# The protocol of Agrobacterium tumefaciens-mediated stable transformation of the 84K Poplar

**Wang Lina**

State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, 51 Hexing Road, Harbin ,150040, China

**Abstract**

A protocol for successful callus induction and plant regeneration from 84K Poplar is described.The stable transformation protocol will promote the functional characterization of genes in woody plants.

**Key words：** *Agrobacterium* Transformation, Plant tissue culture, Explant, Genetic transformation

## 1. Introduction

*Agrobacterium tumefaciens* is a soil phytopathogen that naturally infects plant wound sites and causes crown gall disease in a wide range of plants, eudicot angiosperms and gymnosperms (Daboussi et al., 1989).Agrobacterium tumefaciens-mediated transformation is an efficient genetic transformation method developed in recent 20 years (Li et al., 2013). The method has many advantages, such as simple manipulations, high transformation efficiency and high single-copy rate (Fungaro et al., 1995). It is a powerful tool for plant genetic transformation, gene cloning, and also leads to homologous recombination,which can facilitate gene knockouts (Wu, J et al., 2009).

As previously reported, several genetic transformation methods for84K Poplar have been developed, including electroporation (Brown et al., 1991), particle bombardment (Sunagawa et al., 2001), polyethyleneglycol/$CaCl_2$ (Honda et al., 2000), an amended polyethyleneglycol/$CaCl_2$ method (Koukaki et al., 2003), and restriction enzyme-mediated integration (Irie et al., 2001). Although the successful transformations of several p

lant mediated by A. tumefaciens have been achieved in the past 20 years, the researc h has shown that different plants require unique transformation methods (Pfeifer et al., 1992).

As previously reported, several genetic transformation methods for woody plant h ave been developed, However, these procedures are based upon the many aspects, and they are time-consuming, produce undesirable heterokaryons, unstable transformants, a nd the transformation efficiencies are low (Sandhu et al., 2001).

## 2. Protocol

The basic protocol is divided into five parts:(1) Surface sterilization of explants; (2) Preparation of differentiation media and plant regeneration; (3) Preparation of Agrobacterium for inoculation; (4) Infection and inoculation of leaf pieces; (4) Co-culture stage; (5) Selective cultivation, subculture and rooting culture.

### 2.1 Surface sterilization of explants

84K Poplar budding were collected in the glasshouse of Beijing Forestry University. The leaves were thoroughy washed under running tap water for 1 to 2 h and then cleaned with detergent Tween-20 with the help of a sable hair brush.These were then carried out by soaking the explants in filtered distilled water followed by 70% (v/v) ethanol for 1 min in a sterile beaker. After that, the explants were soaked in 10% sodium hypochlorite solution added with two drops of Tween 20 again (Sigma, USA) for 10 min **(**Yin Jia et al., 2014). The explants were then thoroughly rinsed with sterile distilled water to remove any traces of remaining detergents (Nurul Izzati Osman et al.,2016) .

### 2.2 Preparation of differentiation media and plant regeneration

Using MS as the basic culture medium (Murashige and Skoog, 1962), induction media were used to determine optimal conditions supporting in vitro callus formation of P. forbesii 84K Poplar. Different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0–2.0 mg/L) and kinetin (0.5–2.5 mg/L) were used in the study. Five levels of 2,4-D were tested (1.0, 1.2, 1.5, 1.8, 2.0 mg/l) along with five levels of KT (0.5, 1.0, 1.5, 2.0,

2.5 mg/L). The prepared media were consisted of 30 g/L sucrose and 7 g/L agar and the pH was adjusted to be within 5.6–5.8 by using 1mol/L HCl and NaOH before the addition of plant hormones ( Nurul Izzati et al., 2016).

The GFP gene was PCR-amplified from pCAMBIA1300 by using a pair of prim ers. The PCR fragment was then purified and cloned into pEASYTM-Blunt Cloning V ector (TransGen Biotech, Beijing, China) resulting in plasmid pEASY-GFP. It was veri fied by sequencing. While, both the plasmid pEASY-GFP and the plant expression ve ctor PBI121 were double digested by using Xba I and SpeI. The binary plasmid pBI1 21-GFP contains NosP-NPTII-Nos terminator expression cassette, GFP reporter gene w ith CaMV 35S promoter, T7 terminator and a spectinomycin bacterial selection marker. The binary plasmid was transferred into Agrobacterium strain GV3101. Plasmid integri ty in Agrobacterium was confirmed by PCR amplification with specific primer and do uble digestion identification with Xma I and Spe I (data not shown).

**2.3 Preparation of Agrobacterium for inoculation**

Single colonies were selected from the plate and inoculated in 20 ml YEP liquid medium plus various antibiotics. The bacteria were cultured in a constant temperature shaker at 28° and 200 rpm to OD=0.8-1.0. According to the ratio of 1-2%, the bacterial liquid was transferred into the YEP liquid medium containing antibiotics. After incubation at 28 degrees and 200 rpm for 6 hours to OD about 0.4 (or 0.8), centrifugation at 2000 g and 4 degrees for 10-12 minutes, the bacteria were suspended heavily with sterile 1/3 MS (with sucrose) solution (pH=5.2) (no shaking or suction with a transporter, light hand suspension) for explant infection and transformation.

**2.4 Infection and inoculation of leaf pieces**

Under aseptic conditions, leaves or stem segments of 84K poplar seedlings aged 3-4 weeks with robust growth were selected, and their sizes, shapes and colors were basically the same. Each leaf was cut into 0.5 X 2.0 size by cutting the main vein with the tip of a knife. The main vein was then infected in the differentiation medium for 10min, and then in the OD solution of about 0.4 (or 0.8) for 10min. During this period, the main vein was gently shaken

so that each leaf could be in close contact with *Agrobacterium tumefaciens*.

**2.5 Co-culture stage**

After the leaves were taken out and dried with sterile filter paper, the superfluou s bacterial fluid was spread on the back of the leaves downwards on the differentiatio n medium (MS+ 0.5mg /L, 6-ba +0.002mg/L TDZ+0.1mg/L NAA+80uM acetyleugeno ne), and co-cultured for 2-3d ( modified to 2 days) under the condition of 25 degrees of darkness, indicating that agrobacterium bacteria were around the explants of the le aves.

**2.6 Selective cultivation, Subculture and Rooting culture**

The co-cultured explants were rinsed with 250 mg/L cephalosporin water for 2-3 times and sterile water for 1 time, 3-5 minutes each time, and then transferred to the selective medium (differentiation medium + 250 mg/L cephalosporin + 30 mg/Kana). The medium was changed every 10 days, and the culture was selected under the condition of 16h/8h and 25 degrees of light.

The medium (differentiation medium+250 mg/L cephalosporin + 40-50 mg/Kana) was selected every 10 days to induce differentiation. The transformed adventitious bud gene containing kana resistance gene NPT-II can grow on selective medium, while the non-transformed adventitious bud gene does not contain resistance in vivo, so it can not survive on selective medium.

After 2 months, etc into bud length up to 2 cm, cut and insert with 50 mg/L kana rooting medium to take root in the culture, culture temperature is 25 ℃, 16 h / 8 h light cycle. When the adventitious buds grow to more than 1 cm, the adventitious roots can be cultivated on the rooting medium containing selective pressure, and the adventitious roots can be grown in about two weeks.

The rooting medium was cut and inserted. The culture temperature was 25 C and the light period was 16h/8h. When adventitious buds grow to more than 1 cm, adventitious roots grow on the medium containing selective pressure for about two weeks. After 2 months, the length

of the transformed buds reached about 2 cm. Cut and insert rooting medium with 50mg/L kana. The incubation temperature was 25 and the illumination period was 16 h/8 hours. When adventitious buds grow to more than 1 cm, adventitious roots grow on the medium containing selective pressure for about two weeks.

## Discussion

Plant growth regulators are synthetic molecules used in plants and supplemented at a relatively low concentrations to work as signaling compounds for plant growth and development (Sauer M et al., 2013). The most extensively used and studied class of plant growth regulators in plant tissue cultures are auxin and cytokinin, which is known as a type of auxin has been acknowledged to effectively induce callus formation in many plant species (IkeuchiM et al., 2013). The result from this study revealed that the presence of 2,4-D in the culture media was essentially required to induce callus formation in this species even though the cytokinin was absent. The effectiveness of 2,4-D in inducing the formation of callus is attributed to its main characteristic which can stimulate cell division of plant tissues and strongly suppress organogenesis. It is also considered to be the most potent among any other commonly used auxins (Staba EJ et al., 1980). In the current study, the presence of 2,4-D alone at the concentration of 1.0 mg/L–2.0 mg/L in MS medium was found to delay the formation of calli in which the calli only started to form during the second week of culture (Nurul Izzati et al., 2013). From the preliminary study conducted previously, the supplementation of singly present kinetin in the culture media did not promote callus induction and was not useful in promoting the formation of profuse calli (data not shown). However, the results from this study had shown that the addition of kinetin in the culture media in combination with 2,4-D was fruitful in producing callus. By looking at the trend of callus formation in this study, an increasing induction percentage was noted associated with an increase in kinetin concentration supplemented together with 2,4-D hormone at the concentration of 1.0 mg/L and 1.5 mg/L.

When infecting, do not take too many Agrobacterium in suspension medium, 50 ml of bacterial solution is more suitable. The callus should be placed in suspension medium of

agrobacterium agrobacterium for 15-30 minutes, not too long. The incubation time shall not exceed 72 hours, of course, if you see the agrobacterium grow, you should wash it immediately. And the sealing film must be sealed, to two or three layers, but also sealed with plastic film, to prevent damage to the sealing film, infection of fungi. In addition to measuring OD value, the shaking bacteria should also observe the state of bacteria, such as whether there is precipitation, whether the color is wrong, and whether there is a problem with turbidity.

## Conclusions

In this study, we describe a comprehensive culture method for callus induction and plant regeneration from 84K Poplar.A reliable protocol for inducing callus formation of profuse and friable morphology of 84K had therefore been successfully established.

The optimum treatment for callus induction in this study was identified in MS m edium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The optimum treatme nt for indefinite buds induction in this study was identified in MS medium supplemen ted with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The findings revealed that the supple mentation of kinetin at an optimum concentration and combination with 2,4-D is requi red to produce calli with the desirable morphology.

**Funding**

This work was supported by the The National Natural Science Foundation of China [3180030530].

**Corresponding author email:** wanglina@nefu.edu.cn

**References**

[1] Callus induction and haploid plant regeneration from baby primrose (Pri mula forbesii Franch.) anther culture

[2]Jin J Z, Liang S, Hui C, et al. An efficient Agrobacterium-mediated transformation method for the edible mushroom *Hypsizygus marmoreus*[J]. Microbiological Researc h, 2014, 169(9-10): 741-748.

[3] Ikeuchi M , Sugimoto K , Iwase A . Plant Callus: Mechanisms of Induction and Repression[J]. The Plant Cell, 2013, 25(9):3159-3173.

[4] Jia Y , Zhang Q X , Pan H T , et al. Callus induction and haploid plant regener ation from baby primrose (Primula forbesii Franch.) anther culture[J]. Scientia Horticul turae, 2014, 176:273-281.

[5]Kayani W K, Fattahi M, Palazòn J, et al. Comprehensive screening of influential f actors in the *Agrobacterium tumefaciens*- mediated transformation of the Himalayan elixir: *Ajuga bracteosa*, Wall. ex. Benth[J]. Journal of Applied Research on Medicin al & Aromatic Plants, 2016.

[6]Kalbande B B, Patil A S. Plant tissue culture independent *Agrobacterium tumefacie ns* mediated *In-planta*, transformation strategy for upland cotton (*Gossypium hirsutu m* )[J]. Genetic

[7] Li S , Ridenour J B , Kim H , et al. Agrobacterium tumefaciens-mediated transfo rmation of the soybean pathogen Phomopsis longicolla[J]. Journal of Microbiological Methods, 2013, 92(3):244-245.

[8] M. Liesebach, G. Naujoks. Approaches on vegetative propagation of difficult-to-ro ot Salix caprea[J]. Plant Cell Tissue and Organ Culture, 2004, 79: 239-247.

[9] Osman N I , Sidik N J , Awal A . Effects of variations in culture media and ho rmonal treatments upon callus induction potential in endosperm explant of Barringtonia racemosa L.[J]. Asian Pacific Journal of Tropical Biomedicine, 2016, 6(2):143-147.

[10] Martins P K, Ribeiro A P, Kobayashi A K, et al. A simple and highly efficient Agrobacterium-mediated transformation protocol for Setaria viridis[J]. Biotechnology Reports, 2015, 6: 41-44.

[11] Mallesham Bulle. Agrobacterium tumefaciens-Mediated transformation of Woodfor dia fruticosa[J]. Journal of Genetic Engineering and Biotechnology. 2015, 13(2): 201 -207.

[12] Parons T J, Sinkar V P, Stettler R F, et al. Transformation of Poplar by Agroba cterium tumefaciens[J]. Biotechnology, 1986, 4(6): 533-536.

[13] Rodrigues M B C, Fávaro L C D L, Pallu A P D S, et al. *Agrobacterium* -med iated transformation of *Guignardia citricarpa*: An efficient tool to gene transfer and random mutagenesis[J]. Fungal Biology, 2013, 117(8): 556-68.

[14] Shi Q , Liu P , Wang J , et al. A novel in vivo shoot regeneration system via callus in woody fruit tree Chinese jujube (Ziziphus jujuba Mill.)[J]. Scientia Horticultu rae, 2015, 188:30-35.

[15] Wu J , Philip A. O'Brien. Stable transformation ofRhizoctonia solaniwith a modi fied hygromycin resistance gene[J]. Australasian Plant Pathology, 2009, 38(1):79-84.

[16] Ziemienowicz A. *Agrobacterium* -mediated plant transformation: Factors, applicati ons and recent advances[J]. Biocatalysis & Agricultural Biotechnology, 2014, 3(4): 9 5-102.