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Identification of Excision of Ac Transposable Element in *P. nigra x maximowiczii* Using *Agrobacterium*- mediated Transformation

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Abstract

The Ac (activator) which is one of the well-characterized transposable elements from maize was examined for its transposition possibility to the heterologous plant (*P. nigra x maximowiczii*) genome via *Agrobacterium tumefacience* (LBA4404) mediated transformation system. A number of transgenic plants were successfully recovered after 30 weeks by amount reduction from 50 to 15 g/mL kanamycin for *in vitro* selection to minimize phytotoxic effects and to increase callus growth and regeneration efficiency. Among transgenic plants, 62 out of 106 transgenic poplars (58.5%) showed abnormal phenotypes such as severe serrated leaves and light leaf coloration. Indigo staining with X-gluc proved indirectly the restoration of Gus enzyme function and the presence of Ac in poplar genome by PCR. Southern analysis indicated the transposition and existence of Ac element in poplar genomes. In this research, an *Agrobacterium*-mediated transformation system in poplar species was developed and identified that Ac derived from maize can be excised and transposed into other poplar genomes.

Key words: *P. nigara x maximowiczii*, Activator, transposition, kanamycine, *Agrobacterium* transformation,

Introduction

Native transposable elements (TE) have been identified in many plant species including *Zea mays* and Antirrhinum (Doring and Starlinger 1986; Gierl and Sadler 1992). Molecular structures and mechanisms of TEs have been intensively studied and elucidated in generating stable or unstable mutation in higher plants (Fedroff 1989). With the knowledge of TE characterization, the application of TEs to plant genetics and breeding programs can be possible to isolate unknown gene(s) by tagged TE (Coomer and Feldmann 1993). Once the TE is confirmed stable insertion into the heterologous genomes in the organisms, recovered transgenic plants will be very useful as donors for isolating a specific mutated gene(s) responding to interesting internal or external factors by transposon tagging system. Since DNA sequences of introduced TE are known, an unknown gene inserted by TE can be isolated by using probe with TE. Typical alterations in mutated genes or alleles have been identified by gene tagging systems whose products are phenotypically visible nonfunctional flowers and leaf pigmentation (Doring and Starlinger 1986). Moreover, artificial selection pressure such as environmental stresses can be also applied to isolate specific gene with tagging methods.

Until now, the study of transposition of heterologous TEs has been exclusively reported in potato (Frey et al. 1989), rice (Murray et al. 1991) and *Arabidopsis* (Cardon et al. 1993). In woody plants, an endogenous TE has not been reported until now and only a report showed the application of heterologous TE in woody plant (Aspen) (Fladung and Ahuja 1997). Since woody plants especially for forest trees are extremely difficult to identify and isolate genes through traditional breeding programs because of long generation until time to mature status, the application of TEs in their breeding programs can be the most useful tool for identifying and isolating a specific gene within extreme short time. Earlier study confirmed that excision and transposition of heterologous TE from maize (Activator) could be occurred in the Aspen genome and produced phenotypically unstable mutants such as leaf coloration (Fladung and Ahuja 1997).

The long-term goals for this research were to isolate specific

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genes for woody plants using Ac transposon and to produce novel woody plants with over-expressed woody specific genes. As a first step, this study was to assess the possibility of TE transposition in poplar (*P. nigra x maximowiczii*) genome via *Agrobacterium tumefaciens* to develop Ac mediated gene tagging system and production of transgenic plants with active Ac transposon. The morphological mutations other than variegation on the leaves were also investigated.

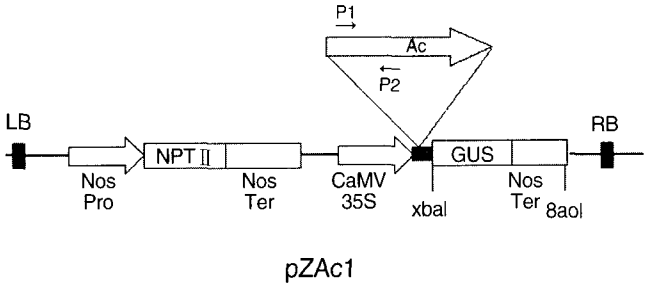
Materials and Methods

Plant materials and culture media

In vitro stocks of *P. nigra x maximowiczii* were initiated from stem segments on 1/2 medium MS (Murashige and Skoog 1962). *In vitro* plantlets were maintained and propagated on basal 1/2 MS in the culture room at 22-25°C with a 16:8 h light/dark cycle. Developed plantlets were further propagated on the same 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 1968) and 0.7% (w/v) agar (Difco-Bacto). For adventitious shoot induction, leaf segments of about 1cm in length and width were cultured on SIM (Shoot Induction Medium: 1/2 MS supplemented with 1 mg/L BA) under the dark condition for 1 week and placed under light condition until adventitious shoots were appeared.

Co-cultivation of leaf segments with disarmed *Agrobacterium tumefaciens* and selection of transformants

The *Agrobacterium tumefaciens* strain LBA4404, a non-oncogenic strain, was used for transformation. The pZAc1 vector plasmid (15 kb, Figure 1) contained a 4.6 kb fragment. This fragement was cloned into the leader sequences of GUS structural gene in the pBI121. Bacterial cultures were grown overnight at 27°C on a rotary shaker (120 rpm) in 50 mL LB (Luria Broth), with antibiotics (kanamycin: 50 μg/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 the diluted 10-fold in liquid regeneration medium with growth regulators. These suspensions were used for subsequent transformation. All leaf explants were precultured for 2 days on SIM without antibiotics at first.



pZAc1

Figure 1. Schematic representation of the binary plasmid pZAc1. Arrows (P1 and P2) indicate Ac internal specific primers for PCR. RB: right border, nos-pro: nopaline synthase gene promoter, npt II: neomycin phosphotransferase coding gene, nos-ter: nopaline syntase termator, CaMV35S: cauliflower mosaic virus 35S promoter, Ac: Activator, LB: left border.

Leaf segments were then immersed in the bacteria solution for short periods (20 min) and blotted with sterile filter paper. And then, these were co-cultivated for additional 48 h on the same medium. Following co-cultivation, the inoculated explants were rinsed with sterile distilled water containing 1 g/L cefotaxime and 1.5 g/L carbenicillin. These were placed on selective SIM (50 mg/L kanamycin, 100 mg/L carbenicillin and cefotaxime), and incubated under the dark for 2 weeks, and then cultured under the light. The bacterial suspension was omitted in control plates.

Leaf explants were subcultured once a week on selective SIM for the first 8 weeks. Control explants were cultured using the same SIM described for the co-cultivated leaf explants. Each developing callus or shoots was excised from the primary explants and cultured on the same medium for additional 5 months. Once adventitious shoots developed, those (1-2 cm in length) were further 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 these conditions were considered to be kanamycin-resistant and transferred to a greenhouse for further growth and development. Transformation frequency was defined as percentage of inoculated leaf explants that produced kanamycin-resistant plantlets.

Molecular analysis of transgenic poplars

The GUS histochemical assay as a rapid way to identify the functional GUS uidA gene expression in the putative transformants was employed. Proliferating calli and shoots formed from explants previously cocultivated with *Agrobacterium*, were incubated overnight at 37°C in X-Gluc (Jefferson et al. 1987). After overnight staining, the explants were washed with 70% (vol/vol) ethanol and observed blue coloration by optical binocular.

A small amount of leaves about 10 mm length and 5 mm width were collected and ground in microcentrifuge tubes containing 45 μL of ddH_2O and 5 μL of 0.5 N NaOH with pestle and microwaved for 5 min. After centrifugation for 3 min at 12,000 rpm, 20 μL of the supernatant was carefully removed,

added in the tubes containing 4 μL of 0.25 N HCl to neutralize pH. After well-mixing, they were placed on the ice until used. Each DNA was then used as a template for PCR reaction. The PCR amplification was carried out in 25 μL of reactions containing 1 μL (about 20 ng) of a template DNA, 1X reaction buffer, 25 pmole Ac primers, 10 mM dNTPs, 25 mM $MgCl_2$, and 1 unit of Taq DNA polymerase.

Primer set utilized was 5′ ATATGCATTTACCTTTCCTA-GATGCG3′ as a forward primer and 5′ GACCCTAGGTA-CAAGAAAATATTGA3′ for a backward primer. The PCR conditions were maintained at 94°C for 1 min 30 sec, followed by 30 cycles of 92°C for 50 sec, 52°C for 50 sec, and 74°C for 50 sec. Cycling was followed with an ending step at 75°C for 5 min followed by 4°C. PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Southern analysis was achieved using PCR products by amplifying randomly selected 19 regenerants. Separated DNA on 1% agarose gel was transferred onto positively charged nylon membrane (Amersham pharmacia biotech). Prehybridization and hybridization were carried out using PhotoGene™ Nucleic Acid Detection System (Gibco BRL, Bethesda, MD) by the manufacturer's protocol. The probe used in the hybridization reaction was the 0.5 kb PCR product amplified from Ac gene and prepared using BioNick™ Labeling System (Gibco BRL, Bethesda, MD) by the manufacturer's protocol.

Results and Discussion

The main obstacles for successful genetic transformation of woody plants are totally depended on establishing uniform and reliable regeneration system. Since induction of adventitious organs requires quite different conditions among species and/or even among tissues from the same species, it is sometimes very difficult to standardize regeneration protocol with a given plant species (Pierik 1987). Especially, many woody plants have proven to be extremely difficult for developing regeneration protocols (Pierik 1987). Since our lab has been established with excellent regeneration protocols from several poplar species including *P. nigra x maximowiczii*, which is one of the most important forest trees, this species has been chosen for developing *Agrobacterium* transformation system and examining possibility of transposition of heterologous transposable element (Ac).

After culturing leaf explants on regeneration medium containing antibiotics for 2 weeks, those started to respond swollen from wounding sites and mid-vein areas. However, visible callus formation appeared on the same medium after 6 weeks under the dark condition. And then, all calli approaching in 5 mm in size were dissected out and transferred to the fresh medium under the light condition resulting in inducing adventitious shoots.

Kanamycin among many other antibiotics has been reported to inhibit morphogenesis in several fruit tree species including *Vitis, Rubus* and much lower concentration (20 g/mL) in *Malus* (Fiola et al. 1990; Gray and Meredith 1992; James and Dandekar 1991). Like other woody plants, poplar tissues used for this research showed strong sensitivity against antibiotics (especially for kanamycin) as a result in retarding callus growth and delaying in regenerating organs (personal observation). Thus reduced concentration from 50 to 15 g/mL was employed after 8 weeks on regeneration medium. Upon reduction of antibiotic concentrations, calli started to induce adventitious organs on modified regeneration medium. After 30 weeks in culture, adventitious shoots were produced from proliferating calli (Figure 2).

Additional selection method for transformed calli was performed using X-gluc histochemical assay during each subculture. Since Ac was inserted into the GUS structural gene (Figure 1), blue coloration from developing or proliferating calli indicated that Ac was transposed to other sites in poplar genomes. Restoration of GUS enzyme function indicated the excision of Ac from GUS structural gene resulting in normal functional GUS enzyme activity. Blue foci were detected after staining with X-gluc of proliferating callus (Figure 3). Only callus showing positive to GUS assay regarded as transformed, and these were subcultured to fresh medium to induce adventitious shoots further.

In case of Aspen (*P. tremuloides*), Ac was transposed to the poplar genomes resulting in generating phenotypic mutation such as diverse variegations (Flaudung and Ahuja 1997).

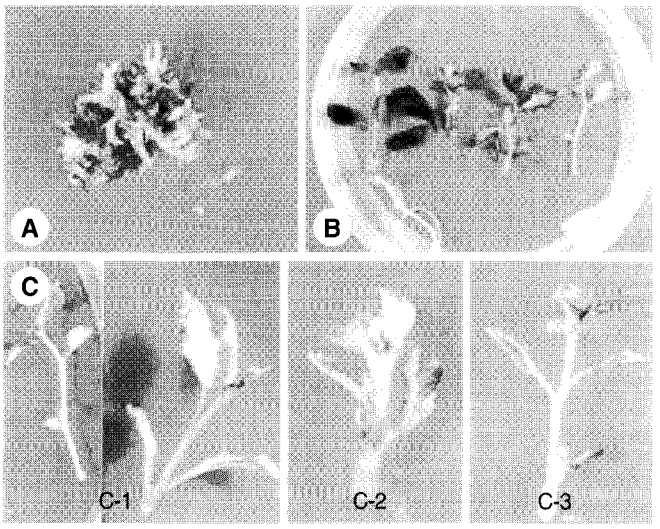


Figure 2. Morphological mutants of transgenic plants derived from *P. nigra x maximowiczii* leaf disks. A: Induction of adventitious shoots from callus; B: Morphological differences among normal and mutants; C: Types of mutants C-1: sharp serration: C-2 cut leaf; C-3: fused leaf.

However, in case of *P. nigra x miximowiczii*, mutated phenotypes showed quite different morphologies than those from Aspen. Severe serrogation, abnormal phenotypes and light leaf coloration appeared to be quiet different when compared with the control (Figure 2). Most abnormal leaves showed cut-fused two leaves, different types of serrated leaf morphologies and light-green coloration of leaves when compared with the control (Table 1, Figure 2). As for the leaf coloration, Ac could be involved in inhibition of chloroplast gene(s) in nucleus resulting in the change of colors from dark to light green. In this research, it was unable to detecte variegation of leaves like Aspen and other plants (Fladung and Ahuja 1997). Thus, it is assumed that abnormal phenotypes from transgenic poplars could be new types of mutation generated by transposon, even though direct evidence of Ac involving these unusual mutations is yet to be proved.

To confirm the existence of Ac in morphologically mutated plants, the total genomic DNAs from randomly selected transgenic plants were isolated and amplified with Ac specific internal primers. Among 9 out of 19 samples showed expected band size (0.5 kb) where Ac excision resulted in the restoration of the GUS gene function (Figure 1). Southern blot analysis of PCR products from transgenic plants showed in most of the independent primary transformants (Figure 4A, B). No hybridization signals were observed in untransformed aspen plants. Amplified band intensity was different from each sample. It was not clear whether these differences were from autonomous replication of Ac or different amount of template genomic DNA for PCR amplification. However, a transgenic poplar plants obviously contained Ac. It was unable to verify that whether Ac insertion to poplar genome was directly related to abnormal phenotypes. More detailed examination should be performed to identify the cause of abnormal alteration of Ac such as anatomy of leaf, light-green and dark-green color, etc.

The use of Ac transposable element has some particular characters during integrating in the plant genomes. If F1 and F2 generations of plant containing Ac will be fixed, it could not be transposed further because of metylation of Ac structural genes but Ac transposition can be restored through *in vitro* regeneration (Lawrence et al. 1993; Lawson et al. 1994). This character is very important for breeders and genetists to isolate mutated gene. Thus, Ac can be used as a powerful mutagen like T-DNA tagging to plant gene. Other character of Ac can transpose on the same chromosome but not others which will be a very useful tool for chromosome study even though it is quite difficult to identify one or two Ac insertion in one chromosome (Izawa and Simamoto 1999). Currently, the confirmation of number of Ac copies in the selected poplar transgenic plants

Table 1. Frequency of morphological mutants observed from poplar transgenic plants.

		No. of shoots	Frequency (%)	Ave. length of shoots (mm)
Normal phenotye		44	41.5	32.2
Abnormal phenotype		62	58.5	12.1
Leaf morphology	Cut	14	13.2	8.71
	Normal	75	70.8	24.3
	Both	17	16	13.9
Serrated of leaves	Sharp	48	45.3	10.5
	Smoth	54	50.9	29.4
	Both	4	3.8	19.3
Leaf color	Green	61	57.5	27.8
	Light green	35	33	10.2
	Both	10	9.4	11.9
Fused leaf		6	5.7	

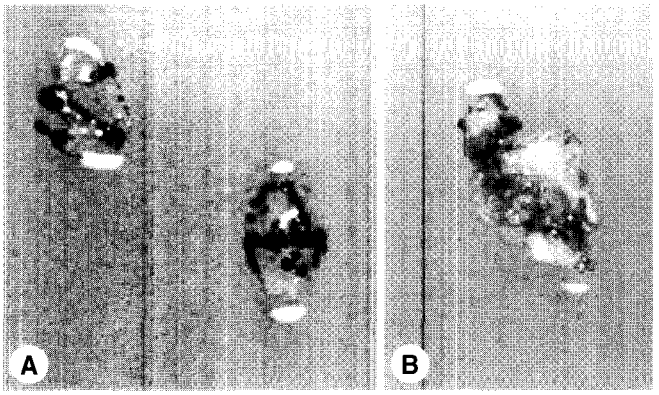


Figure 3. Gus histochemical assay using developed calli after co-cultivating with *Agrobacterium* LBA4404. A: Callus staining with X-gluc from original leaf disks; B: X-gluc staining from proliferating callus detached from leaf disks.

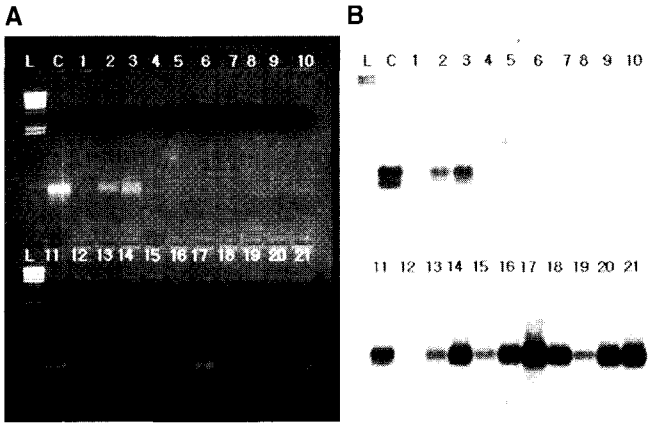


Figure 4. Agarose gel electrophoresis (A) and Southern blot analysis (B) of PCR amplification using Ac internal primers from randomly selected transgenic poplars. 4A and B: Lane L: λ/*Hin*dIII size marker. Lane C: positive control (amplified product from plasmid pZAc1). Lane 1: negative control (amplified product from non-transformed plant). Lane 2 to 21: amplified products from transgenic plants transformed with pZAc1.

and subsequent transposition of Ac transposable elements by second regeneration is under study.

According to the results, Ac autonomously transposed to poplar genome and it can be a useful tool for isolating unknown gene by tagging it. Although there were no direct evidences that Ac directly altered the poplar phenotypic mutations, numerous transgenic poplars with Ac were been obtained. Current data should be considered as preliminary and an attempt to generate second mutation by using poplar transgenic plants is in progress. The availability of reliable and efficient regeneration and transformation systems opens the possibility to transfer useful genes into *P. nigra x maximowiczii* and Ac derived from maize can be transposed into heterologous woody plant genomes which are useful for plant breeders and genetists.

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References

Cardon GH, Grey M, Saedler H, Gierl A (1993) Mobility of the maize transposable element En/Spm in *Arabidopsis thaliana*. Plant J 3: 773-378

Coomber SA, Feldmann KA (1993) Gene tagging in transgenic plant. In Kung SD, Wu R (eds) Transgenic plants, Academic Press, New York 1: 225-240

Doring HP, Stralinger P (1986) Molecular genetics of transposable lements in plants. Ann Rev Genet 20: 175-200

Fedoroff NN (1989) Maize transposable elements in Mobile DNA (Douglas E. Berg and Martha M. Howe) 375-412

Fiola JA, Hassan MA, Swartz HJ, Bors RH, Cc Nicols R (1990) Effect of thidiazuron, light influence, rates and kanamycin on *in vitro* shoot organogenesis from excised Rubus cotyledons and leaves. Plant Cell Tiss Org Cult 20: 223-228

Flaudung M, Ahuja MR (1997) Excision of the maize transposable element Ac in periclinal chimeric leaves of 35S-Ac-rolC transgenic aspen-Populus. Plant Mol Biol 33: 1097-1103

Frey M, Tavantzis SM, Saedler H (1989) The maize En-1/Spm element transposes in potato. Mol Gen Genet 217: 172-177

Gierl A, Saedler H (1992) Plant-transposble elements and gene tagging. Plant Mol Biol 19: 39-49

Gray DJ, Meredith CP (1992) Grape. In Hammerschlag FA, Litz RE (eds) Biotechnology of perennial fruit crops. CAB International, Walling ford 229-262

Izawa T, Simamoto K (1999) Ac/Dsas tools for rice molecular biology. In K. Shimamoto (ed) Molecular Biology of Rice 59-76

James DJ, Dandekar AM (1991) Regeneration and transformation of apple Malus pumilla Mill. In Lindsey K (ed) Plant Tiss. Cult. Manual. Kluwer, Dordrecht B8: 1-18

Jefferson RA (1987) Assayin gchimeric genes in plant: The GUS gene fusion system. Plant Mol Biol Rep 5: 387-405

Murray EE, Rochelea;u T, Eberle M, Stock C, Sekar V, Adang M (1991) Transpositionof the maize activator element in transgenic rice plant. Plant Mol Biol 16: 035-1050

Murashige R, Skoog F (1962) A revised medium for rapid growth and bioasays with tobacco cultures. Physiol Plant 15: 473-499

Lawrence G, Finnegan J, Ellis J (1993) Instability of the L6 gene for rust resistance in flax is correlated with the presence a linked Ac element. Plant J 4: 659-669

Lawson EJR, Scofield SR, Sjodin C, Jones JDG, Dean C (1994) Modification of the 5′ untranslated leader region of the maize Activator element leads to increased activity in *Arabidopsis*. Mol Gen Gent 245: 608-615

Pierik RLM (1987) *In vitro* culture of higher plants. Martinus Nijhoff Puhlishers, Dordrecht

Staba EJ (1969) Plant tissue culture as a technique fof the phytochemist. Rec Adv Phytochem 2: 75-102.