Construction of *Agrobacterium*-mediated High Frequency Transformation System for *Populus tomentosa*[1]

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ABSTRACT ***Populus tomentosa*, as an important economic plant and as a natural host of *Agrobacterium tumefaciens*, has been used as a model plant for tissue culture and genetic transformation study of xyloghyta. In this work, several important factors influencing the efficiency of *Agrobacterium*-mediated transformation and regeneration of plants, such as types of explants, concentration of *Agrobacterium* suspension, wounding treatment on explants, conditions of pre-cultivation and co-cultivation, medium composition etc, were studied, and an *Agrobacterium*-mediated high frequency system was established for *P. tomentosa*. After pre-cultivation in the medium MSy for 48 h, the healthy sterile leaves were cut into discs, co-cultivated with suspension of *A. tumefaciens* LBA4404 at the concentration of 5×10^8 cells/mL for another 48 h, and then transferred onto the selective media containing appropriate antibiotics. The average transformation frequency of kanamysin resistant (Km_r) calli was up to 56.2%, and the ratio of the number of transgenetic plants to the number of explants was up to 19.9%. Only by wounding treatment alone, the transformation frequency was improved by 20~40%. With this transformation system, NP-1, a disease resistant gene, was successfully introduced into *P. tomentosa* and a lot of transgenetic plants were obtained.**

Keywords: ***Populous tomentosa*, *Agrobacterium tumefaciens*, high frequency transformation**

1. INTRODUCTION

Populus tomentosa is a local species of genus *Populus* that exhibits many excellent characters, such as broad adaptability to environment, short growth period and good lignum quality[1]. It is also an important economic plant in North of China. In addition, it is a natural host of *Agrobacterium*[2]. *P. tomentos*, as a model plant, has been the most important materials in the study of genetic transformation for xyloghyta.

Although the methods of tissue culture and techniques of genetic transformation for *P. tomentosa* have been established[2~5], the transformation frequency is very low and can not meet the practical requirement. In this study, we focused on optimizing the transformation system for *P. tomentosa* and the transformation efficiency was greatly improved.

2. MATERIALS AND METHODS

2.1. Plant materials and tissue culture

Sterile plantlet of *P. tomentosa* was from male plant of Yi County line by tissue culture. Three

types of explants, including petioles, leaves and stems of plantlets, were used for transformation. In an attempt to test the inducing transformation frequency, different kinds of media (**Table 1**) were exploited in this research.

The primary medium (MSo) was MS liquid medium[6] plus sucrose 30 g/L, pH 5.8~6.2. Ingredients supplemented to other kinds of media (MSf, MSy, MSg) were shown in **Table 1**.

As three different kinds of selective media, 50 mg/L kanamysin (Km) was added into MSo, MSy or MSf medium, respectively. After co-cultivation, 1 000 mg/L ampicillin (Amp) was also added to the above media respectively.

Table 1 Culture media used in the experiment

Medium	Ingredient (mg/L)			
	6-BA	2,4-D	IBA	ZT
Callus inducing medium (MSy)	1.0	4.0	-	-
Regeneration medium (MSf)	0.25	-	0.25	0.25
Root-inducing medium (MSg)	-	-	0.3	-

2.2. *A. tumefaciens* and plant expression vector

The *A. tumefaciens* strain LBA4404 containing the expression vector pBIC-35sNPI was used for transformation. The expression vector pBIC-35sNPI (Constructed by Group 603, Institute of Genetics, CAS) contained the NOS/NPI chimeric gene and the CaMV35s as promoter. Its Km resistant was pNos-NPTII-Nos3.

2.3. Plant transformation

It was performed essentially as described by Horsch[7] with modified details. Leaf explants from 3-week-old aseptic seedlings were cut into 0.5~1 cm^2 pieces with vein and leaf edge left out. The leaf discs were pre-cultivated in MSf medium for 0 h, 24 h, 48 hand 96 h, respectively, and some were punctured with needles or forceps as wounding treatment while others were left unwounded as control. Then the leaf discs were floated in 20 ml MS liquid medium containing 2 ml of activated *A. tunefaciens* in its log growth phase in a 50 ml Erlenmenyer flask. After about 10~15 min of infection, the explants were blotted with sterile filters and co-cultivation on MSy medium at 25℃ under dark condition for 24 h, 48 h and 96 h, respectively. The leaf discs were washed with sterile water and transferred onto selective media.

The protocol for transformation of stem and petiole was the same as that of leaf disc.

2.4. Plant regeneration

The healthy calli resistant to Km were transferred onto the selective MSf medium in an environmentally controlled growth chamber (25℃, 16/8h day/night). After 2~3 weeks, the shoots at the length of 2~3 cm were transferred onto root-inducing medium (MSg) without any selective agent.

Calli were subcultured once every 4~5 weeks.

2.5. PCR analysis

The genomic DNA of transgenetic and non-transgenetic plants was extracted as described by Fu Rong-zhao, *et al*.[8] Polymerase chain reaction was performed as follows: 3 min at 94℃(1 cycle); 45 sec at 94℃, 1 min at 55℃ and 2 min at 72℃ (22 cycles); and 10 min at 72℃ (1 cycle). The primers (34 bp and 26 bp) were designed based on NP-1 gene sequence.

3. RESULTS AND ANALYSIS

3.1. Difference of transformation frequency in different explants

The leaf discs, stems and petioles of plantlets were used as explants for transformation. Their transformation frequency was shown in **Table 2**. The leaf disc ranked first, the petiole second and the stem last in the transformation frequency among these three explants.

Table 2 Effect of types of explants on the transformation frequency of *P. tomentosa*

Type of explant	No. of explants	No. of Km^r calli (%)	No. of Km^r Plants (%)
Stem	523	13 (2.5)	2 (0.4)
Petiole	458	33 (7.2)	6 (1.3)
Leaf disc	621	76 (12.3)	26 (4.2)

3.2. Effect of pre-cultivation conditions on transformation frequency

Table 3 Effect of different period of pre-cultivation on the transformation frequency of *P. Tomentosa*

Time of pre-cultivation	No. of explants	No. of Km^r calli (%)	No. of Km^r plants (%)
24 h	145	18 (12.4)	11 (7.59)
48 h	150	89 (59.3)	20 (13.3)
96 h	207	67 (32.3)	9 (4.3)

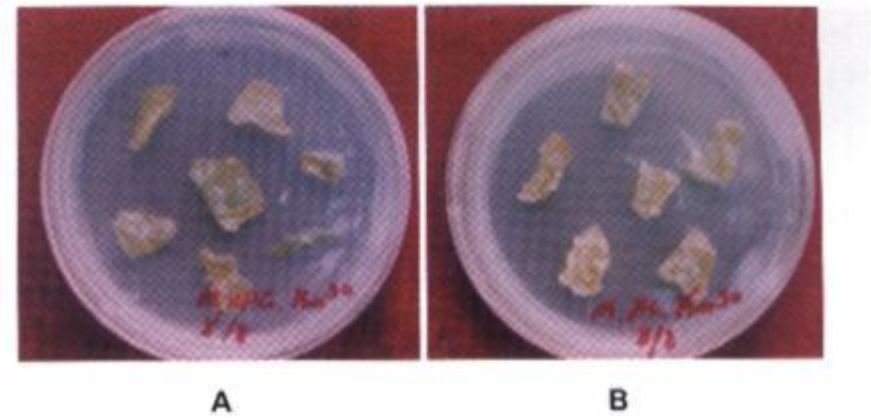


Fig.1 Km^r callus from non-precultivated (**A**) and precultivated (**B**) leaf discs were cultivated on selective medium containing 30 mg/L Km.

When the leaf discs, as explants, were given pre-cultivation treatment before transformation, their transformation frequency was improved significantly compared with those of without pre-cultivation treatment (**Fig.1**). The period of pre-cultivation treatment also affected the transfor-

mation frequency. As shown in **Table 3**, the transformation frequency reached the highest after 48 h pre-cultivation treatment. From **Table 4**, hormone ingredients in the pre-cultivation media also affected the transformation frequency. The transformation frequency was improved greatly while pre-cultivation treatment was conducted on calli inducing medium (MSy).

Table 4 Effect of different pre-cultivation media on the transformation frequency of *P. Tomentosa*

Type of pre-cultivation medium	No. of explants	No. of Km^r Calli (%)	No. of Km^r plants (%)
MSo	250	19 (7.6)	2 (0.8)
MSf	291	36 (12.3)	22 (7.56)
MSy	192	104 (54.2)	20 (10.67)

3.3. Effects of concentration of *A. tumefaciens* suspension and period of co-cultivation

During the transformation, three different concentrations of *A. tumefaciens* suspension (1×10^8, 5×10^8, 1.2×10^9 cells/mL) were used. The highest transformation frequency was achieved when the concentration was 5×10^8 cell/mL (**Table 5**).

Table. 5 Effect of concentration of *Agrobacterium* suspension and duration of co-cultivation on the transformation frequency of *P. tomentosa*

Factors		No. of explants	No. of Km^r calli (%)	No. of Km^r plants (%)
Concentration of *A.tumefaciens* (cells/mL)	1×10^8	380	39 (10.2)	2 (0.53)
	5×10^8	396	88 (22.2)	4 (1.01)
	1.2×10^9	428	32 (7.5)	3 (0.7)
Time of co-cultivation	24 h	331	25 (7.6)	4 (1.2)
	48 h	283	41 (14.5)	12 (4.2)
	96 h	204	2 (0.98)	1 (0.5)

While leaf discs were co-cultivation with 5×10^8 cells/mL of *A. tumefaciens* suspension for different periods of time (24 h, 48 hand 96 h), the best result was obtained by 48 h co-cultivation (**Table 5**). Extending or shortening the period of time reduced the transformation frequency.

3.4. Effect of the ratio of hormones in the selective media

After co-cultivation with *A. tumefaciens*, the leaf discs were cultivated in three different selective media which could induce different tissues. As shown in **Table 6**, the number of Km^r calli derived from leaf discs in MSy medium was the highst and the transformation frequency was the best among the three kinds of media.

Table. 6 Effect of type of selective medium on the transformation frequency of *P. tomentosa*

Type of selective medium	No.of explants	No. of Km^r calli (%)	No. of Km^r plants (%)
MSo	256	6 (2.3)	3 (1.2)
MSf	253	14 (5.5)	6 (2.4)
MSy	245	39 (15.9)	13 (5.3)

3.5. Effect of wounding treatment

It was reported that the leaf disc explants of chrysanthemum with puncturing treatment produced more transformants after infected by *Agrobacterium*[9]. In this experiment, the leaf discs with wounding treatment also presented obviously higher transformation frequency than those without wounding, as shown in **Table 7**.

Table.7 Influence of leaf wounding treatment on transformation frequency of *P. tomentosa* co-cultivation with *A. tumefaciens* strain LBA4404/pBIC-35sNPI

Leaf disc treatment	Pre-cultivation (+/-)	No. of explants	No. of Km^r calli (%)	No. of Km^r plants (%)
N	+	170	89 (52.4)	21 (12.4)
	-	158	34 (21.6)	9 (5.7)
P	+	115	73 (63.4)	20 (17.3)
	-	192	40(20.8)	13(6.7)

Note: **N**: leaf discs without wounding treatment; **P**: leaf discs with wouding treatment; +: precultured; -: non-precultured.

3.6. Amplification of NP-1 gene by PCR

In an attempt to test the effectiveness of this transformation system, six Km^r transformants were selected randomly for PCR analysis. From **Fig.2**, it could be seen that 5/6 of the transformants (83.3%) showed the presence of NP-1 gene as a band of expected size (197 bp), while the control (nontransformants) did not this band. This proved that most of the transformants were the transgenetic plants.

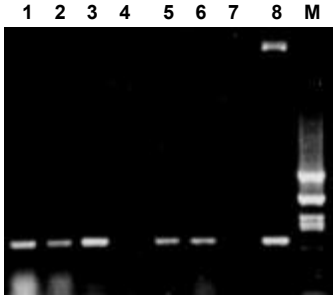


Fig.2 PCR analysis of six transgenetic lines: **1~6**, Transformed plant; **7**, Non-transformed plant; **8**, Plasmid DNA; **M**, Marker DNA.

3.7. Optimized high frequency transformation system for *P. tomentosa*

All of the above results proved that several factors, including the period of pre-cultivation, the infection concentration of *Agrobacterium*, the period of co-cultivation, the hormone ingredients in media and wounding treatment, could influence the frequency of transformation. After comparing these factors, an effective transformation system was established. First, the leaves of *P. tomentosa* were cultivated in MSy medium for a two-day period of pre-cultivation, and cut into discs of 0.5~1.0 cm^2 and then co-cultivated with *Agrobacterium* suspension at the concentration of 5×10^8 cells/mL for 48 h (**Fig.3a**). Then, the leaf discs were transferred onto the selective medium (MSy medium containing 50 mg/L Km). Many Km^r calli would grow up around the leaf discs in

2~3 weeks (**Fig.3b**). Next, Km^r calli were transferred onto the regeneration medium (MSf medium containing 50 mg/L Km and 1 000 mg/LAmp) for shoot formation. The shoot of about 3~4 cm length were excised and put onto the rooting medium (MSg) without any selective antibiotic. Finally, after 3~4 weeks, a complete regeneration plantlet could be harvested (**Fig.3c**). According to our repetitive experiments, the average transformation frequency of Km^r calli by this transformation system was up to 56.2% and the ratio of regenerated transgenetic plants to Km^r calli was 19.9%, which was much higher than those by other systems reported in previous papers.

Fig.3 Plantlets of *P. tomentoa*, regenerated from transformed callus on selective medium containing 50 mg/L Km. **a**, callus; **b**, plantlets; **c**, plant.

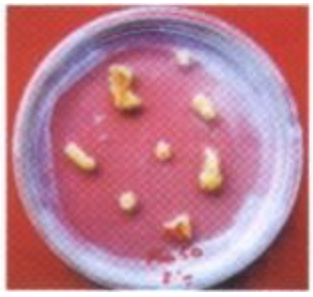
4. DISCUSSION

The aim of this investigation was to establish an efficient transformation system for *P. tomentosa*. Various factors that influence the transformation frequency were optimized. Following is some interesting events occurred in our study.

4.1. The valid concentration of Km as the selective antibiotic

In the previous works of genetic transformation of *P. tomentosa*, people used to employ,

Fig.4 No callus was formed when non-transformed explants were cultured on the medium containing 50 mg/L Km.



Agrobacterium rhizogenes with no selective marker gene or *Agrobacterium* with CAT gene as the

reporter. In this study, we used *Agrobacterium* with NPtII gene in pBIC-35SNP1 expression vector as the selective gene. As we know, plant cells expressing NPtII gene have natural resistance to Km, G418 and neomycin. But the strength of resistance is different among different kinds of plants. So the concentration of antibiotics used for the selection varied from one plant to another. As for the concentration of Km was concerned, it varied from 25 mg/L in sunflower to 400~800 mg/L in wheat. In the genetic transformation of *P. nigra*, the working concentration of antibiotics changed even from one clone to another[10]. The results in our experiment indicated that the formation of calli and the differentiation of petiole and leaf were inhibited completely in the medium containing 30 mg/L Km (**Fig.4**). All three types of explants lost their differentiation capacity as the concentration of Km was increased to 50 mg/L. Therefore, it can be concluded that the optimal concentration of Km as the selective antibiotic is 50 mg/L.

4.2. The effect of Amp on the growth of *P. tomentosa*

In an attempt to restrain the growth of the remaining *Agrobacterium* after infection, 1 000 mg/L Amp was added to the selective media. But in our previous experiments, when 1 000 mg/LAmp was added to the root inducing medium (MSg), it had little effect on the radial growth and rooting of transformants. However, it severely restrained their longitudinal growth, which made the seedlings exhibit shorter internodes and smaller leaves. After 40 days of culture under the same condition, the terminal buds of seedlings stopped developing and the leaves started to shed, which indicated that the seedlings had gone into the dormancy state. When these seedlings were transferred onto the medium containing 300 mg/LAmp, they recovered and developed again. All of these indicate that high concentrations of Amp have obvious negative effects to the growth of *P. tomentosa* transformant seedlings. Therefore, the concentration of Amp should be reduced in root inducing medium in order to get healthy and normal regeneration plantlets.

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