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論　文（Original Article）

Transformation of *Populus alba* and Direct Selection of Transformants with the Herbicide Bialaphos

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Abstract

Genetically transformed *Populus alba* plants were regenerated from calli which were derived from stem segments after co-cultivation with *Agrobacterium tumefaciens* strain GV3101 (pMP90) that harbored a binary vector into which genes for resistance to the herbicide bialaphos (*bar*) and for β-glucuronidase (*GUS*) had been incorporated. The *bar* gene was controlled by the promoter of a gene for nopaline synthase and included the polyadenylation region from the *RbcS-2B* gene of *Arabidopsis thaliana*, which encodes the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. The ability of *Agrobacterium*-treated stem segments to produce calli in the presence of bialaphos, histochemical assays of GUS activity in leaves, and analysis by genomic PCR confirmed the success of transformation. No "escape" plants and no chimeric transgenic plants were obtained in this transformation system, because of the harsh nature of the selection with bialaphos. The morphology of regenerated plants resembled that of the original parental strain.

Key words: *Agrobacterium tumefaciens*, bialaphos, herbicide, *Populus alba*, transformation

Introduction

Genetic engineering has the potential to allow the selective improvement of individual traits in forest trees without the loss of any of the desired traits of the parental line. Using such techniques, we can overcome the difficulties associated with the breeding of long-lived perennials, which require many years for the production of progenies. *Agrobacterium tumefaciens*-mediated transformation has been the preferred method for the introduction of foreign genes into plants. Numerous plant species, including a wide range of woody plant species, are susceptible to infection by *Agrobacterium* (De Cleen et al., 1976). However, many difficulties have been encountered in attempts to regenerate transgenic woody plants and, in many cases, appropriate regeneration systems have not yet been established. The production of transgenic broad-leaved trees, excluding fruit trees, has been limited to only a few genera, which include *Populus* (Fillatti et al., 1987; De Block, 1990; Mohri et al., 1996), *Liquidambar* (Sullivan et al., 1993), *Robinia* (Han et al., 1993; Igasaki et al., 2000), *Betula* (Mohri et al., 1997), *Eucalyptus* (Mullins et al., 1997), *Santalum* (Shiri et al., 1998) and *Pittosporum* (Kondo et al., 2002).

Bialaphos is a tripeptide antibiotic that is produced by *Streptomyces hygroscopicus* SF1293. It consists of phosphinothricin, an analog of L-glutamic acid, and two L-alanine residues. Upon removal of the alanine residues by endogenous peptidases in plant cells, the resulting phosphinothricin inhibits glutamine synthetase, with a resultant rapid accumulation of ammonia that leads to the death of plant cells (Tachibana et al., 1986a, b). Bialaphos is inactivated rapidly in the soil and it does not affect the germination or the growth of crops via the soil. Thus, it has a significant potential for widespread use on arable land. The *bar* gene, cloned from *S. hygroscopicus*, encodes phosphinothricin acetyltransferase, which acetylates the amino group of phosphinothricin, abolishing its herbicidal activity (Murakami et al., 1986; Thompson et al., 1987). Many transgenic plants that retain the *bar* gene are resistant to both bialaphos and phosphinothricin (Toki et al., 1992).

Transgenic poplar plants resistant to phosphinothricin (De Block, 1990; Devillard, 1992; Confalonieri et al., 2000), glyphosate (Fillatti et al., 1987) and chlorsulfuron (Brasileiro et al., 1992) have been reported. To the best of our knowledge, in all reports on the generation of

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herbicide-resistant transgenic poplar, the antibiotic kanamycin has been used for the selection of transgenic plants. However, "escapes" and chimeric calli and regenerated plants frequently appear when kanamycin is used for the selection of transgenic poplar (Mohri et al., 1999). In the present study, we established a new procedure for the *A. tumefaciens*-mediated transformation of *P. alba*, using direct selection with bialaphos. In our transformation system, we obtained no "escape" plants and no chimeric transgenic plants because of the harsh nature of the selection procedure. The transgenic plants that we did obtain exhibited no apparent morphological abnormalities.

Materials and Methods

Plant material

Shoot cultures derived from peeled twigs of mature *P. alba* were maintained on a medium (1/2 MSB5SC medium) that contained half-strength Murashige and Skoog's basal salts (MS basal salts; Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg et al., 1968), 3% (w/v) sucrose, 0.2% (w/v) activated charcoal and 0.4% (w/v) gellan gum. Shoot cultures were incubated at 25 ℃ under cool white fluorescent light (30 μmol·m^{-2}·s^{-1}, 16-h photoperiod) and subcultured at two-month intervals.

Binary vector and bacterial strains

The binary vector pSMAB704 (Fig.1), which contained a *bar* gene and the gene for β-glucuronidase (*GUS*) in its T-DNA region, was used in this study. The construction and characterization of this vector will be published elsewhere (Ichikawa et al., in preparation). The pSMAB704 plasmid was introduced by electroporation into a disarmed strain of *A. tumefaciens*, GV3101(pMP90) (Koncz et al., 1986).

A. tumefaciens were grown overnight at 28 ℃ in liquid Luria-Bertani medium (Sambrook et al., 1989) in the presence of 100 mg/l spectinomycin. For transformation of tissues of *P. alba*, the overnight culture was diluted to 5 x 10^8 cells/ml with a medium (MSS medium) that contained MS basal salts and 5% (w/v) sucrose supplemented with 20 μM acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone; Aldrich, Milwaukee, WI, USA).

Transformation and regeneration

Stem segments from shoot cultures of *P. alba* were vacuum-infiltrated three times for 5 min each in the above-mentioned suspension of *A. tumefaciens* (Horsch et al., 1985). Tissues were then blotted with sterile filter paper and incubated for three days on MSB5S medium, which contained MS basal salts, Gamborg's B5 vitamins, 3% (w/v) sucrose and 0.4% (w/v) gellan gum and had been supplemented with 20 μM acetosyringone. Each segment was washed three times with MSS medium and then once with MSS medium that contained 500 mg/l carbenicillin (disodium salt; Sigma, St. Louis, MO, USA), 500 mg/l cefotaxime (sodium salt; Sigma) and 500 mg/l vancomycin hydrochloride (Shionogi & Co., Ltd., Osaka, Japan). The tissues were blotted with sterile filter paper and placed on MSB5S medium that contained 10 mg/l bialaphos (Meiji Seika Ltd., Tokyo, Japan), 500 mg/l carbenicillin, 500 mg/l cefotaxime and 500 mg/l vancomycin hydrochloride. After calli had been allowed to develop for four weeks, they were excised from tissue segments and transferred to selective shoot-regeneration medium [MSB5S medium supplemented with 10 mg/l bialaphos, 500 mg/l carbenicillin, 500 mg/l cefotaxime, 500 mg/l vancomycin hydrochloride, 0.25 mg/l thidiazuron (Sigma) and 0.05 mg/l 6-benzyladenine]. Rooting of shoots was achieved in selective shoot-culture medium [1/2MSB5SC medium supplemented with 10 mg/l bialaphos, 500 mg/l carbenicillin, 500 mg/l cefotaxime and 500 mg/l vancomycin hydrochloride]. The histochemical and PCR analyses of transgenic *P. alba* were performed done after two months of growth on the selective shoot-culture medium.

Histochemical analysis of GUS activity

The histochemical analysis of GUS activity in transformed *P. alba* was performed as described by Jefferson et al. (1987). Leaves of transformed *P. alba* were incubated overnight at 37 ℃ in a solution of 0.1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) and 50 mM sodium phosphate buffer (pH 7.0). The distribution of GUS activity in leaves was examined after chlorophyll had been extracted with ethanol.

Isolation of genomic DNA and analysis by PCR

Genomic DNA was extracted from transformed *P. alba* plants as described by Murray et al. (1980). The oligonucleotide primers used for PCR detection of the *bar* gene and for that of the *GUS* gene were 5'-ATGAGCCCAGAACGACGCCC-3' (forward) and 5'-TCAGATCTCGGTGACGGGCA-3' (reverse), and 5'-ATGTTACGTCCTGTAGAAAC-3' (forward) and 5'-TCATTGTTTGCCTCCCTGCT-3' (reverse), respectively. The conditions for amplification were 30 cycles of incubation for 30 sec at 94 ℃, 30 sec at 56 ℃, and 120 sec at 72 ℃, with a final extension for 300 sec at 72 ℃.

Results and Discussion

Before attempting the transformation of *P. alba*, we examined the effects of kanamycin and bialaphos on the

survival of stems of *P. alba* that were cultured on shoot-regeneration medium under the light. Survival was determined in terms of the extent of chlorosis of stem segments. Bialaphos at the concentration of 10 mg/l killed stem segments effectively, and no "escape" callus appeared. By contrast, "escape" calli appeared on medium prepared with kanamycin at the high concentration (150 mg/l; data not shown). These results suggested that the *bar* gene might be a suitable selective marker for transformation of *P. alba*. When we tested various type of binary vectors and strains of *A. tumefaciens*, we found that GV3101(pMP90) that harbored the binary vector, pSMAH621 which was constructed to utilize the pVS1 origin of replication for highly stable maintenance in *A. tumefaciens* (Igasaki et al., 2000) gave the highest frequency of integration of the *GUS* gene to the segments after co-cultivation (data not shown). Therefore, we used the GV3101(pMP90) that harbored pSMAB704 (Fig.1), in which the *hpt* gene for hygromycin phosphotransferase in pSMAH621 had been replaced by the *bar* gene, for subsequent studies.

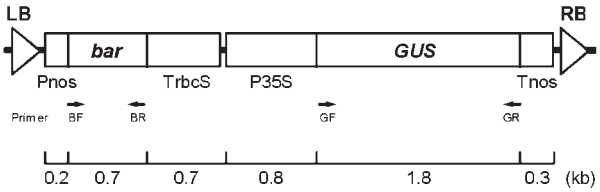

Fig.1. Schematic representation of the binary vector pSMAB704. Arrows (BF, BR, GF and GR) indicate the primers for PCR analysis. LB, Left border of T-DNA; RB, right border of T-DNA; Pnos, promoter of the gene for nopaline synthase; TrbcS, terminator of the gene for small subunit 2B of ribulose-1,5-bisphosphate carboxylase/oxygenase of *Arabidopsis thaliana*; P35S, promoter of the gene for 35S rRNA of cauliflower mosaic virus; Tnos, terminator of the gene for nopaline synthase.

The proportion of stem segments that produced bialaphos-resistant calli after *Agrobacterium* infection was much higher than that of leaf segments (data not shown). Bialaphos-resistant calli derived from stem segments (18 of 589 segments tested) were obtained on the selection medium within four weeks after transformation. The frequency of transformation (approximately 3%) was estimated, assuming that a callus derived from one segment was one genotype. Each bialaphos-resistant callus regenerated about 20 or more adventitious shoots on the selective shoot-regeneration medium. The frequency of regeneration of transgenic plants from bialaphos-resistant calli was close to 100%. The morphological features of the transgenic *P. alba* plants were indistinguishable from those of non-transgenic plants (Figs.2A and 2B). The absence of morphological changes is very important for future genetic engineering of this woody plant because it should allow selective improvement of single traits without the loss of any of the desired traits of parental lines. We selected three transgenic plants (L-1, L-2 and L-3), derived from different lines of calli at random for further analysis.

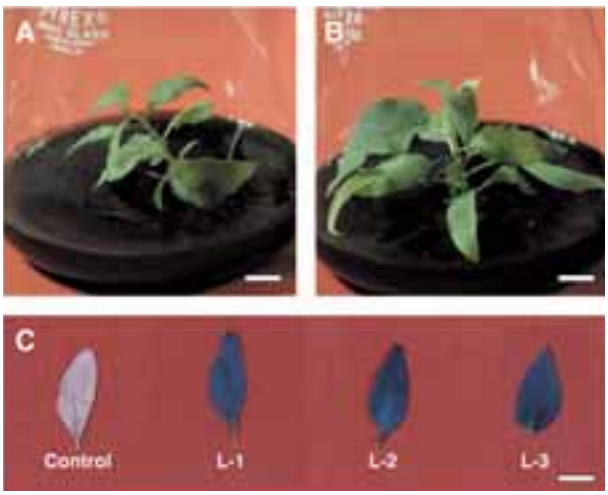

Fig.2. Regeneration of transgenic *P. alba*.
A. A control plantlet. B. A transgenic plantlet. C. Results of histochemical analysis of GUS activity in leaves of transgenic *P. alba*. A leaf of control plant and three leaves of randomly selected transgenic plants (L-1 through L-3) were subjected to histochemical staining for GUS activity. Bars: 1 cm.

Histochemical staining revealed that the leaves of the three transgenic plants were strongly positive for GUS activity (Fig.2C), suggesting that integrated *GUS* gene was expressed at high levels under the control of the 35S promoter of cauliflower mosaic virus (P35S). Successful transformation of *P. alba* was also confirmed by PCR analysis (Fig.3), which showed directly that the *bar* gene (Fig.3A) and the *GUS* gene (Fig.3B) had been introduced into the genome of *P. alba* by the *A. tumefaciens*-mediated transformation.

As described above, we established a simple and reliable procedure for the regeneration of transgenic *P. alba*. To our knowledge, this is the first report of transformation of *Populus* species using direct selection with a herbicide. Kanamycin was used for the selection of transgenic calli and plants in previous studies of herbicide-resistant transgenic poplar (Fillatti et al., 1987; De Block, 1990; Devillard, 1992; Brasileiro et al., 1992; Confalonieri et al., 2000). Moreover, "escapes" and chimeric calli and plants have always appeared when our group has used kanamycin for the selection of transgenic poplar (Mohri et al., 1999). The advantage of using herbicide resistance as compared to antibiotic resistance for selection of transgenic woody

plants is becoming clealy apparent as well as crops (Potrykus, 1990; Toki et al., 1992).

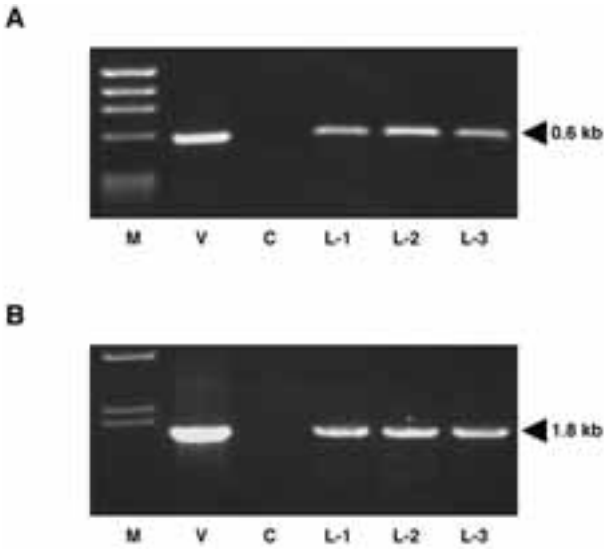

Fig.3. Detection by PCR of integrated *bar* and *GUS* genes in the genome of transgenic *P. alba.* A. PCR amplification of the *bar* gene by a set of primers BF and BR as shown in Fig.1. B. PCR amplification of the *GUS* gene by a set of primers GF and GR. The arrowheads indicate the *bar* gene and the *GUS* gene. M, DNA markers; V, vector; C, control plant; L-1 through L-3, bialaphos-resistant transformants of *P. alba*.

In the present study, we also found that stem segments were the most suitable tissue for transformation with bialaphos selection, as noted in the previous studies of the *A. tumefaciens*-mediated transformation of poplar (Mohri et al., 1996; Confalonieri et al., 2000). These results are, however, inconsistent with those of other studies that identified leaf discs as the best starting material (Fillatti et al., 1987; De Block, 1990; Klopfenstein et al., 1991; Confalonieri et al., 1994). Thus, the most suitable tissue for transformation appears to vary among species of woody plants. The present efficient and reproducible transformation system will allow the selective improvement of single traits in *P. alba* via the introduction of economically relevant genes that regulate, for example, morphological traits, growth, and resistance to insects and disease (Mohri et al., 1999; Igasaki et al., 2000). Furthermore, such transgenic *P. alba* will retain resistance to bialaphos.

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除草剤ビアラホス選抜による*Populus alba*の形質転換

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要　旨

*Populus alba*の茎切片にバイナリーベクターpSMAB704を保持する*Agrobacterium tumefaciens*、GV3101（pMP90）を感染させ形質転換体を得た。pSMAB704はT領域にビアラホス耐性遺伝子（*bar*）とβ-グルクロニダーゼ遺伝子（*GUS*）を保持しているバイナリーベクターで、bar遺伝子の発現はノパリン合成酵素遺伝子のプロモーターで制御されている。また、ポリA付加シグナル領域（ターミネーター）配列としてアラビドプシスのリブロース-1, 5-二リン酸カルボキシラーゼ/オキシゲナーゼのスモールサブユニット*RbcS-2B*遺伝子由来のものを用いている。ビアラホス存在下で形質転換処理した組織片よりカルスが生成・増殖し、植物体が再生・成長すること、植物組織のGUS染色およびゲノミックPCR解析により形質転換の成功を確認した。この形質転換法では、形質転換細胞を厳密に選抜することができるので、エスケープ（非形質転換体）やキメラ個体は出現しなかった。また、形質転換体の外観は、元の個体と同様で形態異常は見られなかった。

キーワード：アグロバクテリウム、 ビアラホス、 除草剤、 *Populus alba*、 形質転換

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