Differential Expression of a Chimeric *nos-npt* II Gene in 9 Years Old Hybrid Poplars (*Populus koreana x P. nigra*)

Eun Woon Noh*, Jae Soon Lee, Young Im Choi, Hyo Shin Lee, Eun Kyung Bae, Ji Hee Lee

Biotechnology Division, Korea Forest Research Institute, Suwon, 441-350 Republic of Korea

Abstract

The expression of a chimeric transgene (*nos-npt* II) has been examined in 9 years old transgenic poplars (*Populus koreana x P. nigra*) growing in a nursery. The expression of the gene in twenty six independentely transformed plants were examined by 1) enzyme (*NPT* II) assay, 2) RT-PCR, and 3) resistance to kanamycin. High *NPT* II activities in young leaves of all the transformed plants were found even without a selection pressure for antibiotics for 9 years. However, the activity varied with the positions of leaves in the stem in that young leaves showed higher activity than did mature tissues. When leaf segments were cultured in the presence of 150 mg/l kanamycin, only those from young leaves produced vigorously growing callus. However, as in the case of *NPT*II assay, the leaf segments from mature leaves did not form callus well on the media. RT-PCR with *npt*II specific primers also showed that amplification products were observed only when RNAs from young tissues were used. The total RNA gel showed that while RNA in young leaves are relatively stable and in a large quantity, those in old leaves were mostly degraded. All the above results suggest that the gene is transcriptionally active only in young tissue even though it is attached to a constituitive promoter. Therefore, the expression of foreign gene in poplar plants seemed to be affected by the metabolic state of the cells and thus vary greatly with the developmental stages and the age of tissue.

Key words: Nos-*npt*II, Gene expression, Kanamycin, Transgene, Poplar, Transformation

Introduction

Plant genetic transformation *via Agrobacterium tumefaciens* vector has now become a routine to produce genetically modified organisms. In many plant taxa, transgenic plants have been successfully regenerated after cocultivation with *A. tumefaciens* vectors (See review by Weising et al. 1988). In the Northern hemisphere, poplars are extensively planted for pulp and fuel wood due to their fast growth. In addition, the ease of regeneration from cell and tissue cultures has made them a model system for plant genetic transformation. Protocols for gene transfer using *A. tumefaciens* vectors have already been developed for poplars (Fillati et al. 1987, Confalonieri et al. 2000, Delledonne et al. 2001). However, since trees grow in the absence of any selection pressure for years, it is necessary to examine the stability and expression pattern of transgenes in the plants (Zambryski et al. 1983). Furthermore, the transgenes have been stably inherited from the plants to their offsprings by Mendelian fashion (Horsch et al. 1984). However, no such information is available in detail with woody species that have a long life cycle. Nevertheless, the information on the expression of the gene in different tissues would be highly useful in trees. Chimeric genes containing coding regions of bacterial origin and regulatory sequences (promoter and enhancer) of nopaline synthase or other plant genes have been constructed and used often to transfer certain selective markers such as kanamycin resistance and hygromycin resistance (An et al. 1985, Becker 1990, Bevan et al. 1983). These chimeric genes have also been used to monitor their expression in the host plants (Scott and Draper 1987). With the availability of several assaying tools, it is possible to compare the level of gene expression in different tissues. Among them are enzyme-linked immunosorbent assay

(ELISA), RT-PCR, and northern hybridization. With the added feature of resistance, these chimeric genes are excellent markers for assaying gene expression in transgenic plants.

Here, we report the expression of a chimeric *nos-nptll* gene in 9 years old transgenic poplars growing in a nursery.

MS: Murashige and Skoog's (1962) medium; 2,4-D: 2,4 dichlorophenoxy acetic acid; NAA: naphthalene acetic acid; BA: 6-benzyladenine; *NPT*II: neomycin phosphotansferase II; PCR: polymerase chain reaction; RT-PCR: reverse transcription PCR

Materials and Methods

Transformation and regeneration of poplars

The transformation of a hybrid poplar (*Populus koreana x P. nigra*) was done during August to October 1993 with a *Agrobacterium tumefaciens* LBA4404 strain carring pBIB-KAN (Becker 1990). The methods of transformation and regeneration of poplar were described elsewhere (Noh et al. 1994). The regenerated plants were acclimatized in a humidity controlled container for 4 weeks before transfer to the greenhouse. After 4 to 5 weeks hardening off in the greenhouse, they were transplanted to a nursery in the first week of May 1994. Every fall, the above ground biomass (i.e. stems and leaves) was removed so that new stems could grow from the stump in spring.

Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from the leaves of 9 years old poplar plants growing in a nursery using a MagExtractor-Plant Genome kit (Toyobo, Japan). Ten μg of the genomic DNA were digested with restriction enzyme *Pst*I overnight. The DNA was then run on 1% agarose gel and transferred to Hybond-XL nylon membrane by capillary transfer method (Southern, 1975). It was then hybridized with ^{32}p-dCTP labelled 800bp *npt*II gene fragment for 12 h. The membrane was washed in 2×SSC and 0.1% SDS (50℃) for 10 min and in 0.2×SSC and 0.1% SDS (50℃) for 30 min followed by exposing to an X-ray film at -70℃.

PCR and RT-PCR

The transgenic plants growing in the nursery were tested for the presence of the transgene by PCR amplification. DNAs were extracted from the leaves of all the transgenic

plants by the methods of Junghans and Mezlaff (1990). Two ng of DNA were used for PCR with the primers specific to *npt*II coding region (Pridmore 1987). They contained the following bases; NPT1: 5'-TTG TCA AGA CCG ACC TGT CC-3' NPT-2: 5'-GAA TCG GGA GCG GCG ATA CCG TAA A-3'. The 30 μl reaction mixture contained 10X Taq DNA polymerase buffer (supplied by Promega Co.), 1.5 mM $MgCl_2$, 200 nM dNTPs, 0.4 μM primer, 2 ng template DNA, and 1.5U Taq DNA polymerase. Thirty five thermal cycles consisting of 20 sec denatuation at 94℃, 40 sec annealing at 55℃, and 90 sec extention at 72℃ were employed to amplify the target sequence.

The expression of the gene was also confirmed by RT-PCR. Leaves were excised from the plants during the second week of August. Total RNAs were extracted by the methods of Verwoerd et al. (1989). For a single tube RT-PCR, the 100 μL reaction mixture contained the same components and concentrations of DNA PCR except 8 U of Rous associated virus 2 (RAV) reverse transcriptase (Takara) and 1 μg of total RNA instead of 2 ng DNA were added. The thermal program consists of 1 cycle of reverse transcription at 42℃ for 60 min followed by the 35 cycles as described in DNA PCR.

RT-PCR products were run on 1% (W/V) agarose gel (in 0.5x TAE buffer) and transferred to Nylon membrane by semi-capillary transfer method (Nakano et al. 1990). The probe for DNA hybridization was a fragment of *npt*II gene. The probe was prepared using commercial DIG-labelling kit in which DIG-dUTP was added to dNTP mixture (Boeringer Manheim Co.). Prehybridization, hybridization, and subsequent immunological detection using DIG-specific antibody were done according to the manual supplied by the manufacturer.

Resistance of transgenic poplars to kanamycin

To test the expression of the *nos-npt*II gene in poplars, the leaves were excised from all 29 clones of 9 years old transformed plants and two untransformed control clones growing in the nursery during the second week of August, 2003. At the time of the experiment, all the plants were about 1 to 1.2 m in height since stems had been cut off and removed each year. Leaves were taken from two different positions of each plant. While the leaves taken from near the shoot apex represent young leaves, those from the middle of the stem were fully matured. They were surface sterilized with 0.1% $HgCl_2$ and rinsed with sterile distilled water. Fifteen to thirty segments in size of ca. 0.5 cm x 0.5 cm were prepared from each leaf and cultured

on callus inducing medium (MS containing 1.0 mg/L 2,4-D, 0.1 mg/L NAA, and 0.01 mg/L BA plus 150 mg/L kanamycin). Callus formation was examined after 4 week culture on the medium.

NPTII assay

NPTII concentration in the leaf tissue was measured by ELISA. Crude proteins were extracted from 200 mg of tissue growen in the nursery and in test tubes by grinding in an extraction buffer (10 mM Tris HCl (pH7.5), 1 mM NaCl, and 0.1 mM EDTA) followed by a centrifugation at 16,000 xg for 5 min. Aliquotes were used to measure the concentration of proteins as well as the enzyme by a commercial *nptII* ELISA kit (CloneTech Co, USA).

Results and Discussion

## Plant regeneration and transfer to a nursery

The frequency of callus formation on the kanamycin medium ranged from 20 to 70% depending on the material (data not shown). For shoot regeneration, about 150 calli formed were transferred to WPM (Lloyd and McCown 1981) plus 1.0 mg/L zeatin, 0.1 mg/L BA and 0.01 mg/L NAA plus 50 mg/L kanamycin when they were 2-3 mm in diameter. About 60 plantlets were regenerated from the calli within 3 weeks of subculturing onto the medium. Thirty two plants survived during the period of acclimatization and subsequent hardening off. They were transferred to a nursery at the institute. After 9 years, twenty nine plants have survived and grown in the nursery.

Southern hybridization and RT-PCR

All the transgenic plants growing in the nursery were positive in PCR amplification, suggesting that the plants still carry the transgene. However, amplification of DNA from control plants failed to produce the target band (Figure not shown). Three transgenic poplars were randomly selected and analyzed by Southern blot hybridization with a DNA probe prepared with *nptII* coding sequence (Figure 1). The result showed that the three transgenic poplars carry a single copy transgene.

RT-PCR performed with the total RNAs from the plants also produced the target sequence. No amplification products were observed when RNAs from untransformed control plants were used. The amplified fragments were verified by hybridization with *nptII* DNA probe (Figure 2). However,

it was very difficult to amplify the bands with the RNAs extracted from mature leaves. The lack of reaction products might be due to interference by some substances carried over from mature leaves during RNA extraction. However,
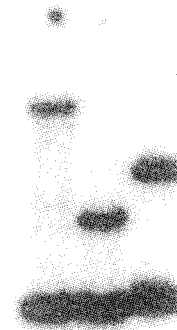
1 2 3 4

Figure 1. Detection of transgene in 9 years old poplars by Southern hybridization with *nptII* specific probe. Ten μg of genomic DNA extracted from the leaves of 9 years old poplar plants were digested with restriction enzyme *Pst*I overnight and run on 1% agarose gel, transferred to Hybond-XL nylon membrane by capillary transfer method and probed with a 800 bp fragment of *nptII* coding sequence. Lanes 1 to 4 represent untransformed control, and 3 transgenic plants, respectively.
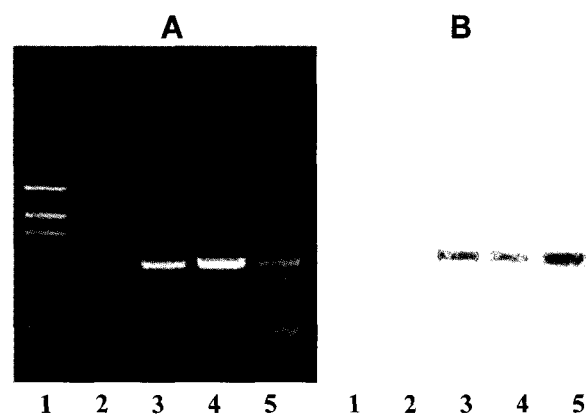

A   B

1 2 3 4 5   1 2 3 4 5

Figure 2. RT-PCR amplificarion and DNA-DNA hybridization with *nptII* specific probe.
A. Amplification of *nptII* sequence from 1 μg total RNAs of transgenic poplars with primers specific to *nptII* coding sequence. Lanes 1 to 5 represent pGEM-DNA size marker, untransformed control, and 3 transgenic plants, respectively.
B. Confirmation of the amplified DNA by blotting to nylon membrane followed by DNA-DNA hybridization with DIG-labelled *nptII* gene probe.
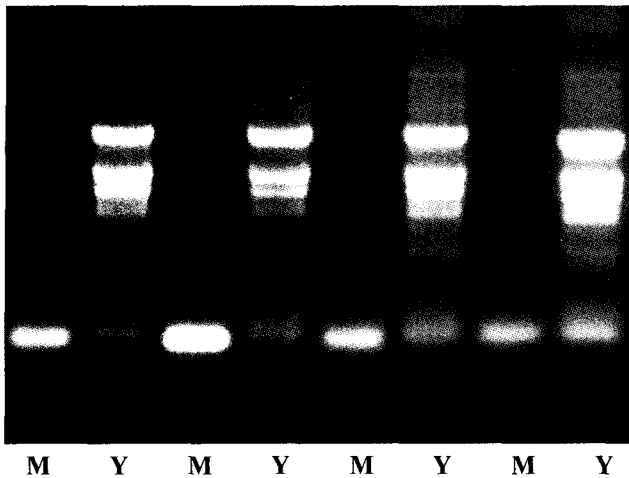
Figure 3. Total RNA profile of young.(Y) and Mature (M) leaves of 3 transgenic poplars. Only young leaves showed active RNA synthesis. The first 2 lanes are untransformed control.

Figure 4. Differential expression of *nos-nptII* gene in transgenic plants revealed by both the frequency of callus induction and *NPTII* activity. A. Frequency of callus induction, and B *NPTII* activity of the leaf fragments taken from test tube grown plants (T), young (A), and mature (B) leaves from 29 fields grown poplar plants on callus inducing medium containing 150 mg/L kanamycin. Error bars represent +/- S. D.

the failure of amplification using the RNAs from the mature leaves is more likely due to the lack of mRNAs in the tissue. Much difference was seen in the amounts of total RNAs between mature and young leaves (Figure 3). Unlike young leaves that contained abundant RNAs, mature tissues appeared not to contain much RNAs, suggesting that these are transcriptionally not active.

Resistance of 9 years old transgenic plants to kanamycin

Within 3 weeks on callus inducing medium containing 150 mg/L kanamycin, calli were visible on the cut edge of the leaf fragments. All transgenic clones formed calli within the period. The calli looked dark red and friable. However, the control clones did not form callus and died off eventually. The red colorization appeared to be a quick indicator of kanamycin resistance since it is the evidence of transcription in the cells in the presence of the antibiotic. An interesting observation is that leaf fragment taken from mature leaves of the transgenic plants did not form callus well (Figure 4A). Only the tissues taken from young and actively growing leaves produced vigorously growing friable callus. Moreover, even in the young leaves, more calli were formed from the cut edge of veins than from other parts of leaves suggesting that veins are metabolically more active.

It is noteworthy that at the time of plant transformation, all the transgenic cells were selected in the presence of 50 mg/L kanamycin after cocultivation with *A. tumefaciens* vector. We tested the resistance of the regenerated plants growing in the test tubes to kanamycin. All of them survived upto 400 mg/L kanamycin. However, untransformed control
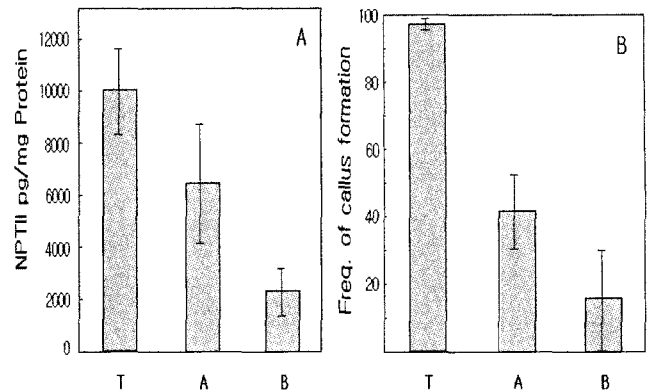
plants died at the concentration of 20 mg/L kanamycin within 3 weeks in culture. The present study demonstrates that the concentration of 50 mg/L kanamycin is enough to select transgenic cells. The cells selected by the concentration exhibit resistance to kanamycin at the concentration of upto 400 mg/L. However, the result was obtained using test tube grown poplar plants that were characteristically very soft and thin. It may be necessary to increase the concentration of kanamycin when explants from greenhouse or field grown plants were used for transformation. The concentration of kanamycin (150 mg/L) used in the present study appeared to be high enough to select transformed cells from field grown plants. No calli were visible at the concentration from untransformed control plants. We believe that this high stringent screening using antibiotics should offer a way to confirm the stability and expression of transgenic plants growing in the field.

NPTII assay

Initially, all the plants assayed showed very high *NPTII* activities when they were growing in culture even though they had been maintained in kanamycin free media for several months. The stability of the transgene could be ascribed to the nature of subculturing since the plants had been maintained by shoot tip culture. Although the plants growing in the nursery also showed high *NPTII* activity, The activity varied with the positions of leaf samples and thus with the growing stages (Figure 4B). While the actively growing leaves near the shoot apex always showed the highest *NPTII* activities, those taken from the middle or lower part of the

branch did not show much. This result was consistent with the kanamycin resistance test in which the young leaf fragments produced more callus than did the mature ones. The difference in the amounts of total RNAs in the tissues also supports the idea that only young tissues are competent and transcriptionally active. Therefore, transcription may not be fully operating in mature leaves since genes may rely upon other cellular components for their expression. Chun and Klopfenstein (1995) also reported the organ specific expression of a transgene (nos-nptII) in a hybrid poplar clone. They also noticed higher NPTII activities in young leaves as well as in petioles. Thus, the nos promoter, although known as constituitive, may not be fully active in mature leaf tissues. Similar results could be easily observable if we look at the histochemical expression of GUS gene in other plant species in which the expression was mainly confined to actively growing meristematic regions (Dong and McHughen 1993). Geier and Sangwan (1996) examined the expression of transgene in transformed Kohleria. They noticed that cells competent for *Agrobacterium* mediated transformation were mainly located in vascular tissues. Histochemical localization of GUS expression in vascular tissue and root tip has been reported in many transgenic plants (Tsai et al. 1994, Hadfi and Batschauer 1994). The localization of gene expression in the vascular tissues suggests that in some cases, expression of gene depend more on the cellular environment than on promoter.

Thus, the transgenic plants offer a good opportunity to study the differential gene expression in plants. In the case of tree species that have distinct phases of juvenile and adult growth, the chimeric nos-nptII gene could serve as an excellent marker for the study of gene expression in different tissues and organs at different development stages. Although the nos-nptII gene serves as a reporter as well as a selection marker in the present study, one drawback of using it may be the difficulty of localizing gene expression in a tissue. The drawback can be bypassed if vectors carrying both nos-nptII and GUS gene are used. Then it will give us more insight into gene expression in trees.

Acknowledgements

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