterile filter paper, and culture in the dark on CIM2 at 25°C for selection of transformed cells.

10. First they were subculture twice every 1 week to CIM2.Then Keep subculture for these transformed calli to proliferate every 2 weeks until 6-8 weeks.

Notes: *Agrobacterium* comes back mostly within the first month. If the recovery of *Agrobacterium* extends to a high degree, CIM2 will be added more Cefotaxime to 500mg/L.

11. Explants with putative transgenic callus were transferred in light at 25°C on SIM1 for 20-30 days. According to the state of callus, the big callus can be excised from the explants and subculture on SIM2 for 30 days.

12. When adventitious shoots appear, the explants promote elongation on SIM3 for 1-2 months in light at 25°C, subculture every 2 weeks.

13. Separate individual shoots and place in RM at 25°C in light. Whole transgenic plantlets with root system were gained after 30 days.
Notes: putative transgenic shoot will grow to 1cm length and separate to inoculate on medium.

14. Cut a small piece of leaf from rooted plantlets for PCR analysis to confirm.

15. Whole transgenic plantlets 3-5 copy were propagated in *In vito* box,then transplant the transgenic plants into soil medium and grow in GH.

Transformation using *in vitro* leaves:

1. *Agrobacterium* cells were grown for 20 hours in LB medium supplemented with the appropriate antibiotics on a shaker at 28°C and 200rpm.Acetosyringone was added to LB medium with final concentration 20um at the same time.

2. *In vitro* leaf were excised using sharp blade and inoculated in an *Agrobacterium* medium.
Notes: A new razor blade should be used for each construct. Agrobacterium media will be put 2ml in the petri dish and keep leaf wet.

3. The inoculated explants were co-cultivation on CIM1 at 25°C in darkness for 2 days.

4. Explants were blotted dry on a sterile filter paper and culture in light on CIM2 .First they were subculture twice every 1 week .Then subculture every 2 weeks until 6 weeks.

5.Following step same as GH leaf.

Agrobacterium medium:

LB, add kanamycin 50mg/L, genmycin 50mg/L, AS 20um

Culture medium

<u>CIM1:</u>

Basal medium: WPM salt and WPM vitamins

BA:0.5mg/L

2,4-D:1.0mg/L

MES:500mg/L

Calcium gluconate: 650mg/L

Sucrose:20g/L

Adjust PH to 5.7 by 1N KOH

Agar:7.0g/L

CIM2:CIM1 autoclaved ,then add filter sterilized

Kanamycin 50mg/L

Cefotaxime 300mg/L

SIM1:

Basal medium: WPM salt and WPM vitamins

MES:500mg/L

Calcium gluconate: 650mg/L

Sucrose: 20g/L

Agar: 7.0g/L, autoclaved , then add filter sterilized

TDZ: 0.5mg/L

Kanamycin 50mg/L

Cefotaxime 300mg/L

SIM2:

Basal medium: MS salt and MW vitamins

L-cystin 1mg/L

L-glutamin 200mg/L

inositol 100mg/L

Sucrose: 30mg/L

Agar: 7.0g/L, Autoclaved , then add filter sterilized.

TDZ 0.02 mg/L

Kanamycin 50mg/L

Cefotaxime 300mg/L

SIM3:

Basal medium: WPM salt and WPM vitamins

MES: 500mg/L

Calcium gluconate: 650mg/L

Sucrose: 20g/L

Agar: 7.0g/L

Kanamycin 50mg/L

Cefotaxime 300mg/L

RM:

Basal medium:WPM salt and WPM vitamins

MES: 500mg/L

Calcium gluconate: 650mg/L

Sucrose: 20g/L

Agar: 7.0g/L

Kanamycin 25mg/L

Cefotaxime 25mg/L