Leaf Age Promotes Agrobacterium-Mediated Transformation of Hybrid Poplar Populus Davidiana Dode × P. Bollena Lauche

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Abstract. Poplar as a woody-plant model is significant to improve the transformation efficiency of poplar. The effect of different leaf age as explants on transformation frequency was studied. The results exhibited that the higher transformation frequency was generated by 30-day-old leaf explants, compared with 60-day-old and 90-day-old leaf explants. The transformation frequency was more than 30%. The results indicated that various of transformation frequency were led by different leaf ages explants under the same conditions. The status of Agrobacterium attachment were observed by scanning electron microscopy (SEM) in the process of transformation. The results exhibited that Agrobacterium attachment were gradually reduced with the increased in leaf ages. Leaf surface at thirty-day-old showed massive Agrobacterium attachment. Our results suggest that younger leaves had higher transformation efficiency than older leaves.

Introduction

Populus davidiana Dode × *P. bollena* Lauche is planted on a significant scale in northeast of China due to its characteristics as graceful shape, cold resistance, fast growth, and suitability to develop transgenic.

Several factors such as the *P. nigra* genotype, *Agrobacterium* tumefaciens strain, bacterial concentration and the presence of acetosyringone on improving the transformation efficiency were systematic analyzed [1,2,3]. Mohri reported that acetosyringone markedly enhanced the efficiency of Agrobacterium tumefaciens-mediated transformation of Japanese white birch [4]. Sunilkumar and Han reported that preincubation of cut leaf tissues, which brought about an increase on transformation efficiency and also increased Agrobacterium vir gene induction [5,6]. Explant source variables studied included leaf disc and stem for transformation [6,7]. Although the effects of some factors on transformation efficiency have been studied, little attention has been paid on explants ages, which can increase the transformation efficiency in our experiments.

In the present study, Populus davidiana Dode × P. bollena Lauche was used for plant transformation. Under the same conditions, the effect of different leaf ages explants on transformation frequency was studied. The status of Agrobacterium attachment of different leaf age explants was observed by scanning electron microscopy (SEM) in the same transgenic conditions. Green fluorescent protein (GFP) as a selection marker was used to monitor the transformation events.

Materials and Methods

Plant Material, Agrobacterium Strain and Plasmid

30-, 60-, and 90-day leaf explants were obtained from Populus davidiana Dode × P. bollena Lauche for transformation. MD-GFP was a marker using for plant transformation. The plasmid pBI121-MD-GFP transferred into the Agrobacterium tumefaciens EHA105, was driven by the CaMV 35S promoter.

Effects of Leaf Ages on Plant Transformation

MS medium was supplemented with the combination of BA (0.3mg/L) and NAA (0.08mg/L) for explants regeneration. And NAA at 0.25mg/L was most effective for root regeneration(100%). 30 mg/L Kan was used for differentiation selection pressure, 40mg/L Kan was used for rooting selection pressure and 200mg/L Cef was used for inhibition in the process of transformation properly. Agrobacterium concentration OD600=0.8-1.0 and infection time 20-30min were used to transformation. Under the same conditions above, the effect of different leaf ages as explants on transformation frequency was studied. Data were subjected to completely randomized design and statistically analyzed using SPSS.

Observations of Bacterium Attachment in the Scanning Electron Microscopy

The leaf explants used for transformation were washed five times for 15s in 0.2 M phosphate-buffer (pH=6.8) containing 0.9% (w/v) NaCl to remove bacteria that were not firmly attached after co-cultivation. Then the leaf explants weresampled and fixed in 50% FAA for 2 days, dehydrated through an EtOH series, coated with platinum and examined by scanning electron microscopy.

Expression of MD-GFP Fusion Protein in Populus of Tissue Culture

The leaf, shoot and root were taken away from transgenic populus of tissue culture and observed in confocal microscopy. Confocal microscopy was performed with a TCS SP2 laser-scanning confocal imaging system (Leica). GFP fluorescent signals were detected with excitations at 488 nm and 543 nm respectively.

Results

Based on the same optimal factors above, the transformation frequency was more than 30% using juvenile leaf explants, which were 30-day-old. And the minimum transformation frequency was 10% using the oldest leaf explants, which were 90-day-old. The result showed that the juvenile leaf explants used for transformation were had more resistance shoots differentiation than older leaf explants shown in Table 1.

Table 1. Frequency of the transformation of *populus davidiana* Dode ×*P. bollena* Lauche with different explant ages

	Explant age	Transformation frequency(%)
1	Thirty-day-old	34.18±1.69a
2	Sixty-day-old	21.05±1.12b
3	Ninety-day-old	10.00±1.09c

Agrobacterium attachment to plant surfaces can be affected by plant tissue age [8]. Attachment of *Agrobacterium* to host plant cells is the most important step in the genetic transformation process. *Agrobacterium* attachment influences the genetic transformation ability. To further confirm that the leaf age was one of the important

influence factors for *Agrobacterium* mediated transformation, *Agrobacterium* attachment with scanning electron microscopy was studied. The results showed that poplar leaf at various stages of development interacted with *Agrobacterium* attachment differently. Massive *Agrobacterium* attachment was observed on the 30-day-old leaf surface, e.g. see Figure 1B. The number of *Agrobacterium* cells attached to leaf surface of 60-day-old was considerably less than that found on the 30-day-old leaf surface, e.g. see Figure 1C. Evenly distributed individual cells of *Agrobacterium* were found to attach on the surface of mature leaf, e.g. see Figure 1D. Under the same conditions, different transformation frequency was generated by the various leaf ages, 30-day-old leaf explants in transformation obviously increased the frequency of transformation. *Agrobacterium* attachment observed in scanning electron microscopy (SEM) showed that leaf surface at thirty-day-old showed massive *Agrobacterium* attachment. Above all, Our results suggest that younger leaves had higher transformation efficiency than older leaves.

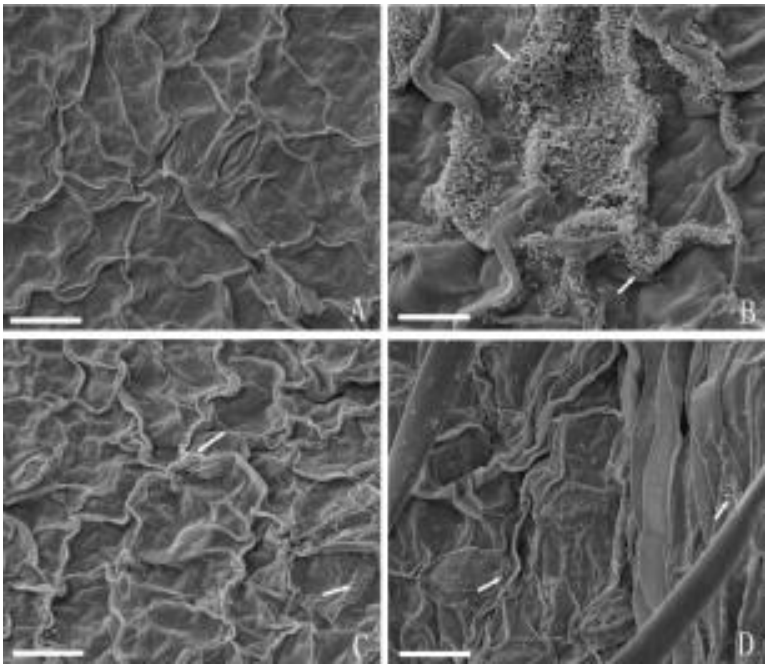

Figure 1. *Agrobacterium* attachment on leaf surfaces of different leaf age explants by scanning electron microscopy.

A. Uninfected leaf as control; B. 30-day-old leaf explants; C. 60-day-old leaf explants; D. 90-day-old leaf explant. (Scale bar 20 μm)

As show in Fig. 2, the GFP fluorescence signals from the leaves (Figure 2C) of transgenic plants were detected, but were not detected in the leaves (Fig. 2A) of non-transgenic plants. The pBI121-MD-GFP plasmid was introduced into hybrid poplars *P. davidiana* Dode × *P. bollena* Lauche.

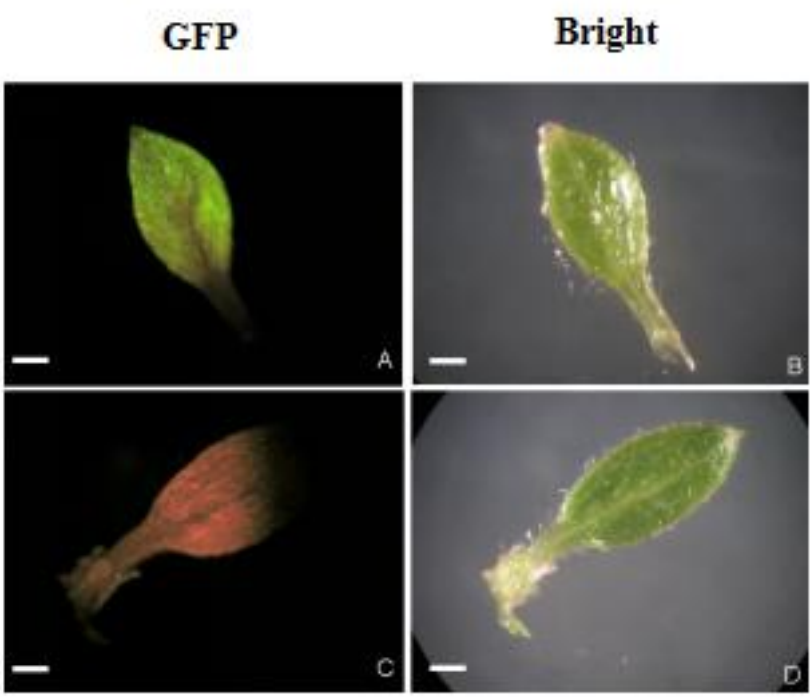

Figure. 2. Expression of the MD-GFP fusion protein in transgenic poplar.

GFP fluorescence of transgenic poplar expressing MD-GFP fusion protein was detected in the leaves (A), Non-transgenic poplar leaves (C). Left column (A, C) was GFP fluorescence images and right column (B, D) was the optical images. Scale bar 0.2 cm.

Discussion

Our data showed that poplar leaf at younger stage was more competent to *Agrobacterium* mediated transformation and this competence was reduced with the age. The possible reason of observed differential response of *Agrobacterium* attachment to different tissues may be due to change in cell wall composition of cells that are at different physiological and developmental stages. As the plant cell gets mature, its cell wall composition changes in such a way that *Agrobacterium* binding sites are lost gradually. Mature cells of intact leaf epidermis having limited number *Agrobacterium* attachment sites have evenly distributed *Agrobacterium* while undifferentiated explants and split shoot apex having young cells with plenty of *Agrobacterium* attachment sites favored development of *Agrobacterium* aggregates [9].

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