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

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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 independently 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 NPTII assay, the leaf segments from mature leaves did not form callus well on the media. RT-PCR with nptII 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 constitutive 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-nptII, 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 selectable 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-nptII 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; NPTII: neomycin phosphotransferase 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 carrying pBIBKAN (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 *PstI* 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 32p-dCTP labelled 800bp nptII gene fragment for 12 h. The membrane was washed in 2 × SSC and 0.1% SDS (50°C) for 10 min and in 0.2 × SSC and 0.1% SDS (50°C) for 30 min followed by exposing to an X-ray film at -70°C.

#### 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 *nptII* coding region (Pridmore 1987). They contained the following bases; NPT1: 5'-TTG TCA AGA CCG ACC TGT CC-3' NPT2: 5'-GAA TCG GGA GCG GGC ATA CCG TAA A-3'. The 30 μL reaction mixture contained 10X Taq DNA polymerase buffer (supplied by Promega Co.), 1.5 mM MgCl2, 200 mM dNTPs, 0.4 μM primer, 2 ng template DNA, and 1.5U Taq DNA polymerase. Thirty five thermal cycles consisting of 20 sec denaturation at 94°C, 40 sec annealing at 55°C, and 90 sec extension at 72°C 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°C 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 Nylor membrane by semi-capillary transfer method (Nakano et al. 1990). The probe for DNA hybridization was a fragment of *nptII* 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-nptII 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 the shoot apex represent young leaves, those from the middle of the stem were fully matured. They were surface sterilized with 0.1% HgCl2 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