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Genetic Transformation of *Populus nigra* × *maximowiczii* Using *Agrobacterium tumefaciens* Harboring Antisense OMT Gene

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Abstract

An *Agrobacterium tumefaciens* LBA4404 (harboring antisense OMT gene)-mediated transformation method has been developed for poplar (*P. nigra* × *maximowiczii*) using prolonging co-cultivation time. Explants on LT (long-term) were induced transgenic calli one month earlier than those from ST (short-term) co-cultivation and remained healthier on LT than ST. With this approach, LT method reduced time to produce transgenic calli. Shoots were successfully regenerated from transgenic calli on SIM (Shoot Induction Medium) and rooted well on the basal medium spontaneously. The presence of antisense OMT gene was verified both by PCR and Southern analysis. Each transgenic poplar was phenotypically indishtinguishable when compared with controls for their growth pattern, leaf morphologies and xylem coloration.

Introduction

There are various gene transfer systems available for delivering foreign genes into plant cells or tissues such as *Agrobacterium*-mediated transfer, microinjection, biolistic gun, electroporation, and laser delivery etc (Potrykus, 1990). Among these, *Agrobacterium* and biolistic-mediated transformation systems have been used most often. Limited *Agrobacterium* host range and transient gene expression via the biolistic gun have been the focus of many investigations (Chaudhary et al., 1998; Denchev et al., 1997; Xiao and Ha, 1997). Recently, numerous advances have been made in the development and optimization of procedures for transformation of plant species (Eady et al., 2000; Niu et al., 2000; Zhang et al., 2000). However, most of them have concerned with developing plant vector systems and mechanical methods in biolistic guns rather than studying plant factors.

Plant cells or tissues must become competent prior to accepting foreign genes. This can be accomplished under specific conditions such as growing cells and tissues on defined media supplemented with plant growth regulators (Christianson and Warnick, 1983; 1987). During this period, they undergo active cell metabolism and division, and are amenable to incorporating delivered DNA into the host plant genome (Potrykus, 1990). It is likely that the frequency of transformation may be closely related to competence of cells for transformation events, resulting in higher frequency of transformation events. Until now, there are no extensive studies on cell competence and its relationship to regeneration and transformation so far.

The ultimate goal of our research is to obtain transgenic poplars with low-lignin contents for commercial paper industry and environmental purposes. As a basic step, this research was to increase the frequency of transformation

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using prolonged co-cultivation time and obtain transgenic poplar (*P. nigra ×maximowiczii*) via *Agrobacterium tumefaciences* harboring an antisense *O-metyltranferase* gene which encodes one of the important lignin biosynthesis enzyme.

Materials and Methods

Plant materials and culture media

In vitro stocks of *Populus nigra ×maximowiczii* were initiated from stem segments *in vitro* on half-strengthen MS medium (Murashige and Skoog, 1962) in a culture room at 22-25℃, with a 16:8 h light/dark cycle. The plantlets were further propagated on the same basic medium but containing 0.5 mg/L BA, through subcultures of stem segments. All media contained 2% (w/v) sucrose (Merck), 100 mg/L myo-inositol, Staba vitamins (Staba, 1969) and 0.7% (w/v) agar (Difco-Bacto). For callus induction, all explants were cultured under the dark condition on 1/2MS medium with 1 mg/L 2,4-D and 0.1 mg/L BA. For adventitious shoot induction from callus, proliferating callus about 0.5 to 1 cm in size was cultured on the 1/2 MS supplemented with 1 mg/L BA but without 2,4-D under the light condition.

Constructions of plasmid and transformation protocols

The *Agrobacterium tumefaciens* strain (LBA4404), a non-oncogenic strain, was used for this experiment. The pBIROMT vector plasmid (12kb, Figure 1) contains a 1.1 kb fragment that was cloned from *P. nigra ×maximowiczii*. Sense OMT gene was cloned into the pBI121 binary vector in reverse orientation replacing with GUS coding gene. The recombinant plasmid pBI121ROMT was subsequently transferred to A. tumefaciens by a freeze-thaw method. Bacterial cultures were grown overnight at 27℃ on a rotary shaker (120 rpm) in 50 mL LB (Luria Broth), with antibiotics (kanamycin: 50 g/mL and streptomycin: 100 μg /mL). When an OD 600 nm of 0.5-0.7 was reached, bacterial suspensions were centrifuged (3000× g, 15 min). The pellets were washed twice and diluted 10-fold in liquid regeneration medium with growth regulators. These suspensions were used for transformation. All leaf explants were precultured for 2 days on CIM (1/2MS medium containing 1 mg/L 2,4-D and 0.5 mg/L BA). They were then subjected to two different transformation protocols as follows:

1) Use of a general transformation method (called ST) which immersed explants in the bacteria suspension for short period (20 min). After blotting with sterile filter paper, the explants were co-cultivated for additional 48 h on the same CIM. Following co-cultivation, the inoculated explants were rinsed with sterile distilled water containing 1 g/L cefotaxime and 1.5 g/L carbenicillin, placed on selective callus induction medium (50 mg/L kanamycin, 100 mg/L carbenicillin and cefotaxime), and incubated in the dark until callus was formed. The bacterial suspension was omitted in control plates.
2) Use of a long co-cultivating period (called LT) for 4 weeks which culture explants on callus induction medium mixed with bacterial suspension. Ten-fold diluted bacterial suspension (100 μL bacterial suspension/100 mL of CIM) which described in the protocol above was mixed with pre-cooled CIM before distributing petridishes. Explants were cultured on CIM without antibiotic for one week and then those were transferred to the same medium with kanamycin and *Agrobacterium* but without carbenicillin and cefotaxime. After one week, explants were subcultured on the same CIM containing *Agrobacterium* and kanamycin. After additional 4 weeks of co-cultivation, the explants were transferred to regeneration medium with kanamycin and cefotaxime 200 μg/mL but without bacterial mixture.

Leaf explants were subcultured every week on selective CIM for the first 8 weeks. Control explants were cultured using the same conditions described for the co-cultivated leaf explants. Each developing callus was excised from the primary explants, cultured on the same CIM for 6 months

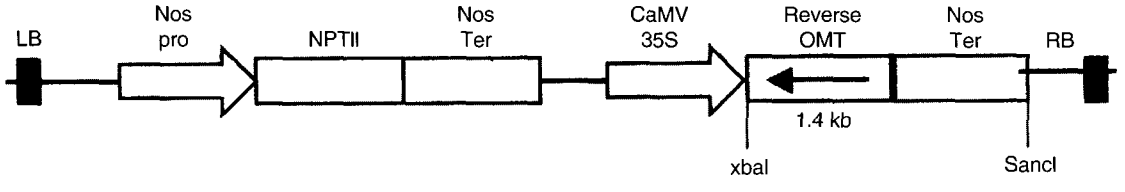

Figure 1. Schematic representation of the binary plasmid pBI-ROMT. Arrows indicate orientation of genes. RB: right border, nos-pro: nopaline synthase gene promoter, nptII: neomycin phosphotransferase coding gene, nos-ter: nopaline syntase termator, CaMV35S: cauliflower mosaic virus 35S promoter, ROMT: reversely orientated OMT coding gene, LB: left border.

additionally, and those were then transferred to selective regeneration medium containing a higher level of BA (1 mg/L) but without 2,4-D. Once adventitious shoots appeared, they (1-2 cm in size) were elongated on the same medium in the absence of growth regulators and reduced kanamycin concentration (from 50 to 15 mg/L).

Regenerated plantlets which rooted under those conditions were transferred to the greenhouse for further growth and development.

Screening of transgenic callus lines and Southern analysis regenerants

PCR analysis of putative transgenic calli for OMT gene was as described by Niu et al. (1998) using the following PCR primres:
sense: GGGGGATCCAAGATTCAACAAG3' and anstisense: 5'GGGGTCGACGGCCTTCTTGCGGAA3' for OMT gene

Each genomic DNA from five randomly selected regenerants was isolated by using Sul and Korban protocol (1996) and Southern analysis was done using OMT PCR products. Separated DNA on 1% agarose gel was transferred onto positively charged nylon membrane (Amersham pharmacia biotech. NJ USA). Prehybridization and hybridization were carried out using PhotoGene™ Nucleic Acid Detection System (Gibco BRL, Bethesda, MD USA) by the manufacturer's protocol. The probe used in the hybridization reaction was the 0.6 kb PCR product amplified from OMT gene and prepared using BioNick™ Labeling System (Gibco BRL, Bethesda, MD USA) by the manufacturer's protocol.

Results and Discussion

Seven days after explants on selective CIM (Callus Induction Medium) containing kanamycin and cefotaxime from ST method, most of non-transgenic parts of the explants became be bleached and ultimately died within 2 weeks while other parts remained healthy (Figure not shown). However, there was no sign of callus induction from the explants from ST method until 3 months on the selective CIM. This delay of callus induction may be due to earlier antibiotic application on CIM. On the contrary, control explants produced calli mostly from wounding sites on CIM within 2 weeks. In the case of LT method, the explants showed somewhat different response than those from ST. The explants became swollen at wound sites after 2 weeks on CIM medium containing *Agrobacterium* without callus induction. Like control (leaf explants on CIM without *Agrobacterium*), the explants showed some responses like swelling from wound sites. However, there was no actual callus induction. Unlike ST explants, those on the CIM with antibiotics but without adding *Agrobacterium* after one month co-cultivation responded rapidly by forming calli. Consequently, leaves on LT started to produce callus within a month on selection CIM. This was one month earlier than in case of ST method.

We do not know why explants from LT responded earlier than did ST explants. However, this difference may be due to increasing larger number of competent cells formed during longer co-cultivation period. Christianson and Warnick (1983) reported that plant cells of a tissue become competent resulting in active cell division and determination for regeneration. High frequency of competent cells finally leads to the higher frequency of regeneration. In our research, competent cells, during ST periods, might not have enough time to be induced resulting in delaying callus induction because of early application of antibiotics but, on the contrary, competent cells from LT may induce enough time to produce callus during 4 weeks' co-cultivation. As a result, calli might be induced earlier than those from ST. According to earlier reports, two to four days' co-cultivation was mostly adapted to induce transgenic plants including other poplars (Confalonieri et al., 1997). However, results from transformation with *P. nigra* × *maximowiczii* showed longer duration with *Agobacterium* may supply enough time to transform cells.

In a practical sense, reduction of time to obtain calli and/or adventitious shoots may be one of the important aspects of tissue culture (Pierik, 1987). The LT explants responded as earlier as one month than those of ST explants resulting in the reduction of the time needed to produce transgenic plants. The LT method can also be very useful to other plant species which show highly antibiotic sensitive characteristics. Earlier application of antibiotics after co-cultivation with *Agrobacterium* might lead cells or tissues to death resulting in lower frequency of transformation. Thus, prolonged co-cultivation from two days to a month results in higher frequency of transformation only with appropriate selective pressures. However, time for co-cultivation should be considered.

The frequency of callus formation from explants showed similar with those of LT explants. However LT explants appeared much healthier than those of ST (Figure not shown). In the case of ST method, frequency of callus formation was 31.6% (142 out of 408 explants), while LT method produced 33% (130 calli out of 390 explants) (Table 1). Even though there is no significant difference on callus

Table 1. Effect of two co-cultivation durations on callus induction obtained after 12 weeks of culture on selective CIM. Control means *P. nigra* × *maximowiczii* leaf samples cultured on CIM only. Control ST is leaf samples cultured on the selective CIM after 2 days' co-cultivation. Control LT is leaf samples cultured on the selective CIM with only 3 weeks after 1 week on CIM with kanamycin.

Treatments		Total no. of leaves	No. of leaves forming callus
Control		50	47 (94%)
ST	Control ST	50	19 (38%)
	2 days	408	142 (31.6%)
LT	Control LT	50	44 (88%)
	30 days	390	130 (33.0%)

induction between two methods, LT methods can be an alternative way because the LT explants were healthier. With minor modification of the protocols such as initial concentrations of bacteria before mixing with CIM and exposure periods of explants to medium containing *Agrobacteria*, increased transformation rate could be achieved.

Upon continuous culture on the selective CIM, resistant calli started to form mostly from wound areas, while control explants showed excellent callus induction and growth on CIM. Unlike herbaceous plants, poplar calli showed extremely slow growth on the selective CIM medium containing kanamycin (50 mg/L). After 10 months' culture on the selective CIM medium, calli managed to reach in a diameter of 1 to 2 cm. To accelerate the callus growth, reduced kanamycin concentrations from 50 to 15 mg/L were employed. As a result, the calli proliferated faster than those from higher concentration of kanamycin. To select transgenic callus lines, PCR amplification before each subculture was performed using samples from proliferating calli. The confirmed transgenic calli were further proliferated on the selective CIM.

To induce adventitious shoots from transgenic calli, each callus was dissected out into 0.5 cm in diameter and cultured on the selective SIM (1/2 MS containing BA and kanamycin) under the light. Within two weeks, some calli showed color change from tarnished yellow to green. However adventitious shoots were appeared after 6 months on SIM. Shoots from calli were then dissected out and cultured on the basal 1/2 MS medium without any growth regulators but with antibiotics to elongate shoots further (Figure 2). Shoot growth was much slower than those of controls. After 6 months' on SIM with every 2 week subculture, about 30 adventitious shoots were developed from callus lines enough to examine morphological differences.

Southern analysis with OMT probe also confirmed the positive signals in all samples but not in controls (Figure 3). PCR amplification of the OMT gene from transgenic plants resulted in the expected band suggesting that they contained introduced OMT gene (Figure 3).

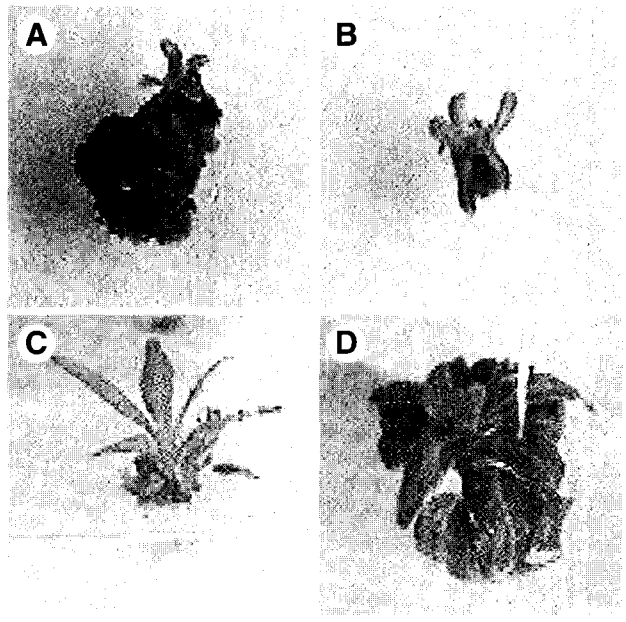

Figure 2. Induction of adventitious shoots from proliferating callus on selective SIM, A; Adventitious shoot was induced from callus on selective SIM after co-cultivating with *Agrobacterium tumefaciences* harboring pBI-ROMT, B, C and D; Developing adventitious shoots on basal 1/2 MS medium without PGR but with kanamycin.

According to the Baucher et al. (1996), transgenic poplars with sense and antisense CAD genes showed peculiar characteristics, such as red-colored xylem, different anatomical xylem structure etc. On the contrary, Dwivedi et al. (1994) reported that there was no morphological and structural difference in the transgenic tobacco plants with antisense OMT gene compared with control plants. In our study, transgenic *P. nigra* × *maximowiczii* did not show any significant morphological differences and red-colored xylem except shoot erectness. Moreover the growth rate for regenerants is faster than those from controls and frequency of rootings are 100% which is consistent with earlier reporter (Hu et al., 1999). However, most shoots of transgenic plants were crooked and weaker than those of controls *in vitro*. Currently, we are investigating further in

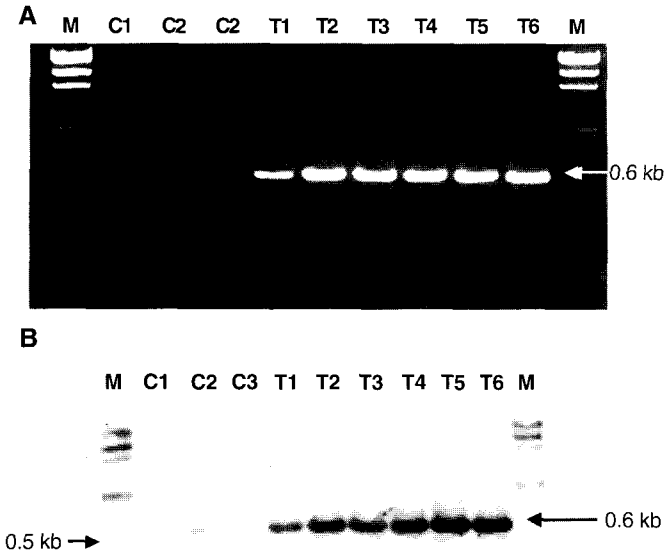

Figure 3. PCR amplification and Southern analysis. A; Agarose gel electrophoresis of PCR products performed with OMT primers. Genomic DNAs were isolated from randomly selected putative transgenic poplars, B; Southern analysis of PCR products from randomly selected putative transgenic poplars. M; Molecular marker (*Hin*dIII cut lamda DNA), C1: PCR without any template DNA, C2: PCR with pBIROMT, C3: PCR with control poplar, T1 to T6: putative transgenic poplars.

the greenhouse if such characteristics were due to low lignin contents. So far after 6 months in growth, those are showing the same characteristics like control poplars meaning that all transgenic plants showed the normal phenotypes and status.

Transformation efficiency can be increased by manipulating either the explants and/or the bacteria, such as by increasing the number of competent plant cells for transformation by pre-culturing explants (Mchughen et al., 1989; Sangwon et al., 1992), by adding phenolic compounds like acetosyringone to activate efficient vir gene induction in the *Agrobacterium* (Stachel et al., 1985). However, there is no report regarding to co-cultivation period to increase transformation efficiency until now. In general, up to for 2 to 4 days co-cultivating duration has been widely employed (Henzi et al., 2000; Dronne et al., 1999; Tzfira et al., 1997). In this report, we developed a method allowing the production of transgenic poplar by simply prolonging co-cultivation periods with *Agrobacterium tumefaciens*. Advantages of our transformation procedure are that it takes less time to obtain transgenic calli resulting in less labor-intensive and more production of transgenic plants.

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