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Transformation of *Populus* Species by an *Agrobacterium* Binary Vector System[1]

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Agrobacterium Binary Vector에 의한 포플러 形質轉換을 위한 基礎硏究[1]

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ABSTRACT

Three clones of *Populus alba* × *P. grandidentata* have been tested for susceptibility to *Agrobacterium tumefaciens* strains A281 and A348. We determined the optimum concentration of kanamycin sulfate for effective selection of leaf disc-derived, transgenic tissues transformed using *Agrobacterium* binary vector pGA472 containing a neomycin phosphotransferase gene (NPT-II) which confers kanamycin resistance. Of the wild type Ti plasmids contained by the two *Agrobacterium* strains, pTiBo542 of strain A281 appears to be best suited to serve as a helper plasmid for binary vector systems. A relatively low concentration (10mg/l) of kanamycin sulfate inhibited adventitious shoot initiation from leaf discs on regeneration medium. Transformed kanamycin-resistant calli were obtained by culturing *Agrobacterium* inoculated leaf discs on selective regeneration medium. The transformed kanamycin-resistant calli continued to grow on regeneration media supplemented with kanamycin sulfate to levels of 50 and 200mg/l. The growth of non-co-cultivated control calli was severely inhibited on regeneration medium containing 50mg/l kanamycin sulfate.

Key words: *transformation*; Populus; *binary vector*; *NPT-II gene*; *genetic engineering*.

要 約

포플러類에 對한 有用遺傳子 삽입에 關한 基礎 硏究로서, 北美의 自然雜種 포플러, *P. alba*×*P. grandidentata* 3 클론을 대상으로 *Agrobacterium tumefaciens* A281과 A348 strain의 감염력을 調査하였다. Kanamycin 抵抗性의 neomycin phosphotransferase (NPT-II) 遺傳子를 가진 *Agrobacterium* binary pGA 472 vector와 이들 組織培養된 세 클론의 잎조각을 함께 培養하여, 形質轉換된 部位를 選拔하기 위해서 kanamycin sulfate의 適正濃度를 調査하였다. A281과 A348 strain 中 A281 strain이 가지고 있는 pTiBo542가 binary vector system의 helper plasmid로서의 역할에 가장 적합한 것으로 나타났다. 비교적

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抵濃度(10mg/l)의 kanamycin sulfate가 잎조각배양으로 부터 植物體 유도를 억제하였다. Kanamycin 抵抗性 遺傳子가 내포된 *Agrobacterium* binary vector와 잎조각을 함께 培養한 後 kanamycin에 대한 抵抗性을 가진 callus가 kanamycin(60mg/l)이 들어있는 식물체 유도배지에서 選拔되었다. Kanamycin 抵抗性 遺傳子에 의해 형질전환된 이들 kanamycin 抵抗性 callus와 정상적인 비교 callus를 kanamycin이 들어있는 植物體 유도배지에서 培養했을때 정상적인 비교 callus는 50mg/l의 kanamycin 濃度에서 生長이 억제된 반면, 形質轉換된 kanamycin 抵抗性 callus는 200mg/l의 kanamycin 濃度에서도 生長을 계속하였다.

INTRODUCTION

Since the first successful plant transformation was reported in 1983(Murai et al., 1983 ; Herrera-Estrella et al., 1983), *Agrobacterium*-mediated gene transfer methods have been developed for several important forest tree species such as *Populus* (Fillatti et al., 1987), *Pinus taeda* (Sederoff et al., 1986), and *Pseudotsuga menziesii* (Dandekar et al., 1987). *Agrobacterium*-mediated gene transfer methods utilize the natural ability of *Agrobacterium tumefaciens* to infect many dicotyledonous and gymnospermous plants (De Cleene and Delay, 1976). The transfer DNA (T-DNA) of the Ti plasmid is transferred to and stably incorporated into the nuclear DNA of cells transformed during the infection process (Chilton et al., 1977 ; 1980 ; Willmitzer et al., 1980). For detailed discussions on the development of *Agrobacterium*-mediated gene transfer methods, the reader is referred to recent reviews by Fraley et al. (1986) and Perani et al. (1986).

The construction of *Agrobacterium* binary vector systems was promoted by the recent discovery that the T-DNA and the virulence (*vir*) region of *Agrobacterium tumefaciens* Ti plasmid could be separated onto two different plasmids without losing the transfer capability of the T-DNA. Binary vector plant transformation systems consist of two plasmids in *Agrobacterium* : the binary vector plasmid and a helper plasmid (Hoekema et al., 1983 ; An et al., 1985). A typical binary vector contains several useful characteristics such as : 1) T-DNA border sequences which are required for successful transfer of DNA from the *Agrobacterium* cell into the plant genome. 2) A wide host range replicon which can replicate in both *Escherichia coli* and *Agrobacterium* cell systems. The desired foreign gene is cloned into the engineered binary T-DNA plasmid between the border sequences. 3) A selectable marker gene that usually confers antibiotic resistance to allow selection of transformed plant material. The typical helper Ti plasmid is an intact wild type or a disarmed (T-DNA deleted) Ti plasmid that usually contains supervirulent (broad host range) *vir* genes. The *vir* genes on a helper Ti plasmid act in *cis* to promote the transfer of the T-DNA on its own plasmid or in *trans* to transfer the T-DNA on the binary plasmid (Hille et al., 1984). Nevertheless, the ability to regenerate plants from leaf segments remains the critical limitation in obtaining transgenic plants for most *Agrobacterium* host species.

Populus species and hybrids are promising candidates as recipients for Ti plasmid-mediated gene transfer (Parsons et al., 1986 ; Fillatti et al., 1987 ; Klopfenstein et al., 1987). Earlier work reports the susceptibility of various *Populus* species to infection by naturally-occurring *Agrobacterium tumefaciens* (De Cleene and Deley, 1976). Phytohormone independent callus growth has been obtained from stem and shoot segments of *Populus trichocarpa* × *P. deltoides* following transformation by wild type *Agrobacterium tumefaciens* strains A281 and A348 (Parsons et al., 1986). Modified T-DNA containing a glyphosate-resistance gene has been incorporated into hybrid *Populus* and expressed (Fillatti et al., 1987).

The objective of this research was to improve transfromation systems for *Populus alba* × *P. grandidentata*. In this preliminary study, we report on 1) the host range of *Agrobacterium tumefaciens* strains A281 and A348 on three clones of *Populus*

alba × *P. grandidentata*; 2) the optimum concentration of kanamycin for effective selection of leaf disc-derived, transgenic tissue of this hybrid poplar that expresses a transferred NPT-II gene; and 3) transformation of hybrid poplar cells with a NPT-II gene.

MATERIALS AND METHODS

Agrobacterium host range study

The host range of *Agrobacterium* strains A281 and A348 was determined on three clones (Crandon, Hansen, and Sherrill) of *Populus alba* × *P. grandidentata*. Shoot cultures of this hybrid poplar were grown *in vitro* on the modified MS medium (Murashige and Skoog, 1962) as previously described by Chun and Hall (1984) and Chun et al. (1986). For the *Agrobacterium* susceptibility tests of the three clones, four *in vitro* propagated shoots (ca. 2 cm in length) of each clone were rooted under intermittent mist in a shaded greenhouse. After four weeks of acclimatizing process, the rooted cuttings were placed in a growth chamber and maintained at a daytime temperature of 25-28℃, with 16 h. photoperiod and a photosynthetically active radiation level of 50-60μE $m^{-2}s^{-1}$ from cool-white fluorescent tubes.

To prepare bacterial cultures for inoculation of plant tissues, single colonies of each *Agrobacterium* strain were isolated and cultured in liquid MGL medium (2.5 g/l yeast extract, 5 g/l tryptone, 5 g/l NaCl, 5 g/l mannitol, 1.16 g/l monosodium glutamate, 0.25 g/l KH_2PO_4, 0.1 g/l $MgSO_4$ $7H_2O$, 1 mg/l biotin at pH 7.0) (An, 1986). The bacterial cultures were grown overnight (to O.D. 600=ca. 1.2) in a shaking incubator at 28℃. Plants were inoculated by syringe puncture. Five to ten inoculations were made per plant stem at 1 cm intervals from the shoot tip. Tumor formation was scored four weeks after inoculation.

Bacterial strains

Wild type *Agrobacterium* strains (A281 and A348) and two *Agrobacterium* strains (6044 and 6048) containing a binary plasmid vector (pGA472) were obtained from Dr. M. P. Gordon at the University of Washington and Dr. G. An at Washington State University, respectively. Although the bacterial strains 6044 and 6048 contain the same binary T-DNA plasmid (pGA472), they each contain a different chromosomal background and different helper plasmids (Table 1). Strain 6044 contains a wild type, supervirulent (broad host range) Ti plasmid (pTiBo542) as the helper plasmid, while strain 6048 contains a disarmed (T-DNA deleted) Ti plasmid (pAL4404). Binary vector pGA472 contains T-DNA borders, a chimeric gene containing a nopaline synthase promoter and the coding sequences for neomycin phosphotransferase (NPT-II), the ColEl replicon, bacteriophage lambda *cos* site and a wide host range replicon (An et al., 1985). The two wild type strains were grown on MGL medium at 28℃, and two bacterial strains that contained a binary vector pGA472 were grown on MGL agar medium containing 20 mg/l kanamycin sulfate and 10 mg/l tetracycline HCl. Liquid cultures were grown overnight at 28℃ with shaking in the same MGL with antibiotics for binary vector *Agrobacterium* and without antibiotics for wild type strains.

Kanamycin concentration test

To determine the optimum concentration of kanamycin for effective selection of transgenic

Table 1. *Agrobacterium* strains and plasmids used for hybrid poplar transformation

Agrobacterium tumefaciens strain	Chromosomal background	Ti plasmid	Binary vector	Selectable markers
A281	C58	pTiBo542	-	-
A348	C58	pTiBo6	-	-
6044	C58	pTiBo542	pGA472	NOS-NPTII
6048	LBA4404	pAL4404	pGA472	NOS-NPTII

tissue, regeneration media containing nine different kanamycin concentrations (0, 10, 20, 30, 40, 50, 100, 150 and 200 mg/l) were tested. The regeneration media was MS medium with 6-benzylaminopurine (BA) 1.0 mg/l, and naphthaleneacetic acid (NAA) 0.1 mg/l at pH 5.7 as previously described (Chun, 1987). Three clones of *Populus alba* × *P. grandidentata* were tested. Six leaf explants per petri dish were cultured to determine the morphogenetic responses on the nine different kanamycin media. These petri dishes were arranged as three replicates of a completely randomized disign with a petri dish serving as the experimental unit. Growth chamber conditions were similar to those during the inoculation of cuttings. Four weeks after culturing, regeneration was measured by recording the visible formation of adventitious shoots, roots, or calli from each leaf disc.

Plant tissue culture and regeneration

Leaf explants were obtained from plantlets derived from axillary bud culture of three *Populus alba* × *P. grandidentata* clones. The initial establishments and proliferation of *in vitro* bud culture of this hybrid poplar have been previously described (Chun and Hall, 1984; Chun et al., 1986). Axillary shoots were produced initially by serial subculture on MS medium with BA 0.4 mg/l (pH 5.7). For adventitious shoot regeneration from leaf discs, MS medium containing BA 1.0 mg/l and NAA 0.02 mg/l was used for the Crandon clone, and BA 2.5 mg/l and NAA 0.02 mg/l was used for the Hansen and Sherrill clones. Leaf explants (entire leaf, terminal half, and basal half segments) were placed in abaxial and adaxial side culture and monitored for shoot, root, and callus formation.

Plant transformation studies

Three clones of *in vitro* cultured *Populus alba* × *P. grandidentata* were used in this study. A binary vector system was utilized for transforming the hybrid *Populus* clones by co-cultivation of leaf segments with *Agrobacterium* strains 6044 and 6048. The basic transformation and selection system used with *Populus* is similar to that used with tobacco (An et al., 1986). Following a 3-day co-cultivation, leaf segments were washed in liquid MS salts, then cultured for seven days on shoot regeneration medium containing cefotaxime sodium salt (250 mg/l) and carbenicillin disodium salt (500 mg/l) to prevent growth of *Agrobacterium*. In subsequent transfers, kanamycin sulfate (60 mg/l) was incorporated into the culture medium to select for regeneration of transformed shoots.

RESULTS AND DISCUSSION

Host range study

The results of the *Agrobacterium* host range study are summarized in Table 2. A different response was observed between the two strains of *Agrobacterium tumefaciens*. Although both A281 and A348 strains have the same chromosomal background of naturally occurring strain C58, strain A281 exhibited an overall higher virulence on the *Pupulus* clones than did strain A348. This is probably due to the supervirulent (broad host range) Ti plasmid (pTiBo542) that strain A281 possesses (Hood et al., 1984; An et al., 1985). Strain A348 contains the Ti plasmid pTiA6. These results are in accordance with a previous report (Parsons et al., 1986), in which A281 produced tumors on greenwood stems of *P. trichocarpa* × *P. deltoides*, but A348 did not. Klopfenstein et al. (1987) also demonstrated that *Agrobacterium* strain A281 exhibited an overall higher virulence on the various *Populus* species and hybrids than did strain A348. Tumor formation was observed 1-2 weeks after

Table 2. Host range of *Agrobacterium tumefaciens* strains A281 and A348 on three clones of *Populus alba* × *P. grandidentata*

Species	Clone	Tumor formation[a] A281	A348
P. alba×*P. grandidentata*	Crandon	25(%)	0(%)
	Hansen	10	0
	Sherrill	50	0

[a]percent tumor formation was calculated as the number of tumors on two plants divided by the number of inoculation sites (5-10/plant).

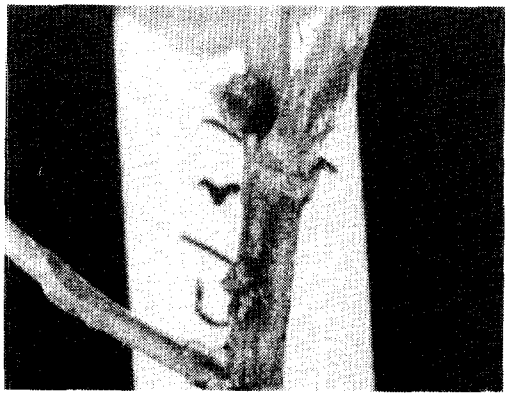

Fig. 1. Gall formation on a two month-old *P. abla* × *P. grandidentata* plantlet inoculated four weeks earlier with *A. tumefaciens* strain A281

inoculation (Figure 1). Four weeks after inoculation, most tumors ranged from 1 to 3 mm in diameter. Opine analysis and culturing of tumors from this experiment was not attempted. The supervirulent Ti plasmid (pTiBo542) of *A. tumefaciens* strain A281 appears to be well-suited to serve as a helper plasmid for binary vector systems designed to transform the three clones of *P. alba* × *P. grandidentata*.

Kanamycin concentration test

The effect of kanamycin concentration on the morphogenetic responses by leaf discs of *Populus alba* × *P. grandidentata* clones is summarized in Table 3. A relatively low level (10 mg/l) of kanamycin sulfate inhibited shoot initiation from leaf discs of the three clones of this hybrid poplar. Higher kanamycin sulfate concentrations (50-100 mg/l) were required to prevent callus formation. Callus often developed on the parts of leaves that were not in direct contact with the medium, but died when subcultured on the shoot regeneration media containing 60 mg/l kanamycin sulfate. Even though root formation at the proximal end of leaf explants was observed frequently at lower kanamycin concentrations (10-50 mg/l), these roots were initiated only on adaxial side cultures and did not contact the medium. Because the morphogenetic responses of these hybrid *Populus* clones are very

Table 3. Morphogenetic responses of leaf explants on regeneration media (BA 1.0 mg/l + NAA 0.1 mg/l) containing various concentrations of kanamycin sulfate

Kanamycin conc.	Morphogenetic responses[a]	Crandon	Hansen	Sherrill
0mg/l	Callus form.	79(%)	93(%)	91(%)
	Shoot init.	24	55	10
	Root init.	10	29	0
10mg/l	Callus form.	33	71	46
	Shoot init.	0	0	0
	Root init.	17	38	9
20mg/l	Callus form.	17	25	17
	Shoot init.	0	0	4
	Root init.	13	13	0
30mg/l	Callus form.	25	38	46
	Shoot init.	0	0	0
	Root init.	17	17	17
40mg/l	Callus form.	25	29	25
	Shoot init.	0	0	0
	Root init.	17	17	17
50mg/l	Callus form.	14	12	14
	Shoot init.	0	0	0
	Root init.	7	7	5
100mg/l	Callus form.	0	0	17
	Shoot init.	0	0	0
	Root init.	0	0	0
150mg/l	Callus form.	0	6	0
	Shoot init.	0	0	0
	Root init.	0	0	0
200mg/l	Callus form.	6	0	0
	Shoot init.	0	0	0
	Root init.	0	0	0

[a]morphogenetic responses of leaf explants were measured as the percentage of leaf explants producing shoots, roots, and calli.

sensitive to kanamycin, this antibiotic should serve as an effective agent to select for trangenic tissue that is expressing a transferred NPT-II gene.

Because the NPT-II gene frequently is used as a dominant marker for selecting transformed plant cells, kanamycin is one of the most frequently used antibiotics for transformation studies (Fraley et al., 1986). Various *Agrobacterium* T-DNA binary vectors contain the NPT-II gene which encodes resistance to the kanamycin through detoxification by phosphorylation.

Fillatti et al. (1987) have demonstrated that 60 mg/l of kanamycin was an affective concentration of the selective antibiotic for selection of transformed *Populus* shoots.

Transformation of leaf discs

Attempts to transfer a NPT-II gene to *P. alba* ×

P. grandidentata clones resulted in the formation of callus in regeneration media containing 60 mg/l kanamycin sulfate. Leaf discs transformed with binary vector 6044, which contains a wild type supervirulent (broad host range) Ti plasmid as the helper plasmid, produced large quantities of kanamycin-resistant calli were first visible 1-2 weeks after placing the leaf discs on selective medium. These kanamycin-resistant calli grew rapidly when transferred to fresh regeneration medium containing kanamycin. The percentages of callus formation by the three clones of the hybrid poplar are listed in Table 4. Sherrill and Crandon clones appeared to produce kanamycin-resistant callus at a much higher frequency than did the Hansen clone.

Leaf discs transformed with binary vector 6048, which contains a disarmed (T-DNA deleted) Ti plasmid (LBA4404), produced only a few calli on one leaf of the Crandon clone and on two leaves of the Hansen clone in areas that were not in direct contact with the selective medium. When these calli were transferred to the regeneration medium containing kanamycin, all calli died. Similar responses have been reported in an earlier study in which kanamycin-resistant shoots were not recovered from explants of *Populus* co-cultivated with a binary vector containing LBA4404 as a helper plasmid (Fillatti et al., 1987). Although leaf discs of the Hansen clone co-cultured with the binary vectors 6044 and 6048 produced six and three shoots, respectively, on the shoot regeneration media containing 60 mg/l kanamycin, these shoots did not grow on the shoot proliferation media (MS+BA 0.3 mg/l) containing 60 mg/l kanamycin sulfate.

Table 4. Growth of poplar transformed with binary vector 6044 (wild type Ti helper plasmid) and 6048 (disarmed Ti helper plasmid) on regeneration media containing 60 mg/l kanamycin. Callus formation was scored after 4 weeks of co-cultivation

		Crandon	Hansen	Sherrill
Strain 6044	No. shoot produced	0	6	0
	No. leaf pieces with callus	8/26 (30.8%)	6/36 (16.7%)	10/32 (31.3%)
Strain 6048	No. shoot produced	0	3	0
	No. leaf pieces with callus	1/39 (2.6%)	2/35 (5.7%)	0/43 (0%)

The putatively transformed calli were cut into small pieces and aseptically weighed (data not shown). These small callus pieces were then transferred to media (MS+BA 1.0 mg/l and NAA 0.1 mg/l) containing 0, 50, 100, 150, and 200 mg/l kanamycin sulfate. The calli of only the Sherrill clone were used for comparisons of callus growth between callus putatively transformed with binary vector 6044 and control callus derived from non-co-cultivated explants. The callus putatively transformed with the binary vector continued to grow at all test levels of kanamycin (Figure 2). A statistical contrast was done between growth of calli grown on 0 mg/l and those grown on all other levels of kanamycin tested for the putatively transgenic calli and non-co-cultivated control calli. The contrast for the putatively transgenic calli showed no difference ($P=0.9174$), but for the non-co-cultivated control calli, the contrast showed a highly significant difference ($P=0.0016$). The non-co-cultivated control calli were severely inhibited

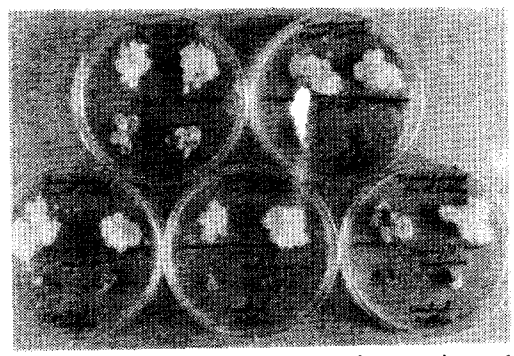

Fig. 2. Growth comparison of transformed kanamycin-resistant calli and untransformed control calli of *P. alba* × *P. grandidentata* Sherrill clone after 4 weeks of culture on regeneration media containing 0, 50, 100, 150, and 200 mg/l kanamycin sulfate. The transformed kanamycin-resistant calli continued to grow at all test levels of kanamycin. The non-co-cultivated control calli were severely inhibited by 50 mg/l kanamycin sulfate

Table 5. Responses of transformed and non-transformed callus growth on the regeneration media containing various kanamycin concentrations. Callus growth was scored as percentage weight increase after three weeks of culture

Kanamycin conc.	Transformed callus (× 100%)	Non-transformed callus (× 100%)
0mg/l	3.02±1.77	1.37±0.64
50mg/l	2.38±0.67	0.31±0.07
100mg/l	3.03±0.55	0.39±0.46
150mg/l	3.34±0.28	0.28±0.24
200mg/l	2.98±0.68	0.31±0.06

by 50 mg/l kanamycin sulfate (Table 5).

In addition, callus transformed with the NPT-II gene grew more rapidly than did control callus even when kanamycin was not used in the media. This result is perhaps attributable to preconditioning of the putative transgenic calli on a high level cefotaxime medium designed to prevent *Agrobacterium* growth during previous subcultures. Mathias and Boyd (1986) have reported that cefotaxime stimulates callus growth of bread wheat (*Triticum aestivum* L EM. Thell). The rapid callus growth of transformed callus also may involve co-integration event with NPT-II gene of the binary vector and intact oncogene of the helper plasmid pTiBo542 (Perani et al., 1986). These phenotypic results are assumed that the NPT-II gene is expressed in the callus tissue of the Sherrill clone. Similar results have been reported earlier in which various herbaceous plants such as tobacco, tomato, potato, *Arabidopsis* (An et al., 1986), white clover (White and Greenwood, 1987), *Stylosanthes* sp. (Manners, 1987) and *Medicage varia* (Deak et al., 1986), were successfully transformed with a NPT-II gene using binary vector pGA472.

At present, our success in regenerating shoots from transformed calli remain limited. In the future, confirmation of gene transfer and whole plant regeneration from transformed calli will be conducted using NPT assay (Reiss et al., 1984), Southern blot analysis (Southern, 1975) and various regeneration techniques.

In our efforts to develop and efficient transformation and regeneration system for *Populus alba* × *P. grandidentata*, we have demonstrated the following ; 1) *Agrobacterium* strain A281 containing pTiBo542 appears to be well suited to serve as a helper plasmid for binary vector systems ; 2) A relatively low kanamycin sulfate concentration (10 mg/l) inhibited adventitious shoot initiation from leaf discs ; and 3) pGA472 is perhaps attributable to transferring NPT-II gene into *Populus* species.

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