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Micropropagation of *Populus trichocarpa* 'Nisqually-1' and genetic engineering *Populus* with CaMV35s-AtWBC19, CaMV35s-AgNt84 and -60CaMV35s-GUS genes

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To the Graduate Council:

I am submitting herewith a dissertation written by Byung-guk Kang entitled "Micropropagation of *Populus trichocarpa* 'Nisqually-1' and genetic engineering *Populus* with CaMV35s-AtWBC19, CaMV35s-AgNt84 and -60CaMV35s-GUS genes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

Zong-Ming (Max) Cheng, Major Professor

We have read this dissertation and recommend its acceptance:

Janice Zale, Feng Chen, Robert Trigiano

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation presented for the Doctoral of Philosophy Degree
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Byung-guk Kang
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DEDICATION

This dissertation is dedicated to my parents, Dong-Won Kang and Seung-Su Roh, my wife Soo-Jin Kim. Their love and support have made the writing of this dissertation possible. Thank you all for your loving support and encouragement. Mom and Dad, I appreciate all you do and for being there for me no matter what the circumstances. And I love my sons, Sean and Brian, for their excitement and joy every time I return home. Finally, I love you Soo-Jin.

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ABSTRACT

In Chapter one, we described development of a highly efficient micropropagation protocol from greenhouse-grown shoot tips of 'Nisqually-1'. The optimal micropropagation protocol involves growing *in vitro* shoots in plant growth regulator free Murashige and Skoog (MS) basal medium supplemented with 3% sucrose, 0.3% Gelrite and 5–10 $g \cdot L^{-1}$ of activated charcoal. Plants grown on this medium were significantly longer, and contained significantly higher concentrations of chlorophyll. In chapter II, we transferred the plant-originated *Arabidopsis Atwbc19* gene encoding an ATP binding cassette transporter which confers resistance to aminoglycoside antibiotics. Transgenic plants were confirmed by polymerase chain reaction (PCR) with Atwbc19-specific primer pair. The expression was confirmed by the reverse transcription PCR. Transgenic plants were tested for aminoglycoside antibiotic resistance. The level of resistance conferred by CaMV35S-*Atwbc19* is similar to that conferred by *npt*II gene. Therefore, plant-ubiquitous *Atwbc19* gene can serve an alternative gene as a plant transformation selective marker gene to current bacterial antibiotic-resistance marker genes and alleviate the potential risk for horizontal transfer of bacterial resistance genes in transgenic plants. In Chapter III, we transformed a *Populus* clone with the enhancer trapping vector, pD991. All 250 transgenic lines were screened and 71 of them (28%) showed positive staining. They showed various patterns of the reporter gene expression, including expression in one tissue and simultaneously in two more tissues. These results confirmed the previously reports that enhancer trap lines can be produced in *Populus*, and these enhancer trap lines can be used for future gene cloning and studying gene

expression in *Populus*. In Chapter IV, we transformed with the heavy metal binding protein *agNt84* gene. Seven putative transgenic lines were confirmed by PCR with the *agNt84* specific primers and two lines shoot tips of transgenic- and non-transgenic plants grown on cadmium (Cd) -containing rooting media to evaluate of Cd resistance. 33% of shoot tips from one line and 44% of those from another transgenic line survived on medium containing 250 mM Cd, respectively, but only 22% of the non-transgenic shoot tips survived on rooting medium with 150 mM Cd at week 8. Also, the Cd analysis by ICP-OES indicated that the transgenic plants which were grown on 100 mM Cd medium accumulated about 45% more Cd in the tissue than non-transgenic plants.

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CHAPTER I

Micropropagation of *Populus trichocarpa* 'Nisqually-1': the genotype deriving the *Populus* reference genome[1]

[1]This chapter has been published in the Plant Cell, Tissue and Organ Culture (PCTOC) in September 2009.

Auther

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ABSTRACT

Populus serves as a model tree for biotechnology and molecular biology research due to the availability of the reference genome sequence of *Populus trichocarpa* (Torr. & Gray) genotype 'Nisqually-1'. However, 'Nisqually-1' has been shown to be very recalcitrant to micropropagation, regeneration and transformation. In this study, a highly efficient micropropagation protocol from greenhouse-grown shoot tips of 'Nisqually-1' was established. The optimal micropropagation protocol involves growing *in vitro* shoots in plant growth regulator-free Murashige and Skoog basal medium supplemented with 3% sucrose, 0.3% Gelrite® and 5-10 $g \cdot L^{-1}$ of activated charcoal. The plants grown in this medium were significantly longer in length and contained significantly higher concentrations of chlorophyll. This highly effective protocol provides a consistent supply of quality leaf and stem materials throughout the year for transformation experiments and other *in vitro* manipulations, therefore eliminating inconsistency due to seasonal and greenhouse environmental variation and the need for repetitive tissue sterilization.

Key words: Activated charcoal · Cytokinin · Gelling agent · Gelrite · Tissue culture · Poplar

Abbreviations: AC – activated charcoal, BA – N^6-benzylaminopurine, Chl *a* – Chlorophyll *a*, MS – Murashige and Skoog, PGR – Plant growth regulator

INTRODUCTION

Populus species are widely used for wood, paper, as an energy source and for other purposes worldwide. They are now considered by the U.S. Department of Energy to be the leading choice for dedicated woody bioenergy crops due to their fast growth, wide adaptation and ease of propagation (Tuskan 1998; Wullschleger et al. 2002). *Populus* is also regarded as the model for woody plant gene function because of the available reference genome of *Populus trichocarpa*, genotype 'Nisqually-1' (Tuskan et al. 2006). However, 'Nisqually-1' can be very recalcitrant to transformation with *in vitro* plant tissue (Ma et al. 2004), and protocols using greenhouse plants require the use of a vigorous sterilization procedure and are subject to limitation of a year-round supply of materials (Song et al. 2006). In addition, seasonal variation may lead to inconsistent plant materials which may produce variant results (Song et al. 2006).

In the process of establishing 'Nisqually-1' *in vitro* for regeneration and transformation research, we have encountered persistent difficulty in maintaining even basic growth of this genotype in various media used for other *Populus* species in culture (Dai et al. 2003; Ma et al. 2004). Despite its ease to root and propagate in the greenhouse and field, *in vitro* shoots remain green for only about one week and then begin to turn chlorotic and necrotic. This limits growth and proliferation and ultimately results in plant death. Rutledge and Douglas (1988) also failed to establish *in vitro* cultures of *P. trichocarpa* and were unable to conduct micropropagation. Furthermore, Nadel et al. (1992) reported severe shoot tip dieback of *P. trichocarpa* and leaf yellowing in three different media, though addition of Ca-gluconic acid and 2-[N-morpholino]

ethanesulfonic acid (MES) transiently reduced the problem. The objective of this research was to develop an effective protocol for growing 'Nisqually-1' *in vitro* so consistent plant material can be produced year-round for transgenic research for poplar functional genomics research, particularly for creating a mutational library and validating gene function. We tested the effects of basal medium, cytokinin concentration, gelling agent and activated charcoal (AC) on microshoot growth of 'Nisqually-1' and thus developed an optimal medium for growing and maintaining this genotype *in vitro*. This protocol enables us now to produce consistent and year-round materials for regeneration, transformation and other *in vitro* manipulation.

MATERIALS AND METHODS

Plant material

In vitro cultures 'Nisqually-1' were established from young shoots of greenhouse-grown plants. Vigorously growing 5-cm shoot tips were excised and then soaked sequentially in a 1% Tween-20 solution for 5 min, 70% ethanol for 1 min and in a 0.525% sodium hypochlorite (Clorox®) solution for 15 min. Explants were triple rinsed with sterile water for 5 min each time. A 2-cm shoot tip was excised from the surface sterilized shoot and placed into a 200-mL baby food jar (Sigma-Aldrich, St. Louis, MO) containing 30 mL of Murashige and Skoog (MS) (1962) basal medium supplemented with MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS), 100 $mg \cdot L^{-1}$ myo-inositol, 3% (w/v) sucrose, 4.4 μM N^6-benzylaminopurine (BA) and 0.8% (w/v) agar (Cat. No. BP1423, Fisher Scientific, Pittsburg, PA). The solution was adjusted to pH 5.8 prior to autoclaving at 120 ºC and 103.5 kPa for 20 min. This was the medium which was used to maintain in vitro aspen (Populus spp.) cultures in our laboratory (Dai et al. 2003). The cultures were maintained in a growth room at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as measured by a Licor LI-250 light meter (LI-COR Inc., Lincoln, Nebraska) held at the top of the culture vessels. All of the stock cultures in the following experiments were maintained under these conditions.

The effects of basal medium and cytokinin concentration on plant growth

To evaluate the effect of basal medium salt on plant growth, 'Nisqually-1' shoots were cultured aseptically on either MS, woody plant medium (WPM) (Lloyd and McCown 1981) or Driver and Kuniyuki walnut (DKW) (Driver and Kuniyuki 1984) medium. In each basal medium, three concentrations of BA were tested: 0.0, 2.2 and 4.4 μM (Table 1.1). All media were supplemented with 0.1% MS vitamins, 100 $mg \cdot L^{-1}$ myo-inositol, 3% (w/v) sucrose and 0.8% (w/v) agar (Cat. No. BP1423, Fisher Scientific, Pittsburg, PA).

The effect of gelling agent on plant growth

Once the effects of basal medium and cytokinin concentration on plant growth were evaluated, MS medium was selected as the basal medium for determining the effect of gelling agent on the growth of 'Nisqually-1' shoots. Two gelling agents were tested: 0.8% (w/v) agar (Fisher Scientific) and 0.3% (w/v) Gelrite® (PlantMedia, Dublin, OH). The culture medium contained either 0.0, 2.2 or 4.4 μM BA (Table 1.2). The surviving 'Nisqually-1' shoots from previous experiments were used in this experiment.

The effect of activated charcoal on plant growth

To evaluate the effect of AC on plant growth, 'Nisqually-1' shoots were cultured on MS basal medium without plant growth regulators (PGRs) and with 0.3 % (w/v) Gelrite®. Four AC concentrations, 0, 3, 5 and 10 $g \cdot L^{-1}$, were tested (Figure 1.1). AC was added to the culture medium after adjusting the pH to 5.8, prior to autoclaving.

Experimental design, data collection and statistical analysis

In the first two experiments (the effects of basal medium and cytokinin concentration on plant growth and the effect of gelling agent on plant growth), four 200-mL baby food jars containing one shoot per jar were used for each treatment, and both experiments were repeated three times. Plant performance was evaluated at 4 weeks for survival rate, leaf chlorosis and overall appearance according to the following numeric criteria: (5-points) plant survived, actively growing, no sign of senescence; (4-points) plant survived, limited growth, lower leaves showing senescence; (3-points) survived, but no growth, showing senescence; (2-points) nearly dead; and (1-point) completely dead. For each treatment, the average of the replicates was given as the performance rating. An additional observation was made at 6 weeks, but data are not shown because many plants died during the last 2-week period. In the experiment that tested AC on plant growth, the explants were aseptically grown in 9-cm diameter x 14-cm high (1000 ml) culture vessels (PhytoTechnology Laboratories). Four explants were used for each treatment and the experiment was repeated three times). The length of each plant was measured every 2 weeks for 6 weeks. The experimental design was a Completely Randomized Design (CRD), and the performance rating and length data were evaluated by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc, Cary, NC).

Chlorophyll content analysis by high-performance liquid chromatography (HPLC)

Since the plants grown in the medium supplemented with AC were significantly longer and appeared much healthier and greener in color than those grown in media without AC, leaf tissues were analyzed for chlorophyll content. Four plants from each of

the four *in vitro* treatments were sampled. We also analyzed the chlorophyll content of plants grown on potting mix without AC in a growth chamber under fluorescent and incandescent lights. 'Nisqually-1' leaf tissues were lyophilized for no less than 48 h (Model 12 L FreeZone; LabConCo, Kansas City, MO) and stored at –80ºC prior to extraction and analysis according to Kopsell et al. (2004) and analyzed according to Emenhiser et al. (1996). Briefly, a 0.1-g sample from each homogenate was re-hydrated with 0.8 mL of ultra pure H_2O and placed in a water bath set at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl-β-8'-apo-carotenoate (Sigma) was added to determine extraction efficiency. After sample hydration, there was an addition of 2.5 mL of tetrahydrofuran (THF) stabilized with 25 $mg \cdot L^{-1}$ of 2,6-Di-*tert*-butyl-4-methoxyphenol (BHT). Samples were then homogenized in a Potter-Elvehjem (Kontes, Vineland, NJ) tissue grinding tube. During homogenization, the tube was immersed in ice to dissipate heat. The tube was then placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was decanted and the sample pellet was re-suspended in 2 mL THF and homogenized again with the same extraction technique. The procedure was repeated for a total of four extractions per each sample to obtain a colorless supernatant. The combined sample supernatants were reduced to 0.5 mL under a stream of nitrogen gas (N-EVAP 111; Organomation Inc., Berlin, MA), and brought up to a final volume of 5 mL with methanol (MeOH). A 2-mL aliquot was filtered through a 0.2-µm polytetrafluoroethylene (PTFE) filter (Model Econofilter PTFE 25/20, Agilent Technologies, Wilmington, DE.) using a 5-mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) prior to HPLC analysis.

An Agilent 1200 series HPLC unit with a photodiode array detector (Agilent

Technologies, Palo Alto, CA) was used for pigment separation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. x 250 mm) 5 μm, 200 Å polymeric C_{30} reverse-phase column (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar pigment compounds. The column was equipped with a guard cartridge (4.0 mm i.d. x 10 mm) and holder (ProntoSIL), and was maintained at 30ºC using a thermostatted column compartment. All separations were achieved isocratically using a binary mobile phase of 11% methyl *tert*-butyl ethanol (MTBE), 88.9% MeOH and 0.1% triethylamine (TEA) (v/v). The flow rate was 1.0 $mL \cdot min^{-1}$, with a run time of 55 min, followed by a 2 min equilibration prior to the next injection. Eluted pigments and chemically similar pigment compounds from a 10 µL injection were detected at 453 (carotenoids and internal standard) and 652 [chlorophyll *a* (Chl *a*)] nm, and data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (ChromaDex Inc., Irvine, CA).

RESULTS

Basal medium and cytokinin effects

Shoots grown on MS medium without BA had higher survival rates at 4 weeks than those grown in MS medium with 2.2 or 4.4 μM BA, or in WPM or DKW medium with or without BA (Table 1.1). Shoots grown on MS medium without BA also appeared healthier and greener and became chlorotic and necrotic about 2 weeks later than those in other media (photographs not shown).

Gelling agent effect

It was clear that significantly more shoots grown on PGR-free medium with Gelrite as the gelling agent survived to week four and had significantly higher performance ratings than those grown in the medium solidified with agar (Table 1.2). The shoots remained green and produced new growth (photographs not shown). In Gelrite-containing media without BA, shoot survival rates were significantly higher than those grown on medium with either 2.2 or 4.4 μM BA, confirming that shoots grew better on PGR-free medium which was more favorable than BA-containing medium.

Activated charcoal effect

Although the shoots grown on MS basal medium solidified with Gelrite had higher survival rates, growth was still limited and not sustained. Addition of AC into Gelrite-containing medium significantly improved the growth of 'Nisqually-1'. In the medium without AC, all 'Nisqually-1' shoots survived to 4 weeks, but at 6 weeks survival

was reduced to 50%. Shoots grew to an average of 2.2 and 2.8 cm at 4 and 6 weeks, respectively (Figure 1.1). In contrast, all of the 'Nisqually-1' shoots grown on AC-containing media survived to week 6, and they grew significantly more than those on the AC-free medium (Figure 1.1). At week 6, the 'Nisqually-1' shoots cultured on the medium with 3, 5 and 10 $g \cdot L^{-1}$ AC grew to 7.8, 9.3 and 8.4 cm, respectively (Figure 1.1). All of the 'Nisqually-1' plants grown on AC-containing medium produced two to three green and healthy shoots (Figure 1.2). Using this medium, we have maintained the 'Nisqually-1" shoots for more than two years (data not presented).

HPLC assay of chlorophyll a

'Nisqually-1' grown on basal MS medium with all AC-containing media appeared to have greener leaves (Figure 1.2). This observation correlated with the chl *a* concentration of the leaves (Table 1.3). 'Nisqually-1' leaves from shoots grown on the medium containing 10 $g \cdot L^{-1}$ AC had the highest concentration of chl *a* [1056.95 µg chl $a \cdot g^{-1}$ dry weight (DW)], much higher than those grown on the medium containing 5 $g \cdot L^{-1}$ AC (779.73 µg chl $a \cdot g^{-1}$ DW), both being maintained in a growth room with 125 µmol $m^{-2}s^{-1}$ fluorescent light. The control plants grown without AC in potting mix in a growth chamber had 507.68 µg chl $a \cdot g^{-1}$ DW (data not presented), which was significantly less than those grown on media containing 5 or 10 $g \cdot L^{-1}$ AC, but higher than those grown on culture media with 0 and 3 $g \cdot L^{-1}$ AC.

DISCUSSION

Although many *Populus* species are generally relatively easy to grow and propagate in the greenhouse as well as in tissue culture (Son et al. 2000; Dai et al. 2003), we have encountered extreme difficulty in maintaining the *in vitro* culture of the genotype 'Nisqually-1' of *Populus trichocarpa* in a common medium that contains cytokinin and is solidified with agar (Dai et al. 2003). In previous reports with *P. trichocarpa*, extensive difficulty of *in vitro* propagation has also been reported (Rutledge and Douglas 1988; Nadel et al. 1992). Rutledge and Douglas (1988) failed to initiate shoots from *P. trichocarpa* meristems and were unable to perform micropropagation. Nadel et al. (1992) was able to reduce leaf yellowing and meristem dieback of *P. trichocarpa* in culture by growing on half strength medium supplemented with Ca-gluconic acid and 2-[N-morpholino] ethanesulfonic acid (MES), but the effect was transient only for a few subculture cycles. In this research, we have developed an effective and efficient method to propagate this important genotype of which the whole genome is sequenced. There are three key factors which contributed to the optimization of the protocol, namely, use of PGR-free MS medium, use of Gelrite as the gelling agent and addition of 5 or 10 $g \cdot L^{-1}$ AC.

The basal medium type can affect the performance of woody plants *in vitro* (Mackay and Kitto 1988; Nadel et al. 1992; Cheng et al. 2000; Dai et al. 2005). Our results showed MS basal medium was more suitable than WPM and DKW for growing 'Nisqually-1'. The MS medium is known for its rich macro- and micro-elements, particularly nitrogen (Murashige and Skoog 1962). Although WPM was developed for

micropropagating woody plants (Lloyd and McCown 1981) and DKW medium was developed for propagating walnut (*Juglans nigra*) (Driver and Kuniyuki 1984), both of these media were not well-suited for growing 'Nisqually-1'. Nadel (1992) also reported that MS medium was a suitable medium, although less effective than ½-strength MS.

Gelling agents can significantly affect the performance of *in vitro* culture (MacCrae and Van Staden 1990; Cheng and Shi 1995). The performance of 'Nisqually-1' in agar and Gelrite media were similar to that observed with Siberian elm [*Ulmus pumila* (Cheng and Shi 1995)], where shoots in agar-solidified medium deteriorated in 1 week, but fully recovered when transferred to Gelrite medium, while those in Gelrite medium deteriorated in 1 week after being transferred to agar-gelled medium. This deterioration phenomenon appears to be specific to 'Nisqually-1' because many other *Populus* species have been grown in agar-containing medium without this problem (Rutledge and Douglas 1988; Nadel et al. 1992; Son et al. 2000; Dai et al. 2003). In other species, agar medium was more favorable than Gelrite medium for shoot subculture of French tarragon [*Artemisia dracunculus* (Mackay and Kitto 1988)] and Asian white birch [*Betula platyphylla* (Cheng et al. 2000)]. Such a dramatic inhibitory effect may be attributed to the species-specific over-sensitivity of 'Nisqually-1' to microelements such as copper (Debergh 1983; Cheng and Shi 1995) in unpurified agar.

Activated charcoal clearly had a significant effect on improving growth of 'Nisqually-1'. Activated charcoal can improve development and growth of many plant species *in vitro* (Pan and Staden 1998), for example, establishment of lisianthus (*Eustoma grandiflorum*) protoplast culture (Kunitake et al. 1995; Teng 1997), spruce (*Picea abies*) somatic embryo development (Pullman et al. 2005), lily (*Lilium longiflorum*) bulb

formation (Han et al. 2004) and embryogenesis of *Brassica oleracea* (da Silva Dias 1999). The beneficial effect of AC is thought to be attributed to its adsorption of inhibitory substances in the culture medium (Fridborg et al. 1978; Weatherhead et al. 1978; Weatherhead et al. 1979). The positive effect of AC is also considered to be due to a reduction of the sometimes toxic effects of cytokinins and auxins (Weatherhead et al. 1978; Zaghmout and Torello 1988; Pan and Staden 1998), therefore, altering ratios of culture medium components and influencing plant growth *in vitro* (Johansson 1983; Druart and Wulf 1993). However, the enhancement of AC on 'Nisqually-1' is unlikely due to such an effect because our best performing medium lacks of exogenous hormones. One of the likely reasons for enhanced growth may be due to the higher content of chl *a*, responsible for greener leaves. Genomic analysis has revealed signaling functions among chlorophyll biosynthetic pathway intermediate compounds which regulate transcriptional production of light-harvesting chlorophyll-binding proteins such as carotene and xanthophyll carotenoids (Lohr et al. 2005). Furthermore, the biosynthesis of chlorophyll molecules is linked to the occurrence and production of light-harvesting complex polypeptides (Xu et al. 2001). Together, genomic and analytical data demonstrate the close connections between chlorophyll and carotenoid biosynthetic pathways. It is widely held that there is a positive correlation between the chlorophyll in leaves and the growth rate of plants (Gupta and Durzan 1984). Moreover, positive correlations between chlorophyll and carotenoid pigment concentrations in plants have been established (Kopsell et al. 2004). Since carotenoid pigments function in light-harvesting and photoprotection, it is also possible that elevated chlorophylls and carotenoids could impart greater fitness to the micropropagated plants. The dark environment provided by

AC may also contribute to enhanced growth by promoting early root growth, thus allowing shoots to absorb nutrients early because ‘Nisqually-1’ produced more adventitious roots and produced roots early (data not presented).

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APPENDIX

Table 1.1. Survival rate and performance rating of *Populus trichocarpa* 'Nisqually-1' after 4 weeks on three basal media with three concentrations of N^6-benzylaminopurine (BA).

Basal medium	BA concentration (μM)	Percentage of shoots surviving after 4 weeks	Performance rating[1,2]
MS	0	75.0	3.875 a
MS	2.2	50.0	2.750 b
MS	4.4	37.5	2.375 b
DKW	0	37.5	2.500 b
DKW	2.2	37.5	2.375 b
DKW	4.4	37.5	2.250 b
WPM	0	50.0	2.875 b
WPM	2.2	37.5	2.375 b
WPM	4.4	37.5	2.125 b

[1]Plants were rated on a 5-point scale: (5) plant survived, actively growing, no sign of senescence; (4) plant survived, limited growth, lower leaves showing senescence; (3) plant survived, no growth, showing moderate senescence; (2) plant nearly dead; and (1) plant completely dead. For each treatment, the average value was given as the performance rating.

[2]The same letters in the different rows indicate that there is no significant difference ($P \leq 0.05$).

Table 1.2. The effect of gelling agent and N^6-benzylaminopurine (BA) concentration on the survival of *Populus trichocarpa* 'Nisqually-1' after 4 weeks.

Gelling agent	BA concentration (μM)	Percentage of shoots surviving after 4 weeks	Performance rating[1,2]
Gelrite	0	100.0	4.875 a
Gelrite	2.2	62.5	3.250 bc
Gelrite	4.4	50.0	3.125 bc
Agar	0	75.0	3.875 b
Agar	2.2	50.0	2.750 c
Agar	4.4	37.5	2.375 c

[1]Plants were rated on a 5-point scale: (5) plant survived, actively growing, no sign of senescence; (4) plant survived, limited growth, lower leaves showing senescence; (3) plant survived, no growth, showing moderate senescence; (2) plant nearly dead; and (1) plant completely dead. For each treatment, the average value was given as the performance rating.

[2]The same letters in the different rows indicate that there is no significant difference ($P \leq 0.05$).

Table 1.3. Visible absorption spectra of Chlorophyll *a* of 'Nisqually-1' by HPLC.

AC treatment ($g \cdot L^{-1}$) [1]	Sample dry wt (g)	HPLC % Recovery	Recovered chlorophyll *a* (µg/g dry weight)
0	0.2812	0.91	48.91
3.0	0.0653	0.74	182.36
5.0	0.0736	0.79	779.73
10.0	0.0484	0.82	1056.95
Control (on soil, without AC)	0.0276	0.83	507.68

[1]Plants were grown on PGR-free MS basal medium, solidified with Gelrite, and supplemented with either 0, 3, 5 or 10 $g \cdot L^{-1}$ activated charcoal (AC). Cultures were placed in a growth room where light intensity was 125 µmol $m^{-2}s^{-1}$ provided by fluorescent light. The control plants were grown on soil without AC in a growth chamber where light intensity was 221 µmol $m^{-2} \cdot s^{-1}$ provided by fluorescent and incandescent lighting.

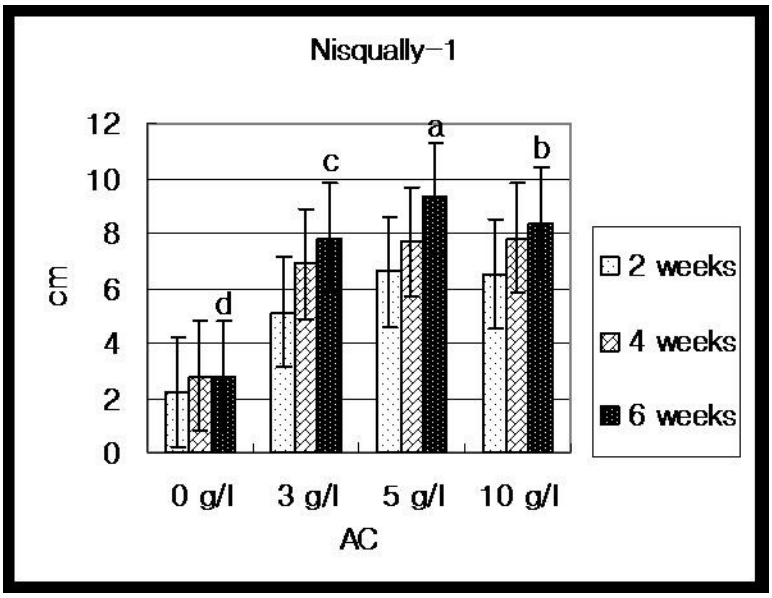

Figure 1.1. Mean length of 'Nisqually-1' shoots grown on MS PGR-free Gelrite medium supplemented with either 0, 3, 5 or 10 $g \cdot L^{-1}$ activated charcoal (AC) after 2, 4 and 6 weeks. Shoots were grown in 9 x 14 cm (1000 ml) plastic vessels in sterile conditions at 25ºC and 16 h photoperiod. Data are the means of 16 shoots. The bar represents the standard error of the mean. The different letters above the bars at six weeks (the final data collection date) indicate the separation of the treatment means.

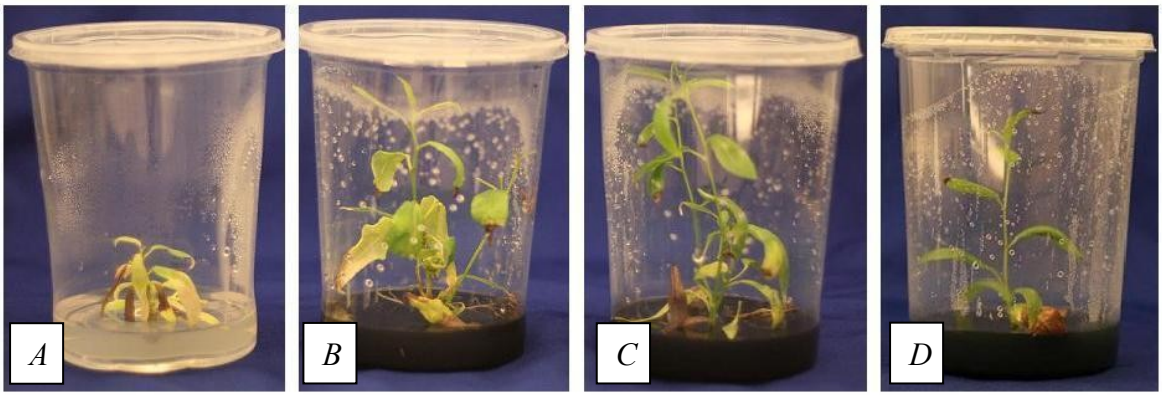

Figure 1.2. ‘Nisqually-1’ grown on MS basal medium, solidified with Gelrite and supplemented with either (A) 0, (B) 3, (C) 5 or (D) 10 $g \cdot L^{-1}$ activated charcoal. Cultures were maintained in a growth room at 25ºC and a 16-h photoperiod where fluorescent light intensity was 125 μmol $m^{-2}s^{-1}$. Photographs were taken at six weeks.

CHAPTER II

Transgenic hybrid aspen overexpressing the *Atwbc19* gene encoding an ATP binding cassette transporter confers resistance to four aminoglycoside antibiotics[2]

[2] This manuscript has been accepted to Plant Cell Reports.

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ABSTRACT

Antibiotic-resistance genes of bacterial origin are invaluable markers for plant genetic engineering. However, these genes are feared to pose possible risk to human health by horizontal gene transfer from transgenic plants to bacteria, potentially resulting in antibiotic-resistant pathogenic bacteria; this a considerable regulatory concern in some countries. The *Atwbc19* gene, encoding an *Arabidopsis thaliana* ATP binding cassette transporter, has been reported to confer resistance to kanamycin specifically as an alternative to bacterial antibiotic resistance genes (Mentewab and Stewart, Nature Biotech 2005, 23:1177-1180). In this report, we transformed hybrid aspen (*Populus canescens* x *P. grandidentata*) with the *Atwbc19* gene. Unlike *Atwbc19*-transgenic tobacco that was only resistant to kanamycin, the transgenic *Populus* plants also showed resistance to three other aminoglycoside antibiotics (neomycin, geneticin, and paramomycin) at comparable levels to plants containing a CaMV35S-*nptII* cassette. Although it is unknown why the transgenic *Populus* with the *Atwbc19* gene is resistant to all aminoglycoside antibiotics tested, the broad utility of the *Atwbc19* gene as a reporter gene is confirmed here in a second dicot species. Because the *Atwbc19* gene is plant-ubiquitous, it might serve as an alternative selectable marker to current bacterial antibiotic-resistance marker genes and alleviate the potential risk for horizontal transfer of bacterial resistance genes in transgenic plants.

Key words: antibiotics resistance genes · aminoglycoside antibiotics · plant-derived selection marker gene · *Populus* · transformation

Abbreviations: *Atwbc19* - *Arabidopsis thaliana* White-Brown Complex homolog 19 gene, *nptII* - neomycin phosphotransferase II gene, BA - N^6 benzylaminopurine, NAA - α-naphthaleneacetic acid, WPM - woody plant medium, MS - Murashige and Skoog medium, GUS - β-Glucuronidase, X-GLUC - 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide cyclohexylammonium salt

INTRODUCTION

For plant transformation, it is necessary to include a selectable marker cassette for selecting transgenic cells that will be induced for regeneration into plants for further characterization (Horsch, Fry et al. 1985; Ow 2007; Rosellini, Capomaccio et al. 2007). Approximately fifty marker genes have been evaluated for their efficiency, biosafety in plant transgenic research, and gene functional validation (Miki and McHugh 2004). Their characteristics, advantages and disadvantages have been intensively reviewed in recent years (Miki and McHugh 2004; Mentewab and Stewart 2005; Ow 2007; Sundar and Sakthivel 2008). Among these marker genes, antibiotic- and herbicide-resistance genes are the most commonly used because of their broad utility, effectiveness as positive selection markers, ease of use, and availability (Miki and McHugh 2004; Sundar and Sakthivel 2008).

Kanamycin is an aminoglycoside antibiotic that was isolated from the soil bacterium *Streptomyces kanamyceticus*. It inhibits both prokaryotic and eukaryotic protein synthesis primarily by binding to the ribosomal complex (Bar-Nun, Shneyour et al. 1983; Wright, Berghuis et al. 1998; Mingeot-Leclercq, Glupczynski et al. 1999), and has been widely used in plant transformation as a selective agent. When plant cells are transformed with the gene encoding neomycin phosphotransferase (*nptII*), which detoxifies the antibiotic (Wright, Berghuis et al. 1998), resistant cells proliferate and nontransformed cells will be inhibited or killed, allowing the transgenic cells regenerate into plantlets (Horsch, Fry et al. 1985). Although kanamycin resistance via aminoglycoside-modifying enzymes is widespread among soil bacteria, a remote risk of

horizontal gene transfer through food chains from transgenic food crops into bacteria in human and animal guts remains a great public concern (Nap, Bijvoet et al. 1992; Heinemann and Traavik 2004; Levy-Booth, Campbell et al. 2007; Pontiroli, Rizzi et al. 2009).

One alternative selectable marker for antibiotic resistance is the *Atwbc19* gene (*Arabidopsis thaliana* White-Brown Complex homolog 19 gene), a kanamycin resistance gene encoding an ATP binding cassette (ABC) transporter, was discovered when its knockout mutants showed much slower root growth on medium containing kanamycin than those of other T-DNA insertional knockout mutants containing *nptII* (Mentewab and Stewart 2005). Further characterization demonstrated that *Atwbc19* can provide resistance to kanamycin at the level of comparable to that conferred by the *nptII* gene when overexpressed in tobacco (Mentewab and Stewart 2005). The *Atwbc19* gene was suggested to be broadly useful for other dicot species, as well as for trees (Mentewab and Stewart 2005), yet there have been no reports in other species so far. In this report, we compared the *Atwbc19* gene with the *nptII* gene, both under control of the CaMV35S promoter in *Populus*, a forest tree which remains relatively undomesticated and has been subject to vigorous regulation and public scrutiny (Heinemann and Traavik 2004; Farnum, Lucier et al. 2007; Strauss, Tan et al. 2009). Our results demonstrated that the *Atwbc19* gene confers a level of kanamycin resistance similar to the *nptII* gene. However, we also found that in *Populus*, the *Atwbc19* gene also confers resistance to three other aminoglycoside antibiotics (neomycin, geneticin, and paramomycin). Therefore, the *Atwbc19* is a useful alternative to the *nptII* gene in *Populus* transformation. This is the

first confirmative report that *Atwbc19* is an effective alternative marker gene to the *nptII* gene (Mentewab and Stewart 2005).

MATERIALS AND METHODS

Plants materials

In vitro aspen shoot cultures were established from actively growing shoots of an aspen hybrid clone [*Populus canescens* x *P. grandidentata*, 36-67-S-1 (pcg)] as described by Dai et al. (2003), and maintained in a culture room at 25ºC under a 16-h photoperiod by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 μmol $m^{-2}s^{-1}$ as measured by a LI-COR LI-250 handheld light meter (LI-COR Inc., Lincoln, NE) positioned at the top of the culture vessels. All explants were aseptically grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.1 % (v/v) MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS), 100 $mg \cdot L^{-1}$ myo-inositol, 3.0% (w/v) sucrose, and 0.3% (w/v) gelrite (Plantmedia, Dublin, OH). All media were adjusted to a pH of 5.7 – 5.8 prior to autoclaving at 120 ºC and 103.5kPa (15 lb in^{-2}) for 20 min. All of the stock cultures in the following experiments were maintained under these conditions.

Vector and Agrobacterium strain

The binary transformation vectors used in this work were *pABC* and *pNPT,* based on pCambia2301 (CAMBIA) having the gene controlled by the constitutive promoter CaMV35s (Mentewab and Stewart 2005). The *Atwbc19* and *nptII* genes were PCR amplified from the initial cDNA clone using forward 5'-GTC TTC CCG GAT TCT TCT CC-3' and reverse 5'-TGT GCT CCT CTT GGA GTG TG-3' primers and forward 5'-GAG GCT ATT CGG CTA TGA CTG-3' and reverse 5'-ATC GGG AGC GGC GAT

ACC GTA-3' primers, which contained SacI-KpnI and XhoI-XhoI sites, respectively (Fig. 2.1). Each binary vector was transferred into *Agrobacterium tumefaciens* strain GV3850 by electroporation. Prior to inoculating aspen leaf segments for transformation, the *Agrobacterium* strain harboring each vector was grown overnight at 28 ºC to a final density of 0.8-1.0 at OD_{600}.

Transformation of aspen using the* Atwbc19 *and nptII genes

For transformation, previously established *in vitro* hybrid aspen plants were transferred from MS medium to woody plant medium (WPM) (Lloyd and McCown 1981), supplemented with 100 $mg \cdot L^{-1}$ myo-inositol, 0.1 % (v/v) MS vitamins, 3.0% (w/v) sucrose, and 0.3 % (w/v) gelrite. Leaves harvested from *in vitro*-grown aspen plants were cut into 0.5 cm^2 segments. One hundred leaf segments were infected by *Agrobacterium* strain GV3850 harboring each vector by immersing them for 30 min in an inoculum solution consisting of 5 ml Lysogeny broth (LB) *Agrobacterium* suspension culture. After infection, the leaf segments were blotted on sterile filter paper to remove the excess bacterial inoculum and then placed on co-cultivation medium [WPM supplemented with 8.9 μM N^6 benzylaminopurine (BA), 5.37 μM α-naphthaleneacetic acid (NAA), and 100 μM acetosyringone]. After 3 days of co-cultivation in the dark, the inoculated leaf segments were washed with WPM liquid medium supplemented with 250 $mg \cdot L^{-1}$ cefotaxime, and transferred to a callus induction medium [(CIM) WPM supplemented with 8.9 μM BA, 5.37 μM NAA, 100 $mg \cdot L^{-1}$ myo-inositol, 0.1 % (v/v) vitamins, 3.0% (w/v) sucrose, and 0.3 % gelrite]. Antibiotics (250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$ cefotaxime) were added to eliminate *Agrobacterium* and 80 $mg \cdot L^{-1}$ kanamycin was used

for selecting transformed cells (Dai, Cheng et al. 2003; 2004). After 3 weeks, induced calli were transferred to fresh CIM for shoot induction. After 5-6 weeks regenerated shoots formed, grew and were transferred to a rooting medium [1/2 MS salts supplemented with 3.0% (w/v) sucrose, 0.3% (w/v) gelrite, 250 $mg \cdot L^{-1}$ carbenicillin, 250 $mg \cdot L^{-1}$ cefotaxime, and 100 $mg \cdot L^{-1}$ kanamycin]. The shoots were later transplanted to sterilized soil after confirmation by PCR. The control leaf segments were treated similarly except that they were infected with LB broth without *Agrobacterium*.

Plant genomic DNA isolation and PCR analysis

To confirm the presence of the transgene in plants that were kanamycin resistant, genomic DNA was isolated from 0.5 g transgenic and control plant leaf tissues using a DNeasy® Plant Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Each sample was taken from shoots growing on rooting medium with 100 $mg \cdot L^{-1}$ kanamycin for 4 weeks to ensure that samples were free from *Agrobacterium* contamination. To detect the *Atwbc19* and *nptII* genes, 50 ng genomic DNA was used for PCR amplification by using the *Atwbc19* primers (forward 5'- GTC TTC CCG GAT TCT TCT CC-3' and reverse 5'- TGT GCT CCT CTT GGA GTG TG -3'), and the *nptII* primers (forward 5'-GAG GCT ATT CTA TGA TG-3' and reverse 5'-ATC GGG AGC GGC GAT ACC GTA-3'). The expected size for the *Atwbc19* gene was 472 bp and that for the *nptII* gene was 679 bp. Amplified fragments were separated on 1 % agarose gels using a DNA 2-KB Ladder (Fisher Scientific Inc., Pittsburgh, PA).

β-Glucuronidase (GUS) histochemical assays

The expression of the GUS gene in the aspen transformants was assayed by histochemical staining. The test was based on the common histochemical GUS assay suggested by Stomp (1992). Tissues were dipped into a GUS assay solution comprised of 50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, and 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide cyclohexylammonium salt (X-GLUC) substrate and incubated for 1 day at 37 °C. The chlorophyll in the petioles and leaves was destained by rinsing with 70% ethanol twice over a 24-h period until all of the chlorophyll was removed and the blue GUS stain was clearly visible (Jefferson, Kavanagh et al. 1987)

Reverse transcription (RT) - PCR

Total RNA was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO) and treated with AMPD1 DNase I (Sigma-Aldrich) to remove any traces of DNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the iScript™ select cDNA synthesis kit (Bio-Rad, Hercules, CA) with oligo dT primers. The RT-PCR reaction was carried out for 1 h at 42 ºC, followed by PCR activation at 95 ºC for 5 min, 35 cycles of amplification (94 ºC for 30 seconds, 55 ºC for 30 seconds, and 72 ºC for 1 min), and a final extension of 10 min in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA).

Confirmation of resistance to aminoglycoside antibiotics by regeneration of leaf tissues of transgenic plants on media with kanamycin, neomycin, geneticin and paromomycin

To confirm whether the *Atwbc19*-transgenic aspen plants were resistant to kanamycin and other aminoglycoside antibiotics, *in vitro* leaf explants from control plants and plants transformed with the *Atwbc19* and *nptII* genes were cultured on CIM that contained various aminoglycoside antibiotics, similar to that described by Kapaun and Cheng (1999). Several concentrations of kanamycin (0, 50, 100, 150 and 200 $mg \cdot L^{-1}$), neomycin (0, 50, 100, 200 and 400 $mg \cdot L^{-1}$), geneticin (0, 1.25, 2.5, 5.0 and 10.0 $mg \cdot L^{-1}$) and paromomycin (0, 50, 100, 200 and 400 $mg \cdot L^{-1}$) were added to the media to compare the effects of each specific antibiotic on regeneration. For each antibiotic, three replicate plates were used, each with five 0.5 cm^2 leaf segments. Each experiment was repeated three times. Regeneration frequency was defined as the percentage of leaf segments that produced shoots in each antibiotic-containing medium. The experimental design was completely randomized and the regeneration frequency was evaluated by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

Confirmation of transformed plants with the Atwbc19 and nptII genes by selection medium

Among the 100 leaf segments that were infected with *Agrobacterium* that contained either the *Atwbc19* or the *nptII genes*, 60% and 63% of the leaf segments, respectively, formed calli. Of those kanamycin-resistant calluses, 58% and 60%, respectively, regenerated into shoots on medium containing 80 $mg \cdot L^{-1}$ kanamycin (Figures 2.2A, 2.2B, respectively, and Table 2.1). While non-infected control leaf segments formed few calli, when placed on medium with 80 $mg \cdot L^{-1}$ kanamycin, they did not produce shoots (data not shown). The seven *Atwbc19*- and nine *nptII*-transgenic plants (Table 2.1) were transferred into pots after confirmation by PCR and all transgenic plants appeared morphologically normal (Fig. 2.2D). There were no significant differences among 9-week-old transgenic and nontransgenic plants in plant height and number of leaves (data not shown).

Confirmation of transgenic plants by PCR

Both the *Atwbc19* (Fig. 2.3, left panel) and the *nptII* (Fig. 2.3, right panel) genes were PCR-amplified using their specific primer pairs, yielding the expected band sizes of 0.47 kb and 0.67kb, respectively.

β-Glucuronidase (GUS) histochemical assays

GUS gene expression, indicated by blue staining, was detected in the aspen transformants containing the *Atwbc19* gene and the *nptII* gene. GUS expression was not detected in non-transgenic control plant tissues (Fig. 2.4).

RT-PCR analysis of transcription level of the Atwbc19 and nptII genes in transformed plants

RT-PCR was performed on two *Atwbc19*-transgenic lines to confirm the expression of the *Atwbc19* transgene. As shown in Fig. 2.5, the expected 472 bp bands for the *Atwbc19* gene (left panel, lanes 3 and 4) and 679 bp bands for the *nptII* gene (right panel, lanes 5 and 6) were clearly visible. A non-transformed aspen plant sample was used as the control which showed no amplification (lane 2). Total RNA was also screened by PCR without reverse transcriptase to ensure preparations were free of DNA contamination.

Confirmation of transgenic plants by retesting regeneration ability in media containing aminoglycoside antibiotics

Transgenic plants were retested for resistance to kanamycin and other aminoglycoside antibiotics by regeneration on aminoglycoside antibiotic-containing media. Leaf segments from *Atwbc19*- and *nptII*-transgenic plants were regenerated on medium with 100 and 150 $mg \cdot L^{-1}$ kanamycin, respectively, while a non-transformed control plant was regenerated on medium with only 50 $mg \cdot L^{-1}$ kanamycin. *Atwbc19*- and *nptII*-transgenic plants were regenerated on media with 200 $mg \cdot L^{-1}$ neomycin, 5 $mg \cdot L^{-1}$

geneticin, and 100 mg·L^{-1} paromomycin, respectively, while the leaf segments from a non-transformed plant were regenerated on media with 50 mg·L^{-1} neomycin, 1.25 mg·L^{-1} geneticin, and 50 mg·L^{-1} paromomycin. Transgenic plants with either the *Atwbc19* gene or the *nptII* gene showed comparable regeneration ability at similar levels of antibiotics, which was about 2-3 times of the antibiotic level used for the non-transformed control plants (Fig. 2.6 and Table 2.2).

DISCUSSION

Marker genes of bacterial origin that confer antibiotic resistance, such as *nptII*, are commonly used for plant transformation, especially for dicotyledonous species (Bevan, Flavell et al. 1983; Horsch, Fry et al. 1985; Miki and McHugh 2004; Sundar and Sakthivel 2008). Although these bacterial antibiotic resistance marker genes are regarded by many regulatory agencies to be safe (Miki and McHugh 2004), there is a remote probability of horizontal gene transfer between transgenic plants and soil microorganisms or bacteria in human and animal guts that might cause unpredictable consequences to ecosystems or human health (Heinemann and Traavik 2004; Nielsen and Townsend 2004). These strong public concerns of antibiotic resistance genes of bacterial origin in transgenic plants has prompted development of alternative means to remove them from commercial products, such as deletion of the marker gene after selection (Luo, Duan et al. 2007; Ow 2007; Darbani et al. 2007), using plant native genes as selective marker genes (Rommens, Humara et al. 2004; Mentewab and Stewart 2005), and use of non-bacterial antibiotic-resistance genes as marker genes (Mentewab and Stewart 2005).

The *Atwbc19* gene is a plant gene that confers resistance to kanamycin at levels similar to the bacterial *nptII* gene, but, in transgenic tobacco, not to other aminoglycoside antibiotics (Mentewab and Stewart 2005). However this gene's potential as a plant-derived antibiotic resistance marker gene has not been explored in other plant species. Using a well-established protocol for aspen transformation (Dai, Cheng et al. 2003; 2004) with *nptII* as a selective marker gene which confers resistance to aminoglycoside antibiotics, we compared the *Atwbc19* and *nptII* genes in aspen transformation. PCR-

amplification, RT-PCR on gene expression, GUS staining, and shoot regeneration from leaves of transgenic plants on antibiotic-containing media together suggest that the transgenes are stably integrated into the aspen genome, expressed, and functionally conferring resistance to four aminoglycoside antibiotics (kanamycin, neomycin, geneticin, and paromomycin). The results confirmed that transformation efficiencies with the *Atwbc19* gene were, as shown by time for callus formation, shoot formation, level of resistance to kanamycin, and normal morphologies of transgenic plants, similar to transformation efficiencies when the *nptII* gene was employed. Since *Populus* species are generally less domesticated, and gene flow is common via natural hybridization between planted and natural *Populus* stands, the release of transgenic *Populus* into the environment has been subject to vigorous regulatory review and public scrutiny and opposition (Heinemann and Traavik 2004; Farnum, Lucier et al. 2007; Strauss, Tan et al. 2009). Therefore, use of the plant-native *Atwbc19* gene as a selective marker might, at least, partially alleviate the public concerns of field deployment of transgenic *Populus*.

Unlike the *Atwbc19*-transgenic tobacco which is resistant only to kanamycin, the *Atwbc19*-transgenic *Populus* were resistant to kanamycin and other three aminoglycoside antibiotics: neomycin, geneticin, and paromomycin. Mentewab and Stewart (2005) suggested that the *Atwbc19* gene forms a homodimer, but there is a possibility that it forms a heteromeric complex with another half-molecule ABC transporter. Several mammalian half-molecule ABC transporters form homodimers, including the breast cancer resistance protein, the human homolog of *Atwbc19*, which confers resistance to the anticancer antracyclines and mitoxantrone (Kumie, Satomi et al. 2002). In *Drosophila melanogaster*, the white, scarlet, and brown proteins can form heterodimers that mediate

the transport of pigment precursors into the cells responsible for eye color (Dreesen, Johnson et al. 1988). Since whether *Atwbc19* forms homodimers or heterodimers has not been determined in transgenic tobacco (Mentewab and Stewart 2005) or transgenic *Populus*, it is unknown why *Atwbc19*-transgenic tobacco plants are resistant only to kanamycin while *Atwbc19*-transgenic *Populus* plants are resistant to multiple aminoglycoside antibiotics. It could be possible that *Atwbc19* behaves differently in tobacco and *Populus* ; for example, it could form heterodimers with endogenous *Populus* WBCs to enlarge the substrate specificity in poplars. Regardless of narrow- or broad-resistance to aminoglycoside antibiotics, our research confirms that the *Atwbc19* gene is an alternative to the bacterial *nptII* gene as a plant transformation selective marker gene. The broad utility of this plant-derived antibiotic resistance gene as a selective marker gene might be applicable in other dicotyledonous plants and coniferous trees.

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APPENDIX

Table 2.1. Confirmation of aspen transformation with the *Atwbc19* and *nptII* genes by antibiotic selection medium and PCR

Gene	Leaf segments with kanamycin-resistant calluses	Shoots from kanamycin-resistant calluses	Kanamycin-resistant shoots confirmed with PCR
Atwbc19	60/100 (60.0 %) a	35/60 (58.3 %) a	7/35 (20.0 %) a
NptII	63/100 (63.0 %) a	38/63 (60.3 %) a	9/38 (23.7 %) a
Control	20/100 (20.0%) b	0/20 (0.0%) b	not applicable

Explants were grown on WPM supplemented with 250 $mg \cdot L^{-1}$ carbenicillin and cefotaxime, and kanamycin (80 $mg \cdot L^{-1}$ for callus and 100 $mg \cdot L^{-1}$ for shoots). The same letters in different rows indicate that there is no significant difference ($p \leq 0.05$)

Table 2.2. Regeneration frequency (%) from leaf tissues of *Atwbc19*- and *nptII*-transgenic plants and non-transformed control plants on media containing a range of concentrations of aminoglycoside antibiotics

	Kanamycin ($mg \cdot L^{-1}$)				
	0	50	100	150	200
Atwbc19	84 b	65 a	36 a	0 b	0
nptII	91 a	67 a	40 a	18 a	0
Control	89 ab	33 b	0 b	0 b	0
	Neomycin ($mg \cdot L^{-1}$)				
	0	50	100	200	400
Atwbc19	89 a	69 a	44 a	13 b	0
nptII	87 a	73 a	49 a	24 a	0
Control	91 a	27 b	0 b	0 c	0
	Geneticin ($mg \cdot L^{-1}$)				
	0	1.25	2.5	5.0	10.0
Atwbc19	91 a	62 a	29 a	11 a	0
nptII	89 a	73 a	36 a	20 a	0
Control	89 a	18 b	0 b	0 b	0
	Paromomycin ($mg \cdot L^{-1}$)				
	0	50	100	200	400
Atwbc19	89 ab	62 a	33 a	0	0
nptII	87 b	56 a	29 a	0	0
Control	96 a	36 b	0 b	0	0

Plants were grown on WPM supplemented with kanamycin, neomycin, geneticin or paromomycin. Cultures were maintained in a growth room at 25ºC and a 16-h photoperiod where fluorescent light intensity was 125 µmol $m^{-2}s^{-1}$. The same letters in different rows indicate that there is no significant difference ($p \leq 0.05$).

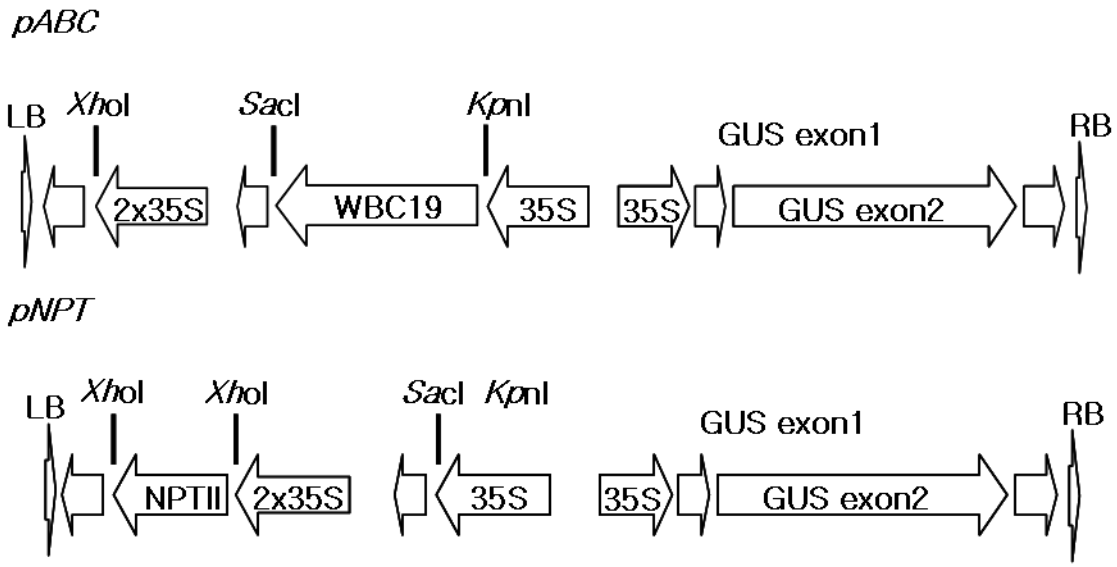

Figure 2.1. The T-DNA portion of the binary vectors *pABC* and *pNPT*: the binary vectors containing the *Atwbc19* and *nptII* gene, respectively, used to transform aspen. The vector also contains GUS as a storable marker under the control of the CaMV35S promoter.

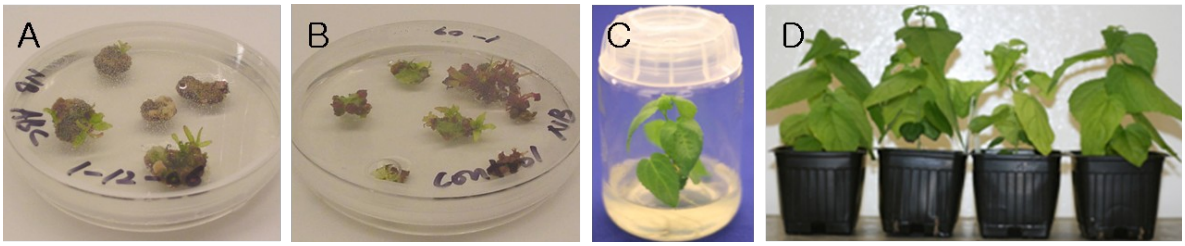

Figure 2.2. Transformation of aspen with the *Atwbc19* and *nptII* genes. (A) and (B) Adventitious shoots formed from calluses that were infected with *Agrobacterium* strain GV3850 containing the *Atwbc19* and *nptII* genes, respectively, on 80 $mg \cdot L^{-1}$ kanamycin-containing medium. (C) The *Atwbc19* transgenic plant was cultured on rooting medium (1/2 MS plus antibiotics). (D) *Atwbc19*- (left two) and *nptII*- (right two) transgenic plants in pots.

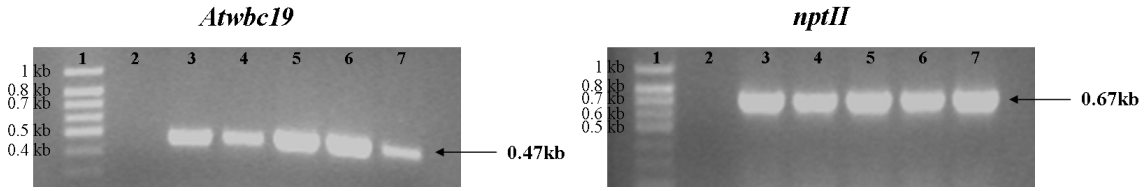

Figure 2.3. PCR-amplification with the *Atwbc19*- (left panel) and the *nptII*- (right panel) specific primers (*Atwbc19*-specific primers: forward 5'-ACTGCAGGTACCATGAATC TATCACTCAGCGG-3' and reverse 5'-TGTCCCCGTTTTTATCCAG-3'; the *nptII*-specific primers: forward 5'-GAGGCTATTCTATGATG-3' and reverse 5'-ATCGGGAG CGGCGATACCGTA-3' primer). Left panel: lane 1: 2-Kb marker, lane 2: non-transgenic control plant; lanes 3-7; five independent transgenic lines with the *Atwbc19* gene. Right panel: lane 1: 2-Kb marker, lane 2: non-transgenic control plant; lanes 3-7; five independent transgenic lines with the *nptII* gene.

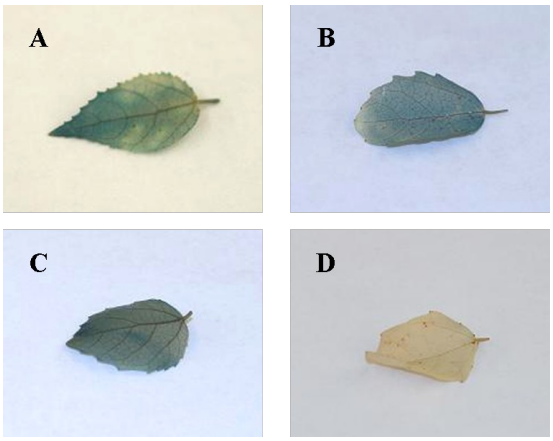

Figure 2.4. Histochemical analysis of *β*-Glucuronidase (GUS) gene expression in transgenic plants. Leaves were stained with X-GLUC (A) and (B) Transformed with *Atwbc19*; (C) Transformed with *nptII*; (D) non-transgenic control plant showing absence of blue color.

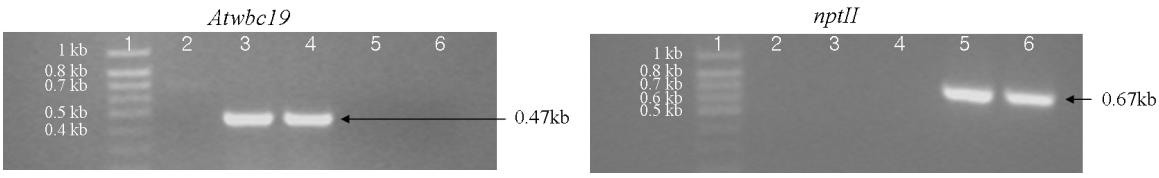

Figure 2.5. Expression of the *Atwbc19* (left panel) gene and the *nptII* (right panel) gene analyzed by RT-PCR. RT–PCR analysis of selected transcripts in leaf tissues of aspen. Left panel: lane 1: 2-Kb marker, lane 2: non-transgenic control plant, lanes 3 and 4: two independent transgenic lines with the *Atwbc19* gene. Lanes 5 and 6: two independent transgenic lines with the *nptII* gene. Right panel: lane 1: 2-Kb marker, lane 2: non-transgenic control plant; lanes 3 and 4: two independent transgenic lines with the *Atwbc19* gene. Lanes 5 and 6: two independent transgenic lines with the *nptII* gene.

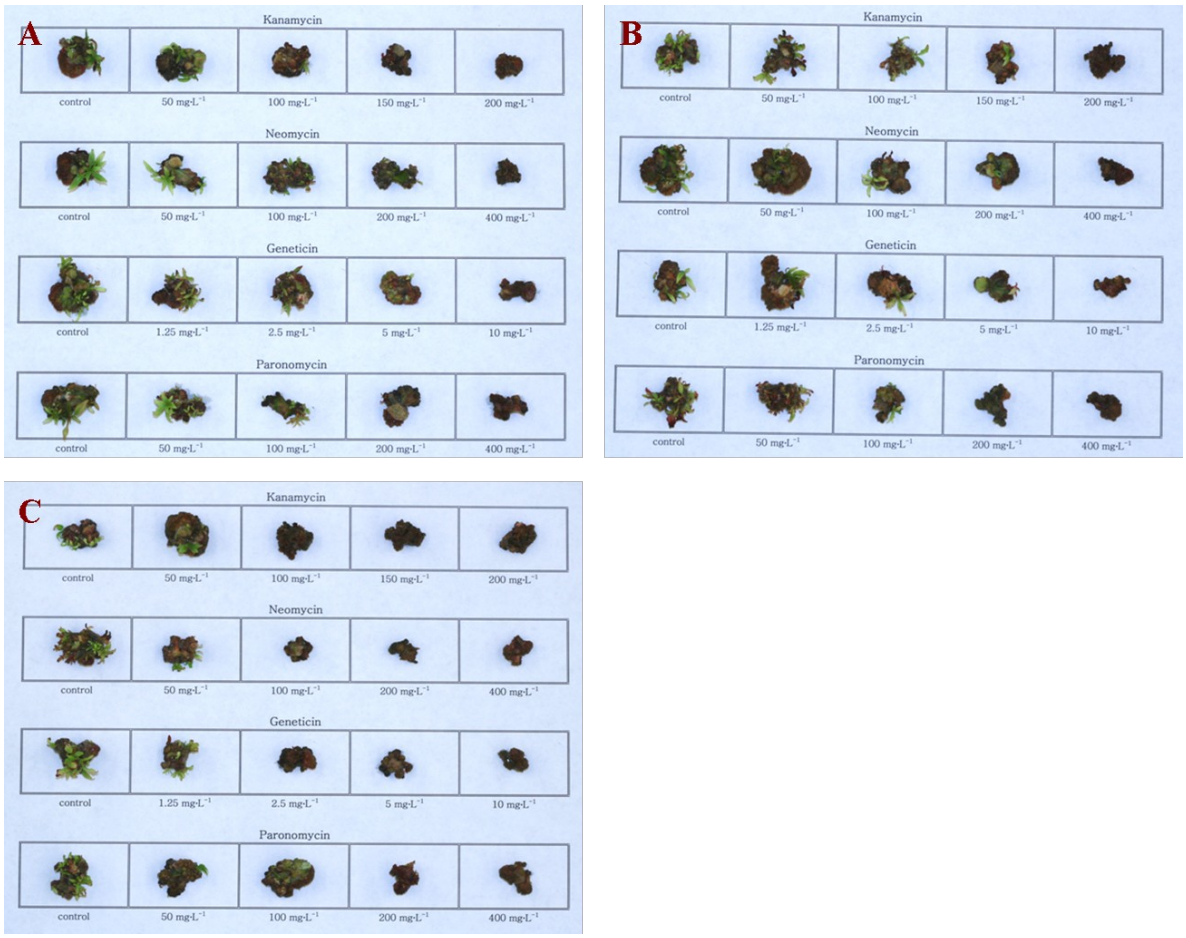

Figure 2.6. Regeneration of leaf tissues of *Atwbc19*- (A) and *nptII*- (B) transgenic plants and non-transformed plants (C) on media containing various concentrations of four different aminoglycoside antibiotics.

CHAPTER III

Establishment of Aspen Enhancer Trap Lines for Functional *Populus* Genomics

ABSTRACT

Enhancer trapping is an insertion-based strategy based on detecting gene expression, instead of detecting mutant phenotypes. In this report, using previously established *Agrobacterium tumefaciens*-mediated transformation system, we transformed a *Populus* clone with the enhancer trapping vector, pD991, which contains a minimal -60 CaMV promoter fused to the *uid*A (GUS) reporter gene and the *npt*II gene conferring resistance to kanamycin. A total of 250 transgenic lines were confirmed with polymerase chain reaction with the GUS specific primers. The *in vitro* rooted, 4-5 leaf-stage plants were used in histochemical GUS staining assay. All 250 lines were screened and 71 of them (28%) showed positive staining. They showed various patterns of the reporter gene expression, including expression in one tissue and simultaneously in two more tissues. These results suggest that the GUS gene with the minimal promoter was inserted into the genome where the enhancers are nearby. The rest of them did not show the GUS staining probably because 1) they were not inserted in the positions with nearby enhancers, or 2) they were not inserted in the positions with the genes which the enhancer act on were developmentally regulated so they were expressed at the stage or tissues which were used in the GUS staining. These results confirmed the previously reports that enhancer trap lines can be produced in *Populus*, and these enhancer trap lines can be used for future gene cloning and studying gene expression in *Populus*.

Kew words: Agrobacterium tumefaciens · a minimal -60 CaMV · pD991 · GUS · *npt*II · enhancer trap · *Poplulus*

Abbreviations: GUS - β-Glucuronidase, X-GLUC - 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide cyclohexylammonium salt, *nptII* - neomycin phosphotransferase II gene,

INTRODUCTION

Limitations of current research methods in understanding popular breeding and biology

Trees are the main life form in many ecosystems and comprise greater than 90% of the earth's terrestrial biomass. Managed and unmanaged forests throughout the United States, and indeed the world, provide recreational and environmental benefits such as carbon confiscation, renewable energy supplies, watershed protection, enhanced air quality, biodiversity and habitat for endangered species (Spies et al., 2002). However, despite the importance of trees for natural ecosystems and the world economy, little is known about tree biology in comparison with the detailed information available for crop plants and model organisms such as *Arabidopsis*. The approach by traditional genetics in forestry are very limited by the large plant size, long generation time and outcrossing mating of most trees. Thus, the community of forest science needs to derive much benefit from a comprehensive genomics research program. So, forest biologists will have the essential resources to begin a large scale, thorough genetic analysis that produce traits useful in the pursuit of basic science questions, to foster the development of improved plant materials for the forest product industry.

General aspect of poplar genomic projects

Populus is a genus that is widely distributed over the Northern Hemisphere, and the genus comprises about 35 species of poplar, cottonwoods, and aspens, grown for their economic use for wood, fiber, and energy. *Populus* species are also utilized in

environmental protection projects for their ability to clean contaminated soil and ground water (Zsuffa et al., 1984; Stealer et al. 1996). They grow fast, are generally easy to propagate vegetatively, and are adapted to various soil types found in different geographical regions: from boreal to sub tropical and from mountains to riparian. Furthermore, the genus *Populus* has many advantages as a model system for forest tree molecular biology (Bradshaw et al., 2000).

Populus is particularly well suited to serve as the tree genome model for several reasons. Its genome has been completely sequenced (Tuskan et al., 2006). With a relatively small genome, the haploid genome size of *Populus* is only 485 Mbp, similar to rice and only 4X greater than that of *Arabidopsis*, but ~40X smaller than those of pines. All *Populus* are diploids (2n=2x=38) that can be easily hybridized. Since the hybrids are fertile, advanced generation pedigrees can be generated, and many of such pedigrees are available. Its rapid growth allows meaningful measures of important traits to be taken within a few years after establishment of genetic trials. This early assessment allows manipulations to be evaluated across common genetic backgrounds; destructive sampling (e.g., wood quality assessment) can be carried out without loss of the original tree. Many *Populus* species and genotypes are amendable for high-throughput transformation and regeneration which will permit detection and characterization of gene function in transgenic trees. *Populus* is the only tree genus with all of these features in this regard. Genetic maps have been constructed in *Populus*, including the first identification of "quantitative trait loci" (i.e., DNA markers associated with phenotypic traits). Most importantly, many scientists around the world have used poplar as a model organism to study forest tree morphology, physiology, biochemistry, ecology, genetics and molecular

biology, which collectively have made considerable advancement in *Populus* biology, and this trends will accelerate in the future with the reference genome available.

Poplar genomics – understanding poplar tree biology

Many tree species have large and complex genomes, making any type of genetic characterization or manipulation more difficult than *Arabidopsis* or even in monocotyledonous crop plants (Taylor, 2002). The genome sequence is a basic research tool for studying all aspects of tree biology. Knowledge of the genome sequence is essential in identification of genes related to commercially important traits such as growth, and genes involved in biological processes related to adaptation to the environment. Genetic and physical maps using markers on the genetic linkage groups, combined with the known genome sequence support the generation of a cooperated genome sequence-physical map-genetic map resource. The goal of *Populus* genomics research is to understand the genetic and molecular basis of all biological processes. The sequence can thus be used to identify genes related to commercially economically and ecologically important and relevant traits.

Sequence project

The sequencing of the complete genome of *Populus* has been completed by the Department of Energy-Joint Genome Institute (DOE-JGI) (Tuskan et al., 2006). The availability of the *Populus* genome sequence has provided researchers many new opportunities to compare the genome of an herbaceous plant with the genome of a perennial plant. From the comparison of the *Populus* genome with the *Arabidopsis*

genome, it should be apparent if a gene of interest belongs to a known gene family or represents a class of genes that is unique to trees. These unique genes will provide insights into the developmental and evolutionary processes that distinguish perennial woody plants from herbaceous plants. In addition, the analysis of expressed sequence tag (EST) databases of poplars with special characteristics, in comparison to the whole genome sequence of *P. trichocarpa*, will point to genes that may be involved in the adaptation of trees to extreme environments, such as deserts habitats with frequent fires. The orthologs of genes that have been identified in *Arabidopsis* and that have commercial applications in poplar can be isolated using the poplar sequence information. Finally, availability of the whole genome sequence marks the post-genomic era of forestry research, including the analysis of the full complement of proteins and metabolites of *Populus* (Ellis et al., 2004).

Functional genomics

Functional genomics is the study of the function of all genes of an organism. The vast wealth of data produced by genomic projects can be utilized in order to describe gene functions and interactions. The creation of transgenic lines with enhanced levels of gene expression is the most straightforward way to determine gene function (Wullschleger et al., 2002). Identification of specific disease-resistant genes, for instance, could allow the researcher to produce increased numbers of plants that are resistant to disease. Light, temperature, water accessibility, and salinity, to name a few, are important environmental response factors. Plant hormones are other important factors. It already has been discovered that the plant hormone, ethylene, affects a wide variety of plant

processes, including fruit ripening, leaf and flower wilting and leaves' color changing (Chandler 1990). Scientific studies involved in functional genomics uses mostly high-throughput techniques to characterize the abundance of gene products such as mRNA and proteins.

T-DNA tagging

One of most widely used approaches for large-scale functional gene analysis in plants is random insertional mutagenesis. Both T-DNA and transposons can be used as insertional mutagens in plants (Ramachandran et al., 2000). Transposon tagging has become a powerful tool for isolating new genes since the controlling element was first recognized by McClintock (Fedorff et al., 1984; 1993). A number of genes have been isolated using endogenous transposons as tags (Sundaresan, 1996). The first successful cloning of a plant gene was achieved via the Activator-Dissociation (Ac/Ds) transposon system (Fedorff et al., 1984; 1993). Other transposon systems, such as Enhancer/Suppressor-mutator (En/Spm) and Mutator (Mu), have been used for cloning several genes of maize. The non-autonomous Ds element has been transposed in the presence of Ac transposase via the direct gene transfer method. Germinal transposition of Ds was observed at high frequency in the R_2 progeny when a transgenic plant containing the Ds element was crossed with a transgenic plant carrying Ac transposase under the control of the cauliflower mosaic virus (CaMV) 35S promoter. A wide spectrum of mutations affecting growth morphogenesis, flowering time and disease resistance were observed in the Ds population.

Insertion mutagenesis using *Agrobacterium*-mediated T-DNA integration into

plant genomes has proved to be very successful (Leehan et. al, 1997). The exact insertion site is then determined by reference to the genome sequence, obviating the need for genetic mapping and associated sexual reproduction which are both laborious as well as time-consuming. Insertions in individual genes are thus indexed with mutant plant phenotypes and together provide a dynamic resource serving the scientific community. This approach has the advantage of simplicity as each transformant yields a stable insertion in the genome and does not need additional steps to stabilize the insert (Ramachandran et al., 2000). In Arabidopsis, a model dicot species, numerous genes have been isolated from T-DNA-tagged lines. T-DNA tagging has become an important tool in plant physiology and molecular biology research. T-DNA insertion might result in an obvious change in phenotype in the host plant. T-DNA-tagged lines are useful resources for studying gene function. Increasingly, T-DNA tagging is also being used to characterize genes in other plant species. For example, activation tagging involves the random insertion of a strong promoter element that can result in overexpression of genes near the insertion site, and resulting developmental phenotypes are dominant (Kardailsky et al., 1999). The sequence of the tag provided a landmark allowing its isolation of the mutated gene. T-DNA insertion is being increasingly used to determine gene function (Ricard, 2002).Thus, it may be anticipated that this method of gene tagging will play an important role in investigating gene function in plant developmental processes that are not easily accessible with Arabidopsis (Ricard, 2002). One must determine whether these phenotypes are induced by plant transformation or by the T-DNA insertion itself. Once the mutant phenotype co-segregates with T-DNA, the sequence flanking T-DNA can then be isolated by PCR-based methods such as thermal asymmetric interlaced (TAIL) PCR,

inverse PCR and plasmid rescue system (Weigel, 2000). TAIL PCR entails consecutive reactions with nested sequence-specific primers and a shorter arbitrary degenerate primer. Many of these methods require special steps before PCR such as Southern blot analysis to determine suitable restriction sites, followed by manipulations such as restriction cutting and ligation or tailing (Liu et al., 1994).

However, lethal mutations may be induced during the T-DNA transformation process. Chromosome rearrangement or modification might also occur during transformation (Jeon et al., 2001). Conventional strategies, such as T-DNA mutagenesis or transposon tagging, are not efficient for analyzing the function of redundant genes. In addition, these strategies will not be able to identify the genes that are required during multiple stages of a life cycle and whose loss of function results in early embryonic or gametophytic mortality (Weigel et al., 2000). Such mutations do not always produce an obvious phenotype due to various factors such as functional redundancy. Many genes tagged in the *Arabidopsis* genome don't generate a visible phenotypic change. Therefore, complementing technologies are needed for assessing the function of the remaining genes. In such cases, increasing the expression level or ectopically expressing a gene can provide dominant gain of functional mutations that produce informative mutant phenotypes. A strategy to generate mutants as a consequence of increased or expanded expression of a tagged gene is known as 'activation tagging' (Ramachandran et al., 2000).

Gene and Enhancer traps

Many insertions can integrate within transcriptional units so that promoters, enhancers, exons, and introns can be tagged. In such cases, if the tag contains marker genes, endogenous patterns of expression of tagged sequences can be monitored (Ricard et al., 2002). This kind of tagging is called entrapment tagging. The entrapment tagging system allows for monitoring gene activity by creating fusions between tagged genes and a reporter gene, such as ß-glucuronidase (GUS) and green fluorescent protein (GFP). Insertion of the promoterless reporter will not destroy the normal gene. Three entrapment systems are available: enhancer, promoter and gene trap. The system for entrapment can be transferred into plant cells as a part of T-DNA or transposons (Jeon et al., 2001).

Gene and enhancer traps are powerful tools for isolation and characterization of tissue-specific novel plant genes. Gene and enhancer trapping are alternative methods for insertion-based gene discovery that both reference genome sequence data and produce a dominant expression phenotype (Springer, 2000). Gene trap reporter genes (promoter traps and exon traps) do not have a functional promoter, while enhancer trap reporter genes have a minimal promoter element preceding a reporter gene. The reporter gene, which mimics the normal expression pattern of a gene at the insertion site, has been shown for *Arabidopsis* gene and enhancer trap lines (Gu et al., 1998; Pruitt et al., 2000). The isolation of genomic sequences flanking of the insertion site is using a novel PCR – based method and sequenced, and alignment of the flanking sequence with the genome sequence allows immediate mapping of insertions (Sundaresan et al., 1995). Insertion sites in genes can thus be indexed with expression phenotypes. The collection of gene or enhancer trap lines will be used to screen for genes expressed in specific cell or tissue

types (Groover et al., 2003). The pD991 enhancer trap vector contains the -60 CaMV minimal promoter fused to the *uidA* (GUS) gene. In the enhancer trap vector, the GUS gene will be driven by a minimal promoter, which, however, by itself will not be strong enough to drive GUS gene expression. If an enhancer trap gene inserts near the expressed gene, though, transcriptional enhancers will enhance the minimal promoter to drive detectable GUS expression. The expression of a gene at or near the insertion site will be reflected by GUS expression. Previous studies of enhancer trap-transformed plants also exhibited reporter gene expression. For example, Campisi et al. (1999) found that 31% of the transgenic Arabidopsis enhancer trap lines exhibited reporter gene expression in the inflorescence of those plants. Similarly, Groover et al. (2004) reported that 38% of transgenic poplar gene or enhancer trap lines exhibited reporter gene expression in various tissues.

In this report, we transformed a *Populus* clone with a GUS gene with a minimal promoter in the enhancer trap vector pD991, and produced 250 transgenic lines, of which 71 of them showed various GUS expression patterns, confirming that enhancer trapping strategy can be used in *Populus* functional study.

MATERIAL AND METHODS

Plants materials and culture conditions

In vitro aspen hybrid shoot cultures had been previously established from actively growing shoots of an aspen hybrid ['Clone 6' *Populus canescens* x *P. grandidentata*, 36-67-S-1 (pcg)] (Dai et al.2003). The cultures were maintained in 200-mL culture jars (Sigma-Aldrich, St. Louis, MO) containing 30 mL of Murashige and Skoog [MS (Murashige and Skoog, 1962)] basal medium supplemented with MS vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS), 100 $mg \cdot L^{-1}$ myo-inositol, 3% (w/v) sucrose and 0.5% (w/v) charcoal (Sigma-Aldrich). All jars were placed in a growth room at 22-25°C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 $\mu mol \bullet m^{-2} \bullet s^{-1}$ as measured by a Licor LI-250 light meter (LI-COR Inc., Lincoln, Nebraska) held at the top of the culture vessels. All of the cultures in the following experiments were maintained in these conditions.

Vector and Agrobacterium strain

The enhancer trap vector pD991 was kindly provided by Dr. Tom Jack of Dartmouth College (http://www.dartmouth.edu/~tjack/index.html). The vector was a derivative of the binary plant transformation vector pCGN1547 (Mcbride et al., 1990). The vector pD991 contains the neomycin phosphotransferase gene (*nptII*) which confers kanamycin antibiotic resistance for selection of putatively transformed plants. The vector pD991 contained the -60 cauliflower mosaic virus (CaMV) minimal promoter fused to

the *uid*A (GUS) gene. The -60 CaMV promoter sequences in the vector were located about 350 base pairs from the T-DNA right border sequence (Fig. 5.1). By itself the -60 CaMV promoter directs very low levels of transcription, and in the absence of a closely linked enhancer element, the level of GUS activity is undetectable. However, in the presence of the enhancer element, the GUS can be detected by GUS staining using the chromogenic substrate X-GLUC. Prior to inoculating aspen leaf segments for transformation, the *Agrobacterium* strain harboring the vector was grown overnight at 28 ºC to a final density of 0.8-1.0 at OD_{600}.

Transformation of aspen using the pD991 gene

For transformation, previously established *in vitro* hybrid aspen plants were transferred from MS medium to Woody Plant Medium (WPM) (Lloyd and McCown 1981), supplemented with 100 $mg \cdot L^{-1}$ myo-inositol, 0.1 % (v/v) MS vitamins, 3.0% (w/v) sucrose, and 0.3 % (w/v) gelrite. Leaves harvested from *in vitro*-grown aspen plants were cut into 0.5 cm^2 segments. Leaf segments were infected with *Agrobacterium* strain GV3850 harboring pD991 by immersing them for 30 min in an inoculum solution consisting of 5 ml Lysogeny broth (LB) *Agrobacterium* suspension culture. After infection, the leaf segments were blotted on sterile filter paper to remove the excess bacterial inoculum and then placed on co-cultivation medium [WPM supplemented with 8.9 µM N^6 benzylaminopurine (BA), 5.37 µM α-naphthaleneacetic acid (NAA) and 100 µM acetosyringone]. After 3 days of co-cultivation in the dark, the inoculated leaf segments were washed with WPM liquid medium supplemented with 250 $mg \cdot L^{-1}$ cefotaxime, and transferred to a callus induction medium [(CIM) WPM supplemented

with 8.9 μM BA, 5.37 μM NAA, 100 mg·L^{-1} myo-inositol, 0.1 % (v/v) vitamins, 3.0% (w/v) sucrose and 0.3 % gelrite]. Antibiotics (250 mg·L^{-1} carbenicillin and 250 mg·L^{-1} cefotaxime) were added to eliminate *Agrobacterium* and 80 mg·L^{-1} kanamycin was used for selecting transformed cells (Dai et al., 2003; 2004). After 4 weeks, induced calli were transferred to fresh CIM for shoot induction. After 5-6 weeks regenerated shoots formed, grew and were transferred to a rooting medium [1/2 MS salts supplemented with 3.0% (w/v) sucrose, 0.3% (w/v) gelrite, 250 mg·L^{-1} carbenicillin, 250 mg·L^{-1} cefotaxime and 100 mg·L^{-1} kanamycin]. The shoots were later transplanted to sterilized soil after confirmation by PCR. The control leaf segments were treated similarly except that they were infected with LB broth without *Agrobacterium*.

Plant genomic DNA isolation and PCR analysis

To confirm the presence of the transgene in plants that were kanamycin resistant, genomic DNA was isolated from 0.5 g transgenic and control plant leaf tissues using a DNeasy® Plant Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Each sample was taken from shoots growing on rooting medium with 100 mg·L^{-1} kanamycin for 4 weeks to ensure that samples were free from *Agrobacterium* contamination. To confirm the transgene presence in regenerated kanamycin-resistant plants, 50 ng genomic DNA was used for PCR amplification by using GUS primers (forward 5'-GAG GCT ATT CTA TGA TG-3' and reverse 5'-ATC GGG AGC GGC GAT ACC GTA-3'). The expected size for the GUS genes were 335 bp. Amplified fragments were separated on 1% agarose gels using a DNA 1-KB Ladder (Fisher Scientific Inc., Pittsburgh, PA).

Reverse transcription (RT)- PCR

Total RNA was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO) and treated with AMPD1 DNase I (Sigma-Aldrich) to remove any traces of DNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the iScript^{TM} select cDNA synthesis kit (Bio-Rad, Hercules, CA) with oligo dT primers. The RT-PCR reaction was carried out for 1 h at 42 ºC, followed by PCR activation at 95 ºC for 5 min, 35 cycles of amplification (94 ºC for 30 seconds, 55 ºC for 30 seconds, and 72 ºC for 1 min) and a final extension of 10 min in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA).

ß-Glucuronidase (GUS) histochemical assays

The expression of the GUS gene in the aspen transformants was assayed by histochemical staining. The test was based on the common histochemical GUS assay suggested by Stomp (1992). Tissues were dipped into a GUS assay solution comprised of 50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl and 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide cyclohexylammonium salt (X-GLUC) substrate and incubated for 1 day at 37 °C. The chlorophyll in the petioles and leaves was destained by rinsing with 70% ethanol twice over a 24-h period until all of the chlorophyll was removed and the blue GUS stain was clearly visible (Jefferson et al., 1987)

RESULTS

Confirmation of transformed plants with the pD991 gene by selection in Kanamycin-containing medium

Leaf segments infected with *A. tumefaciens* that contained the pD991 enhancer trap vector produced callus two weeks after inoculation. The calluses formed from at the edge of explants of the cut surface on WPM supplemented with 8.9 μM BA, 5.37 μM NAA, 100 μM acetosyringone, 80 $mg \cdot L^{-1}$ kanamycin, 250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$ cefotaxime (Fig. 5.2A). Then shoots emerged 5-6 weeks after callus formed (Fig. 5.2B). When shoot were induced 2-3 cm long with 3-4 leaves on the selection media, shoots were transfer to the new WPM supplemented with same as above media. When shoots were induced 3-4 cm long with 5-6 leaves on second selection media, shoots were transferred to the MS rooting media which were supplement with 100 $mg \cdot L^{-1}$ kanamycin, 250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$ cefotaxime (Fig. 5.2C). Transgenic lines were transferred into pots and all transgenic plants appeared morphologically normal (Fig. 5.2D).

Confirmation of Transgenic Plant by PCR

Transgenic lines transformed with pD991 vector were confirmed by PCR. All transgenic lines with pD991 that showed up kanamycin resistance on the rooting media were confirmed by PCR. The transgene was PCR-amplified using GUS specific primer pairs, yielding the expected band sizes of 0.34 kb (Fig. 5.3).

RT-PCR analysis of transcription level of the pD991 gene in transformed plants

The Fig. 5.4 shows the RT-PCR on pD991-transgenic lines to confirm the expression of the pD991 transgene. The expected 335bp bands were clearly visible. A non-transformed aspen plant sample was used as the control which showed no amplification (Fig. 5.4. line 2). Total RNA was also screened by PCR without reverse transcriptase to ensure preparations were free of DNA contamination.

Characterization of the expression patterns by β-Glucuronidase (GUS) histochemical assays

The GUS expression patterns in these 250 lines were characterized. Approximately 28 % (71 out of 250 lines) of the transgenic plant exhibited GUS gene expression, indicated by blue staining. All 250 lines were determined by the GUS staining. The different staining patterns were observed in transgenic lines (Fig. 5.5 and Table 5.1).

Discussion

Enhancer trapping is an alternative insertion-based strategy which is based on detecting gene expression, instead of detecting mutant phenotypes (Springer, 2000; Groover et al., 2004). An enhancer trap vector carries a report gene with minimally functional promoter. When the reporter gene is inserted under a functional indigenous promoter, it will be expressed in a manner that mimics the normal expression pattern of a gene at the insertion site. With a large experiment, many such transgenic lines with a target functional gene can be generated, indexed and categorized. Groover et al. (2004) demonstrated the potential of enhancer trap as a new resource for novel gene discovery for poplar. Using the enhancer trapping vectors carrying the *β*-glucuronidase (GUS) reporter gene, they generated 455 enhancer trap primary transformants. Many of these inserted genes are expressed during primary and secondary vascular tissues, although other kinds were also found (Groover et al., 2004). They also found that significant overlapping genes in responsible for development and function of secondary vascular tissues of stems and primary vascular tissues in other organs of the plant. Using the thermal asymmetric interlaced PCR and sequencing, Groover et al. (2004) amplified and identified the chromosomal DNA of the insertion sites by reference to the *Populus trichocarpa* genome sequence. They further isolated full-length cDNAs for five genes of interest, including a new class of vascular-expressed gene tagged by enhancer trap line cET-1-pop1-145. These *Populus* gene and enhancer trap lines have been established and are available for screening (http://www.fs.fed.us/psw/programs/ifg/genetraps.shtml). However, there has been no report using this strategy to generate trap lines that respond

to environmental stresses or biotic challenges.

In this report, using a well-established protocol for aspen transformation (Dai et al. 2003; 2004), we generated 250 kanamycin resistant lines with an enhancer trap vector pD991 (Campisi et al., 1999). All 250 transgenic plant lines were confirmed by PCR using specific primers of the GUS gene which showed kanamycin resistance on the rooting media (Fig. 5.2D). GUS expression in 71 out of 250 lines (28 %). Not all of the transgenic plants that were confirmed by PCR using specific GUS primers (Fig. 5.3) were stained by GUS and some showed varying GUS expression patterns in their tissues (Fig. 5.4). This is because the PCR detection method detect the presence of the inserted genes, but the staining for GUS can only detected when the gene is inserted nearby an enhancer which hallow the expression.

Although pD991 transgenic aspen lines showed different GUS expression patterns than the expression patterns of Arabidopsis from the Jack study (Campisi et al., 1999), the transgenic aspen with pD991 also showed very specific patterns. Jack's lab used T2 transgenic Arabidopsis seeds to characterize the specific lines. However, studies of aspen with enhancer trap gene can be difficult to generate similar patterns because aspen is woody plant and would take many years to get seeds for the next generation. The different GUS staining patterns suggest that the gene was inserted in different locations, either in close proximity to, or distant from, an enhancer. Those plants that tested PCR-positive for GUS but at the same time did not exhibit GUS staining, were probably not inserted into a position with the enhancers nearby. It may also be possible that the genes which the enhancers(s) act on were developmentally regulated, so that they were expressed only at the stage or tissues which were used in the GUS staining.

This research further demonstrated that it is possible to generate a large number of enhancer trap lines through Agrobacterium-mediated transformation. These GUS-expressing transgenic lines can be further studied to determine the specific locations of gene expression using PCR-based methods such as thermal asymmetric interlaced (TAIL) PCR, inverse PCR and plasmid rescue system (Weigel, 2000) which are used to isolate the sequence flanking T-DNA with which the mutant phenotype co-segregates. TAIL PCR entails consecutive reactions with nested sequence-specific primers and a shorter arbitrary degenerate primer. Many of these methods require special steps prior to PCR such as Southern blot analysis to determine suitable restriction sites, followed by manipulations such as restriction cutting and ligation or tailing (Liu et al., 1994).

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APPENDIX

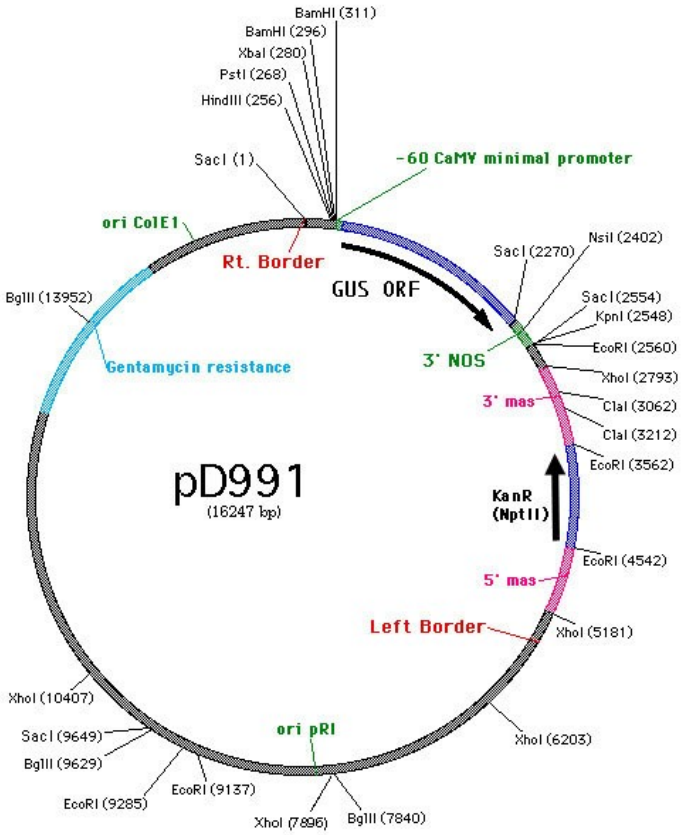

Figure 3.1. The enhancer trap vector pD991

The enhancer trap T-DNA vector pD991 was a derivative of the binary plant transformation vector pCGN1547 (Mcbride et al., 1990). This plasmid contains the -60 cauliflower mosaic virus (CaMV) minimal promoter fused to the *uid*A (GUS) gene with the *npt*II gene as a selective marker gene.

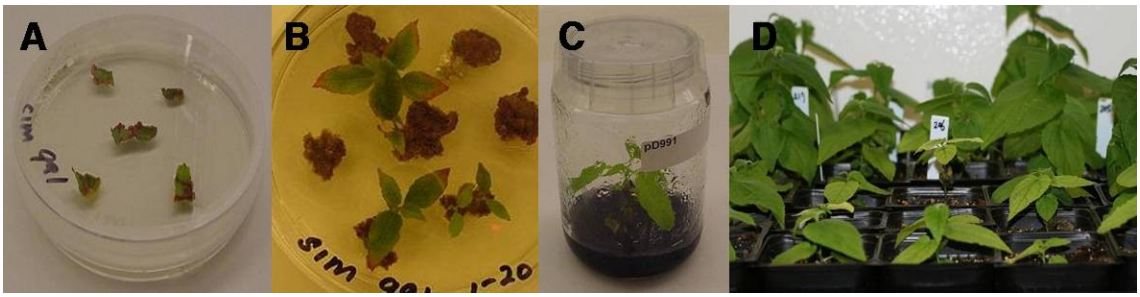

Figure 3.2. Transformation of aspen with the pD991 vector.

(A) Callus initiation was observed two weeks after inoculation that were infected with *Agrobacterium* strain GV3850 containing the pD991 on 80 mg·L^{-1} kanamycin-containing medium. (B) Adventitious shoots formed 5-6 weeks after callused formed. (C) A pD991 transgenic plant was cultured on rooting medium (1/2 MS with 3% charcoal plus antibiotics). (D) Transgenic plants in pots.

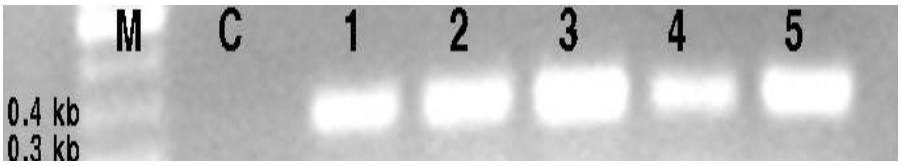

Figure 3.3. PCR-amplification of pD991 gene. PCR-amplification with the GUS-specific primers: forward 5'-GAGGCTATTCTATGATG-3' and reverse 5'-ATCGGGAG CGGCGATACCGTA-3' primer. M: 1-Kb marker, C: non-transgenic control plant; lanes 1-5; five independent transgenic lines.

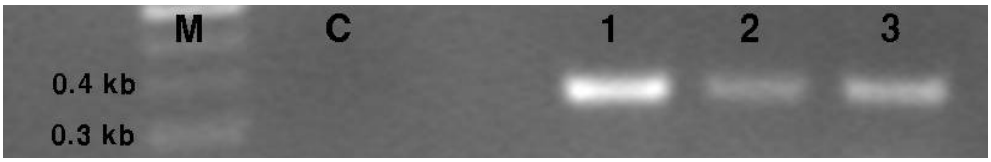

Figure 3.4. Expression of the transgenic plants analyzed by RT-PCR. RT-PCR analysis of selected transcripts in leaf tissues of aspen. M: 1-Kb marker, C: non-transgenic control plant, line 1-3: independent transgenic lines.

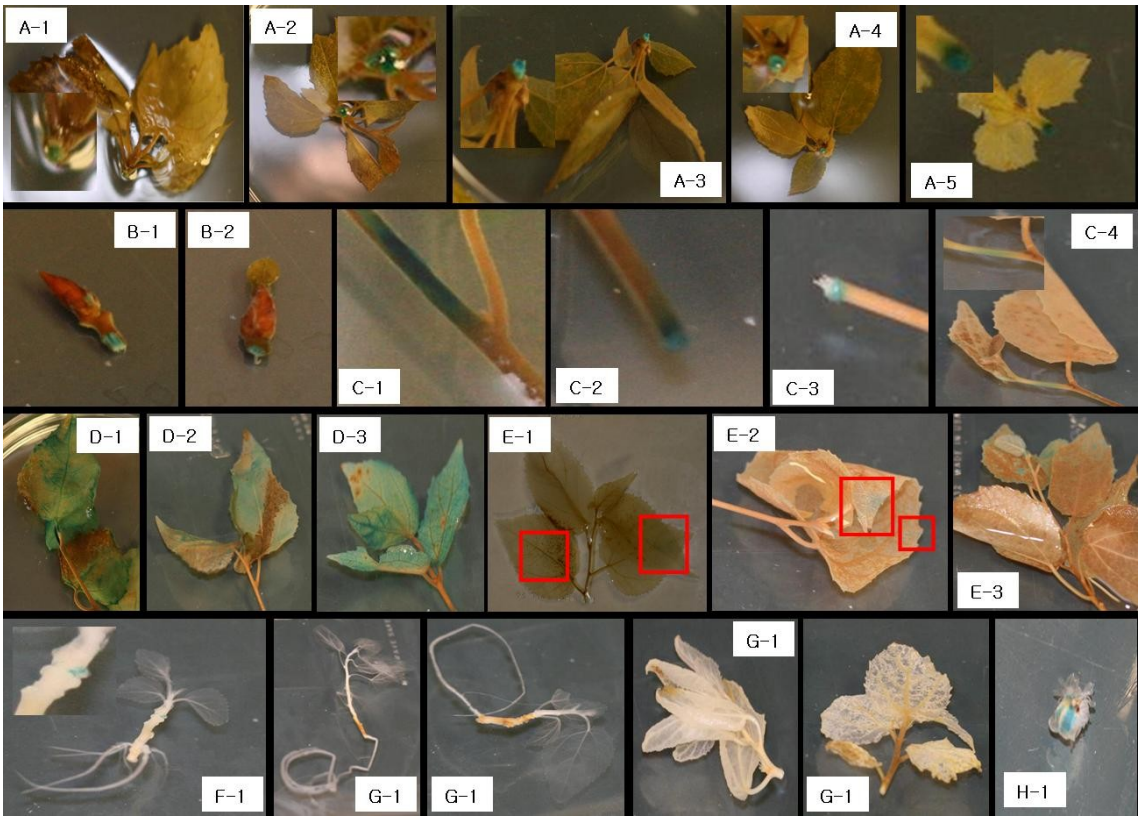

Figure 3.5. Histochemical analysis of *β*-Glucuronidase (GUS) gene expression patterns of transgenic plant lines. All lines were confirmed by kanamycin resistance and PCR amplified using the GUS-specific primer.

(A) expression in stipule, (B) expression in shoot tip bud, (C) expression in stem, (D) expression in entire leaf, (E) expression in part of leaves, (F) expression in the axil, (G) expression not-, (H) expression on callus stage

Table 3.1. Categories of 71 enhancer trap aspen lines by histochemical analysis of *β*-Glucuronidase (GUS) gene expression*

Staining Location	Number of Lines	% of Staining Lines
expression in stipule	19	26.8
expression in shoot tip bud	8	11.3
expression in stem	12	16.9
expression in entire leaf	15	21.1
expression in part of leaves	12	16.9
expression in axil	5	7
Total	71	100 %

*: These 71 lines were positive in GUS staining, which were part of the total of 250 transgenic lines which have been confirmed with PCR with the GUS-specific primer pair.

Chapter IV

Transformation of *Populus* with *agNt84*, a Novel Heavy Metal Binding Protein Gene

ABSTRACT

Heavy metals released by various industries such as mining, factories and agriculture contaminate soils and fresh and ground water, causing serious threat to the environment and human health. Traditional remediation methods of heavy metal are expensiveness, time consuming and environmentally destructive. Phytoremediation is an effective clean-up technology for contaminated sites which is cost effective, non-intrusive, aesthetically pleasing. In this report, we transformed hybrid aspen (*Populus canescens* x *P. grandidentata*) with the heavy metal binding protein *agNt84* gene. Seven transgenic lines were confirmed with polymerase chain reaction (PCR) with the *agNt84* specific primers and two lines shoot tips of transgenic- and non-transgenic plants grown on Cd-containing rooting media to evaluate of Cd resistance. 33% of shoot tips from one line and 44% of those from another transgenic line survived on medium containing 250 mM Cd, respectively, but only 22% of the non-transgenic shoot tips survived on rooting medium with 150 mM Cd at week 8. Also, the Cd analysis by ICP-OES indicated that the transgenic plants which grown on 100 mM Cd medium accumulated about 45% more Cd in the tissue than non-transgenic plants. Although *agNt84* transgenic and non-transgenic aspen were not produced root on Cd resistance test media, the transgenic plant with heavy metal binding protein showed the enhancement of Cd tolerance and accumulation.

Key words: Cadmium · Transformation · Poplar · Inductively Coupled Plasma Emission spectroscopy (ICP-OES)

Abbreviations: CaMV 35s – Cauliflower Mosaic Virus 35s, PCR - Polymerase chain reaction, *nptII* - neomycin phosphotransferase II gene, BA - N^6 benzylaminopurine, NAA - α-naphthaleneacetic acid

INTRODUCTION

Heavy metals such as cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), and selenium (Se) are released into the environment by various industries including mining and agriculture, threatening the environment and ultimately, human health (Nicholson et al., 2003; Zhenbin and Shuman 1997). Contamination of soils, sediments and water with toxic chemicals and heavy metals is one of the major problems facing the world today (Qing-Ren et al., 2003). Over the past decade of industrial growth, it has been recognized that environmental pollution is far greater than was previously assumed and that many contaminants are present in the environment (Kirk et al., 2002; Burgos et al., 2005). Some heavy metals are strongly retained in the soil, with little leaching, which can adversely affect soil property and fertility (Zhenbin and Shuman 1997). The toxicity of these heavy metals depends on the concentration and their speciation. They can exist in the environment for hundreds of years or more after they are emitted as they are not biodegradable. Thus, heavy metals tend to bioaccumulate as they move up the food chain (McGrath et al., 1997; Chen et al., 1998; Abollino et al., 2002).

Traditional methods of removing heavy metal from soils, such as soil washing, are usually very expensive, laborious and risk spreading contaminated soil during removal (Comis, 1995, 1996; Dushenkov et al., 1996; Marchiol et al., 2004; Walker et al., 2003; 2004). Phytoremediation, also referred to as bioremediation, botanical bioremediation and green remediation, is a good strategy that can be used to clean up the environment by rendering soil contaminants nontoxic. This technique can be considered part of the so-called assisted natural remediation and plays an important role in the

restoration of the physical, chemical and biological properties of contaminated soils (Ernst 2000; Perez de Mora et al., 2005). Due to the acute toxicity of many contaminants, there is an urgent need to develop low-cost, effective and sustainable methods to detoxify the soil that contains them, or completely remove them from the environment.

Although many metals (e.g. Cu, Fe, Mn, Ni, Zn) are essential for proper plant function and nutrition, excessive concentrations can be toxic to plants. An important characteristic of tree species that makes them suitable candidates for remediation is their large above- and below-ground biomass and long life cycle (McIntyre 2003). The ideal plant species to be selected/utilized for phytoremediation purposes is one that has a high biomass production, is sufficiently hardy and competitive in the climate where it is to be used and has a good phytoremediation capacity to start with. Poplar trees (*Populus* spp.) fit this description well (Brian et al., 2004; Prasad et al., 2003). Most species in this generus are well known for their extensive root system, high water uptake, rapid growth, and large biomass production. Found in most global environments, poplar trees have a high nitrogen requirement, high transpiration rate and a deep root system (2 to 5 m) with an extensive fine root mass at the soil surface to maximize nutrient uptake (Stealer et al, 1996; Taylor 2002). Thus, poplars possess compelling characteristics that make it a suitable plant for phytoremediation.

In genetic engineering of poplar plants, a foreign gene of DNA is stably inserted into the genome of a poplar cell, which is then regenerated into a transgenic plant. Historically, *Agrobacterium*-mediated transformation has been one of the major methods used for the transformation of plants (Bradshaw and Stettler 1993; Bradshaw et al., 2000; Confalonieri et al., 1995; De Block 1990; Han et al., 1995; 2000). Using genetic

engineering methodologies, it is possible to transfer the appropriate gene(s) for hyperaccumulation traits into poplar plants. A number of heavy metal-binding proteins, such as cytochrome *c* oxidase, albumin, metallothionein, prion protein (PrP), matrilysin, and non-heme iron-containing metalloproteins have been studied and well characterized (She et al., 2003). Examples include transgenic Indian Mustard with glutathione synthetase (Zhu et al., 1999, Belimov et al., 2004) and *Arabidopsis thaliana* with *merB* (Bizily et al., 1999; Kim et al., 2006). These transgenic plants with various metal-binding protein genes have been reported to increase plant tolerance and heavy metal uptake in hydroponics (January et al., 2007) or agar-based cultivation. *Brassica napus* with endophytic bacteria (Sun et al., 2007). Also, some woody species have already been employed for pollution from municipal waste in Europe (Merkle 2003). Nicotiana tabacum (Janoušková et al., 2004; Yazaki et al., 2006) has been transformed with the *iaaM* gene to increase root mass to extract more metals from soil.

The agNt84 metallohistin is a class of small plant metal-binding proteins that have the capacity to bind multiple atoms of Ni^{+2}, Zn^{+2}, Co^{+2}, Cu^{+2}, Cd^{+2} and Hg^{+2} (Gupta et al., 2002). They were discovered as nodule-specific cDNAs expressed at high levels in actinorhizal host plant cells newly infected by the nitrogen-fixing symbiont *Frankia* (Pawlowski et al., 1997). Genes from plants with a metal-hyperaccumulating ability to tolerate these toxins could be transferred to a fast-growing poplar plants for enhanced phytoremediation. They have a high metal-binding capacity protein molecule and the ability to bind metals from solution or by exchange on a solid matrix. High levels of expression in metabolically active cells do not appear to interfere with cell metabolism, increasing the likelihood that heterotopous overexpression of these proteins may be

possible (Krishnamachary et at., 2003).

The objectives of this study are to genetically engineer *Populus* with the *agNt84* gene, which encodes for a metallohistin protein, and to evaluate its potential for removing and detoxifying heavy metals from contaminated soil and water.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

In vitro aspen hybrid shoot cultures had been previously established from young shoots of an aspen hybrid [*Populus canescens* x *P. grandidentata*, 36-67-S-1 (pcg), or 'Clone 6'] (Dai et al., 2003). The cultures were maintained in a growth room at 22-25°C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as measured by a Licor LI-250 light meter (LI-COR Inc., Lincoln, Nebraska) held at the top of the culture vessels. All of the cultures in the following experiments were maintained in these conditions.

Vector and Agrobacterium Strain

The binary vector (GeneBank accession No. U69156.1) was used to clone agNt84 gene under control by the constitutive promoter CaMV35S, resulted in pBin-*AgNt*84. The *AgNt84* gene was PCR-amplified from the initial cDNA clone using forward 5'- CTT TGC TGT TGT GCT CCT CA -3' and reverse 5'- TGG TTT CAG TTT CGG TTT CTG -3' primers, cloned into the *Bam*HI and *Sac*I restriction sites (Figure 4.1). The plasmid also contains the neomycin phosphotransferase gene (*nptII*) which confers kanamycin antibiotic resistance for selecting putative transgenic plants. The binary vector was transferred into *A. tumefaciens* strain GV3850 by electroporation.

Cell Culture of Bacterial Inoculum

Cells of *A. tumefaciens* strain GV3850 containing the binary plasmid pBin*AgNt84* were grown in 20 ml Luria Bertani (LB) liquid medium containing 100 $mg \cdot L^{-1}$ kanamycin and 100 $mg \cdot L^{-1}$ rifampicin at 28 ºC for 16 hrs on a shaker at 225 rpm. Next, the cells were collected by centrifugation at 3500 rpm for 10 min, re-suspended in 50 ml LB medium with 100 µM acetosyringone and cultured at 28 ºC for 5 hrs to an optical density of 0.8-1.0 at OD_{600}.

Transformation of Aspen Using the pBinAgNt84 Gene

For transformation, previously established *in vitro* hybrid aspen plants were transferred from MS medium to woody plant medium (WPM) (Lloyd and McCown 1981), supplemented with 100 $mg \cdot L^{-1}$ myo-inositol, 0.1 % (v/v) MS vitamins, 3.0% (w/v) sucrose, and 0.3 % (w/v) gelrite. Leaves of *in vitro* grown aspen plants were cut into 0.5 cm sections and infected by immersion in the *A. tumefaciens* bacterial inoculum solution for 1 hour. After infection, the leaf segments were blotted on sterile filter paper to remove the excess solution and then placed on co-cultivation medium [WPM supplemented with 8.9 µM N^6 benzylaminopurine (BA), 5.37 µM α-naphthaleneacetic acid (NAA), and 100 µM acetosyringone]. After 3 days of co-cultivation in the dark, the inoculated leaf segments were washed with WPM liquid medium supplemented with 250 $mg \cdot L^{-1}$ cefotaxime and transferred to a callus induction medium [(CIM) WPM supplemented with 8.9 µM BA, 5.37 µM NAA, 100 $mg \cdot L^{-1}$ myo-inositol, 0.1 % (v/v) vitamins, 3.0% (w/v) sucrose, and 0.3 % gelrite]. Antibiotics (250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$

cefotaxime) were added to eliminate *Agrobacterium* and 80 $mg \cdot L^{-1}$ kanamycin was used for selecting transformed cells (Dai et al. 2003; 2004).

After four weeks, induced calli were transferred to fresh CIM for shoot induction. After 5-6 weeks regenerated shoots formed, grew and were transferred to a rooting medium [1/2 MS salts supplemented with 3.0% (w/v) sucrose, 0.3% (w/v) gelrite, 250 $mg \cdot L^{-1}$ carbenicillin, 250 $mg \cdot L^{-1}$ cefotaxime, and 100 $mg \cdot L^{-1}$ kanamycin]. The shoots were later transplanted to pots containing sterilized soilless potting mix after confirmation by PCR. The control leaf segments were treated similarly except that they were infected with LB broth without *Agrobacterium*.

Plant Genomic DNA Isolation and PCR Analysis

The kanamycin resistant plants were verified by polymerase chain reaction (PCR) to confirm the presence of the transgene. Genomic DNA was isolated from 0.5 g young leaf tissue of both transgenic and control plants using a DNeasy® Plant Maxi Kit (Qiagen) according to the manufacturer's protocol. Each sample was taken from *in vitro* plants that had been growing on rooting medium for 4 weeks to ensure that samples were free from *A. tumefaciens* contamination. To detect the *agNt84* gene, 50 ng of genomic DNA was used for amplification by PCR using the *agNt84* primer sets, which could yield 251 bp internal fragments. Primers were as follows: *agNt84*, 5'- CTT TGC TGT TGT GCT CCT CA -3' forward and 5'- TGG TTT CAG TTT CGG TTT CTG -3' reverse primers which contained *Bam*HI and *Sac*I, sites respectively. The amplification was subjected to 2 min at 95°C for denaturation. Forty cycles were performed as follows: denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5

min. The expected size for the *agNt84* gene was 251 bp. Amplified fragments were separated on a 1% (w/v) agarose gel containing ethidium bromide alongside a 2-KB DNA Ladder (Fisher Scientific Inc., Pittsburgh, PA) and visualized under UV light.

Reverse Transcription (RT)- PCR

Total RNA was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO) and treated with AMPD1 DNase I (Sigma-Aldrich) to remove any traces of DNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the iScript™ select cDNA synthesis kit (Bio-Rad, Hercules, CA) with oligo dT primers. The RT-PCR reaction was carried out for 1 h at 42 ºC, followed by PCR activation at 95 ºC for 5 min, 35 cycles of amplification (94 ºC for 30 seconds, 55 ºC for 30 seconds, and 72 ºC for 1 min), and a final extension of 10 min in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA).

Confirmation of Resistance to Cadmium by Shoot Tips of Transgenic Plants on Rooting Media with Cadmium

To compare the effects of Cd on *in vitro* plants and to confirm whether the *agNt84*-transgenic aspen plants were resistant to Cadmium (Cd), *in vitro* shoot tips from control plants and plants transformed with the a*gNt84* gene were cultured on rooting media that contained either 0, 10, 25, 50, 75, 100, 150, 200, 250, 300 or 400 mM of Cd. For each treatment, three replicate jars were used, each containing one 3 cm shoot tip. Each experiment was repeated three times. Cadmium resistance was evaluated as the percentage of shoot tip segments that survived in each Cd-containing rooting medium.

The experimental design was completely randomized (CRD), and the performance rating and survival data were evaluated by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc, Cary, NC).

Cadmium Analysis by Spectro CIROS ICP-OES

Transgenic- and non-transgenic plants were collected after the test of confirmation of resistance to cadmium on rooting media with cadmium. The collected samples were dried on the dry-over at 65 ºC for 48 hours to completely remove the water. Plant tissue samples (0.2 g each) were digested with 10 ml conc. HNO_3 on hot plate for 30 min. Cd concentration were determined by Spectro CIROS Inductively Coupled Plasma Emission spectroscopy (ICP-OES) (Fitchburg, MA) in Dr. Michael Essington's laboratory in the Department of soil Sciences and Environmental engineering, University of Tennessee.

RESULTS

Transformation of Plants with the agNt84 Gene

Putative *agNt84*-transgenic plants were obtained via *A. tumefaciens* mediated transformation. Callus formed on the cut surface of explants on WPM supplemented with 8.9 µM BA, 5.37 µM NAA, 100 µM acetosyringone, 80 $mg \cdot L^{-1}$ kanamycin, 250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$ cefotaxime. Callus initiation was observed two weeks after inoculation and shoots emerged 5-6 weeks after callus formation. After the induced shoots were 2-3 cm long with 3-4 leaves, they were transferred from the selection media to fresh media (Fig. 4.2A and 4.2B). When the induced shoots were 3-4 cm long with 5-6 leaves, they were transferred to MS rooting media supplemented with 100 $mg \cdot L^{-1}$ kanamycin, 250 $mg \cdot L^{-1}$ carbenicillin and 250 $mg \cdot L^{-1}$ cefotaxime (Fig. 4.2C and 4.2D). Seven transgenic lines, all appearing morphologically normal, were transferred into pots containing autoclaved soilless potting mix (Fig. 4.2E and 4.2F).

Confirmation of Transgenic Plants by PCR

Seven putative *agNt84*-transgenic lines, as indicated by kanamycin resistance on rooting media, were confirmed by PCR using specific primers of derived from the *agNt84* gene. PCR amplification yielded the expected band sizes of 251 bp. No amplification was observed in non-transformed controls (Fig. 4.3).

Analysis Transcription Level of the agNt84 Gene in Transgenic Plants by RT-PCR

RT-PCR was performed on *agNt84*-transgenic lines to confirm the expression of the transgene. Three transgenic lines were analyzed by RT-PCR with respect to the degree

of *agNt84* gene expression (Fig. 4.4). As shown in Fig. 4.4 lines 3, 4 and 5, the expected 251 bp bands for the *agNt84* gene were clearly visible in the RT-PCR product. Non-transformed aspen plants were used as the controls (Fig. 4.4 Line 2). The total RNA was tested for DNA contamination by PCR without reverse transcriptase, to ensure preparations were free of DNA contamination. These results indicate that all transgenic plants have the transcriptionally active *agNt84* gene inserted in their genomes.

Rooting of Transgenic Plants in Cadmium-containing Media

Transgenic plants were tested for resistance to Cd by culturing on rooting media supplemented with various concentrations of Cd (Fig. 4.5). Shoot tips from two *agNt84* transgenic plant lines survived on rooting medium with 250 mM Cd, while a non-transformed control plant survived on medium with only 150 mM (Fig. 4.5 A-3 and 4.5 B-5 and Table 4.1).

Cadmium Analysis by Spectro CIROS ICP-OES

Transgenic plant grown on rooting medium with 100 mM Cd showed significant high Cd concentration in plant tissue (492.259 $mg \cdot kg^{-1}$) than non-transgenic plant (338.614 $mg \cdot kg^{-1}$). Cd content in the transgenic plant cultured on 150 and 250 mM Cd media were 906.716 and 1146.028 $mg \cdot kg^{-1}$, respectively and non-transgenic plant cultured on 150 and 250 mM Cd media were 834.596 and 1103.234 $mg \cdot kg^{-1}$, respectively (Fig. 4.6).

DISCUSSION

Using a well-established protocol for aspen transformation (Dai, Cheng et al. 2003; 2004) with *nptII* as a selective marker gene which confers resistance to kanamycin, seven plants transformed with the *agNt84* gene were initially screened by kanamycin resistance, then confirmed by PCR using specific primers of *agNt84* (Fig. 4.3). The transgenic plants with the *agNt84* gene showed normal morphologies (Fig. 4.2E and F) which were similar to previous transformation study, especially for dicotyledonous species (Bevan, Flavell et al. 1983; Horsch, Fry et al. 1985; Miki and McHugh 2004; Sundar and Sakthivel 2008). Inserted T-DNA confirmed by PCR-amplification (Fig. 4.3), RT-PCR confirming gene expression (Fig. 4.4) and shoot tips of transgenic and non-transgenic plants grown on Cd-containing rooting media (Fig. 4.5 and Table 4.1) showed that the transgene is stably integrated into the aspen genome, expressed and functionally conferring resistance to Cd.

Shoot tips of transgenic lines 1 and 2 survived to week 8 on medium containing 250 mM Cd and they survived significantly longer than non transgenic plants. At week 8, 33% of shoot tips from one line and 44% of those from another transgenic line survived on medium containing 250 mM Cd, respectively, but only 22% of the non-transgenic shoot tips survived on rooting medium with 150 mM Cd at week 8 (Fig. 4.5 A-3, B-5 and Table 4.1). According to the Cd analysis by ICP-OES, transgenic plant grown on medium with 100 mM Cd accumulated 492.259 mg/kg Cd in the tissue but non-transgenic plant had 338.614 mg/kg Cd in plant tissue (Fig. 4.6). Transgenic plants accumulated about 45% more Cd than non-transgenic plants. Although transgenic and non-transgenic aspen

were not produced root on Cd containing medium, transgenic plants with heavy metal binding protein showed the enhancement of Cd tolerance and accumulation.

Approximately 400 species of plant have been reported to hyperaccumulate metal ((Prasad et al. 2003). The plant species best suited for phytoremediation is one that has a high biomass production, is sufficiently hardy and competitive in the climate where it is to be used, and has a good heavy metal-binging capacity (Pilon-smiths. 2002). Genes from plant with a metal-hyperaccumulating ability to tolerate these toxins could be transferred to a fast-growing plant species for enhanced phytoremediation (Vrian et al., 2004). Therefore, the genetically engineered *Populus* with the *agNt84* gene may hold promise for phytoremediation

The small glycine- and histidine-rich protein, agNt84, encoded by nodule-specific cDNAs isolated from *Alnus glutinosa* nodule cDNA libraries (Gupta et al., 2002). The metal binding protein, *agNt84*, has a high capacity for binding to multiple metal atoms of Ni^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+},Cd^{2+}, and Hg^{2+}. Also, it was reported that agNt84 involved in transport/storage of metal during the infection process (Obertello et al., 2003).

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APPENDIX

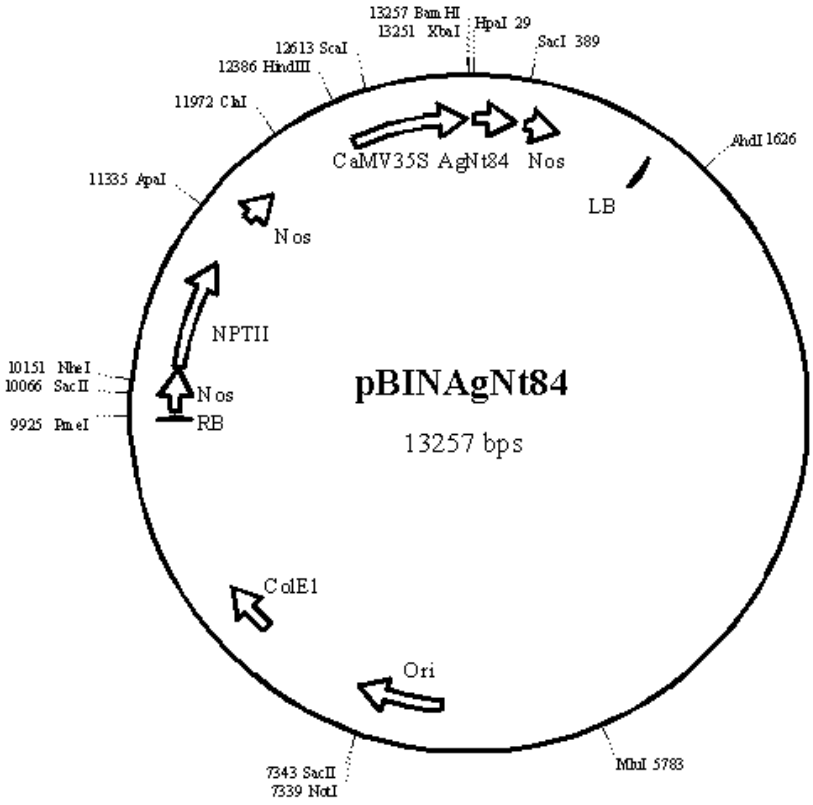

Figure 4.1. Plasmid map of pBIN*AgNt84*. The binary plasmid pBIN*AgNt84* was transformed into *A. tumefaciens* GV3850. This plasmid contains the CaMV 35s promoter driving the expression of *agNt84* gene with the *npt*II gene as a selective marker gene.

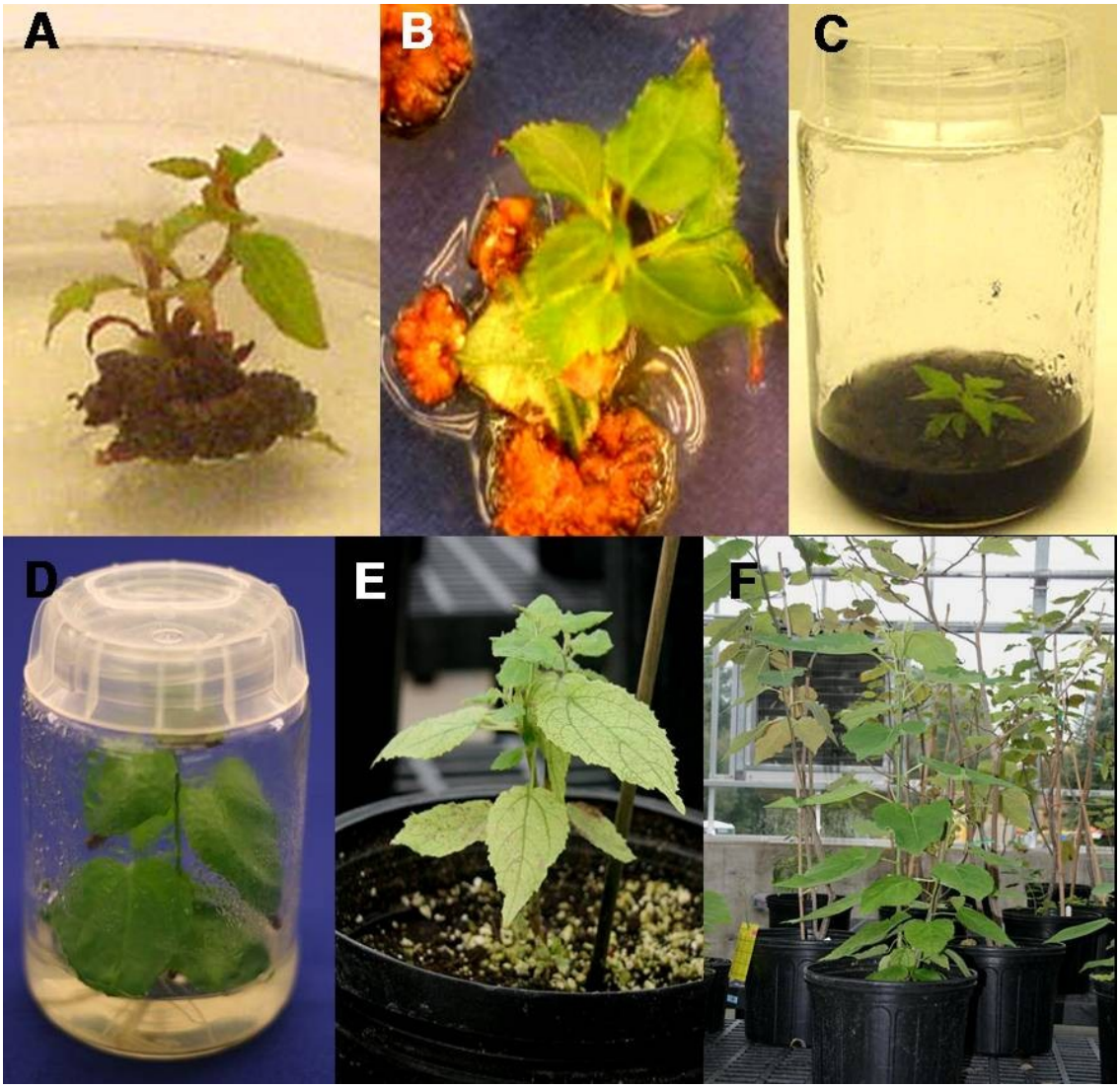

Figure 4.2. Transformation of aspen with the *agNt84* gene. (A) and (B) Adventitious shoots formed from callused leaf segments that were infected with *Agrobacterium* strain GV3850 containing the *agNt84* gene on 80 mg·L^{-1} kanamycin-containing medium. (C) and (D) The *agNt84*-transgenic plants were cultured on rooting medium (1/2 MS plus 100 kanamycin). (D) and (E) The *agNt84*-transgenic plants in pots.

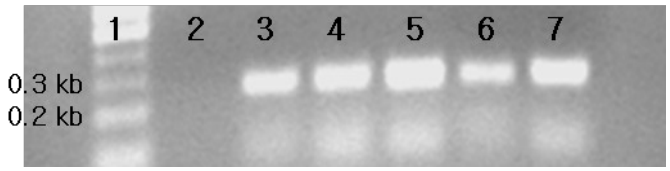

Figure 4.3. PCR Amplification of *agNt84* gene. PCR was performed with the *agNt84* Forward and Reverse primers designed from the *agNt84* gene sequence. Amplification produced approximately 251 bp product representing the *agNt84* gene. Lane 1: 1-Kb marker; Lane 2: non-transgenic control plant; Lanes 3-7: five independent transgenic lines with the *agNt84* gene.

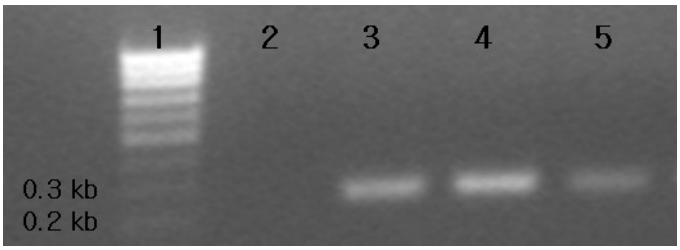

Figure 4.4. Expression of the *agNt84* gene analyzed by RT-PCR. RT–PCR analysis of selected transcripts in leaf tissues of aspen. Lane 1: 1-Kb marker; Lane 2: non-transgenic control plant; Lanes 3, 4 and 5: three independent transgenic lines with the *agNt84* gene.

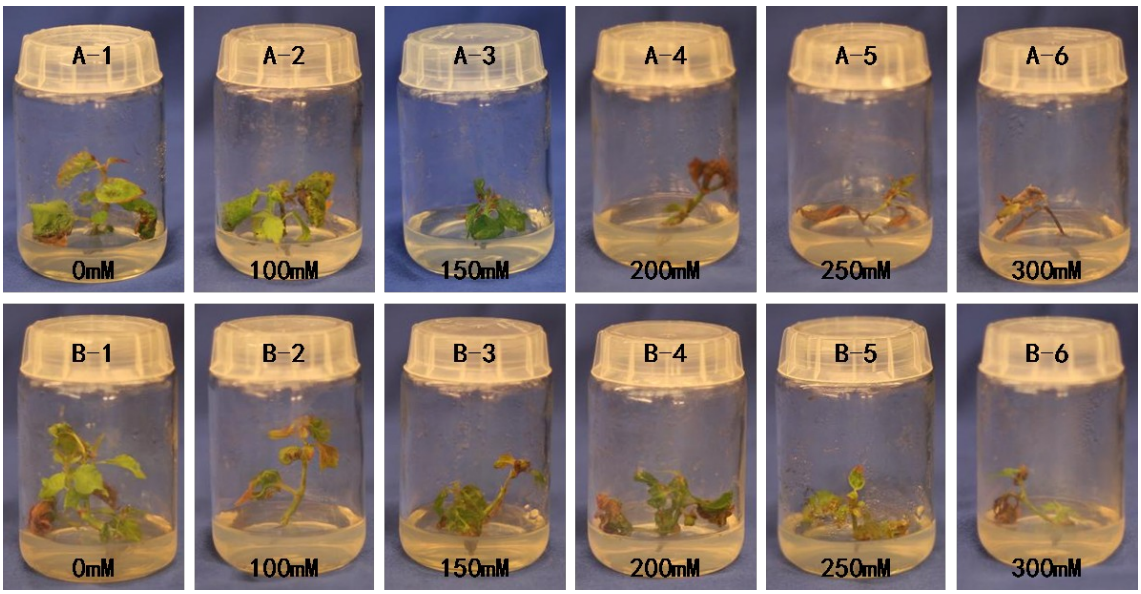

Figure 4.5. Growth of transgenic plants and non-transgenic plants in cadmium-containing media. Shoots were grown on MS basal medium supplemented with 0, 100, 150, 200, 250 or 300 mM cadmium. Cultures were maintained in a growth room at 25ºC and a 16-h photoperiod where fluorescent light intensity was 125 µmol $m^{-2}s^{-1}$.

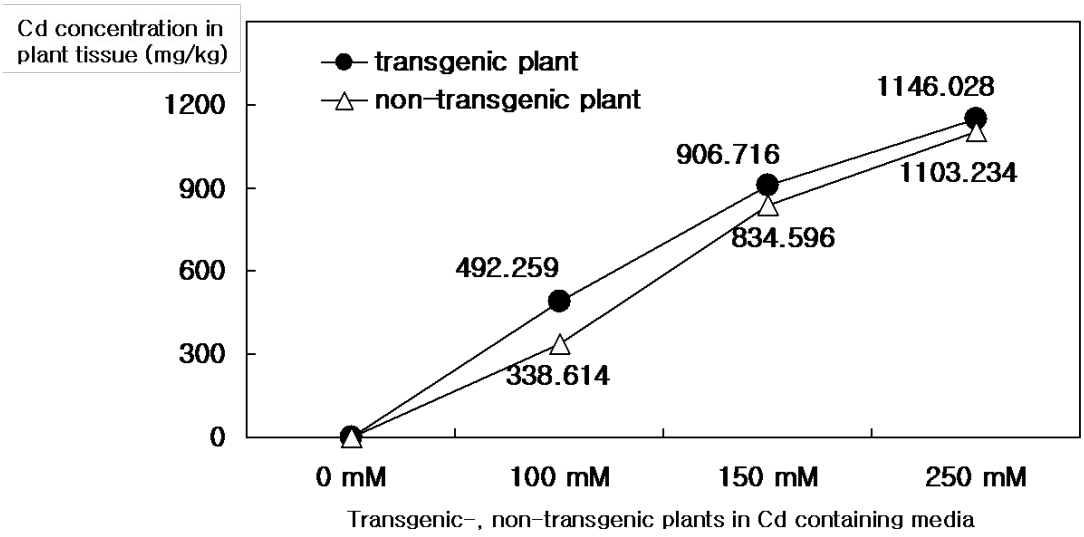

Figure 4.6. Visible absorption spectra of cadmium from *agNt84* transgenic plants by ICP-OES

The *in vitro* shoot tips of transgenic- and non-transgenic plant were grown on MS basal medium supplemented with 0, 100, 150 or 250 mM cadmium. Cultures were maintained in a growth room at 25ºC and a 16-h photoperiod where fluorescent light intensity was 125 μmol $m^{-2}s^{-1}$.

Table 4.1. Percentage of shoots of transgenic plants and non-transgenic plants survived in cadmium-containing media.

Cd ($mg \cdot L^{-1}$)	Transgenic Line 1		Transgenic Line 2		Control	
	4weeks	8weeks	4weeks	8weeks	4weeks	8weeks
0	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)
10	9/9 (100%)	8/9 (89%)	9/9 (100%)	9/9 (100%)	9/9 (100%)	9/9 (100%)
25	9/9 (100%)	9/9 (100%)	9/9 (100%)	8/9 (89%)	8/9 (100%)	8/9 (89%)
50	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)
75	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)
100	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)	8/9 (89%)	6/9 (67%)
150	9/9 (100%)	8/9 (89%)	9/9 (100%)	8/9 (89%)	8/9 (89%)	2/9 (22%)
200	9/9 (100%)	6/9 (67%)	9/9 (100%)	5/9 (56%)	6/9 (67%)	0/9
250	9/9 (100%)	4/9 (44%)	9/9 (100%)	3/9 (33%)	5/9 (56%)	0/9
300	8/9 (89%)	0/9	8/9 (89%)	0/9	3/9 (33%)	0/9
400	6/9 (67%)	0/9	5/9 (56%)	0/9	1/9 (11%)	0/9

Plants were grown on MS supplemented with various concentration of cadmium. Cultures were maintained in a growth room at 25ºC and a 16-h photoperiod where fluorescent light intensity was 125 μmol $m^{-2}s^{-1}$. The same letters in different rows indicate that there is no significant difference ($p \leq 0.05$).

VITA

Byung-guk Kang was born in Seoul, South Korea on April 4, 1972. He went to KyungHee University in 1992 and enlisted in the Korean Army from 1993 to 1994. After his military service, he received a B.S. in Agriculture in 1999 and M.S. in the Graduate School of Biotechnology in 2001. From 2001 to 2003, he served plant molecular genetic Lab. technician at Plant Metabolism Research Center, KyungHee University. From 2003 to 2009, he has been in Ph.D. program of the Department of Plants, soils and insects at the University of Tennessee, Knoxville and in going to receive a Ph.D. degree in December 2009.