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ABBREVIATIONS

A	adenosine
ARS	autonomous replication sequence(s)
ATP	adenosine triphosphate
<i>bar</i>	gene coding phosphinothricin acetyltransferase
bp	base pair(s)
C	cytidine
CEN4	the minimal centromere domain of chromosome 4
CT	chromosome/transgene
CTAB	cetyltrimethyl ammonium bromide
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequencing tag
G	guanosine
<i>hpt</i>	gene coding hygromycin phosphotransferase
IPCR	inverse polymerase chain reaction
kb	kilobase(s) or 1000 bp
NADH	nicotinamide-adenine dinucleotide reduced form
nt	nucleotide(s)
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
S/MAR	scaffold/matrix-attachment regions
T	thymidine
TAE	4.84 g Tris base/1.142 ml glacial acetic acid/2 ml 0.5M EDTA (pH8.0) per liter
T-DNA	transfer DNA
tRNA	transfer RNA
W	adenosine, or thymidine

INTRODUCTION

Gene transfer techniques, including particle bombardment, are important tools in genetic engineering both for the analysis of genes and for practical uses. Bombardment-mediated transformation has been shown to be the most versatile and effective way for creating a variety of transgenic organisms that include microorganisms, mammalian cells, and a large number of plant species (Christou et al., 1988; Klein et al., 1988). In our laboratory, transformants of *Arabidopsis thaliana* (Seki et al., 1991; Sawasaki et al., 1994; Takahashi and Morikawa, 1996), cultured tobacco cells (Iida et al., 1990), *Marchantia polymorpha* (Irifune et al., 1996), and haploid plants of *Nicotiana rustica* (Nishihara et al., 1995) were reported.

Arabidopsis has emerged as the most widely used and facile experimental organism for studies of a broad range of problems in development, metabolism, genetics, environmental adaptation, pathogen interactions, and many other areas. Although it is a typical higher plant in most respects, *Arabidopsis* has an unusually small genome (10^8 bp/haploid), can be readily grown in confined laboratory environments, has convenient genetic properties such as a short generation time and prolific seed production. For these and related reasons, biological and genetic information about *Arabidopsis* is in a phase of explosive growth, and *Arabidopsis* has been chosen as the major organism for plant genome projects.

Exploitation of novel selectable marker genes for stable transformation of *Arabidopsis* will be informative for the development of basic research on transgenic plants. The *bar* gene encoding phosphinothricin acetyltransferase (PAT) from *Streptomyces hygroscopicus* (Murakami et al., 1986) has been shown to be a useful selectable marker for stable transformation of various monocots (Gordon-Kamm et al., 1990; Toki et al., 1992; Vasil et al., 1992) and dicots (De

Block et al., 1987; De Block et al., 1989; Saito et al., 1992). To my knowledge, however, no reports on stable transformation of *Arabidopsis* with this gene have been published. The hygromycin phosphotransferase gene (*hpt*) has also successfully been used for transformation of various plants such as rice (Shimamoto et al., 1989) and *A. thaliana* (Lloyd et al., 1986; Takahashi and Morikawa, 1996).

Nevertheless gene transfer techniques, including particle bombardment, are used for the crop engineering, the basal mechanisms are not almost known. For example, there is no information on the integration mechanism or the site of integration of transgenes introduced by particle bombardment. *Arabidopsis* is a very useful plant with which to analyze the integration site of transgenes because its genome size is much smaller than that of plants such as rice and maize (Meyerowitz, 1989).

The eukaryotic genome in the nucleus is thought to form a chromatin structure that has important roles in replication, transcription, and repair processes (reviewed by Berezney et al., 1995). According to the current model, chromatin is organized in chromosomal loop domains, the basal parts of which are attached to a proteinaceous scaffold, also referred to as the nuclear matrix (Paulson and Laemmli, 1977; Berezney and Coffey, 1974). Accordingly, the responsible DNA sequences have been called 'scaffold-attachment regions' (SARs) or 'matrix-attachment regions' (MARs). These S/MAR elements insulate the chromatin domains from neighboring loops with respect to their chromatin structure and torsional state. Their attachment to the nuclear matrix is mediated by component proteins that have a high, specific affinity *in vitro* for SAR sequences (Mirkovitch et al., 1984; Amati et al., 1990; Hall et al., 1991). Moreover, S/MAR has been found close to one T-DNA end in a transgenic petunia plant (Dietz et al., 1994) and within target sites of retroviral vectors (Mielke et al., 1996).

I describe here my studies on exploitation of novel selectable marker genes for stable transformation of *Arabidopsis* and on genomic DNA sequences flanking with transgene introduced by particle bombardment.

In chapter I, I separately introduced plasmid DNA's pARK22 (constructed in this study), pCaMVNEO (Fromm et al., 1986) and pCH (Matsuki et al., 1989), each of which respectively contains the *bar*, *nptII* and *hpt* genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator, into root sections of *Arabidopsis* using our pneumatic particle gun (Iida et al., 1990), and compared the yield of drug-resistant calli from the bombarded root sections. I find that the *bar* gene gave the best yield of the resistant calli in section I and observe the regeneration in section II. I also characterize transgenic *Arabidopsis* plants having this gene in the genome in section III. I observe morphology of three transgenic plants in section IV.

In chapter II, I describe the results of sequence analysis of junction region and provide experimental evidence that the junction regions bind to nuclear matrices. In section I, I show cloning of transgene and junction regions in transgenic *Arabidopsis*. Characterization of sequence of junction regions are introduced in section II. In section III, I find that junction regions can bind nuclear matrices isolated from tobacco. I compare the homology of junction regions with database in section IV. In section V, I find the characteristics of sequences at the chromosome/transgene junctions and terminals of the transgene.

In chapter III, I discuss the integration model for the transgene introduced by particle bombardment.

MATERIALS AND METHODS

For CHAPTER I

Plant materials

Seedlings of *A. thaliana* ecotype C24 (provided by Dr. D. Valvekens, Laboratorium voor Genetica, Rijksuniversiteit Gent, Belgium) were grown, and root sections were prepared as described previously (Seki *et al.*, 1991b). Briefly, 4- to 6-week old seedlings of *A. thaliana* that had been aseptically grown on germination medium (GM) containing B5 salts and vitamins (Gamborg *et al.*, 1968), 3% sucrose and 0.6% agarose under 16 h light/8 h dark at 26°C were harvested, and roots were excised and cut into sections (0.5-1.0 cm long). Approximately 200 root sections were spread in a circle (35 mm diameter) on a filter paper (ADVANTEC TOYO No. 2, 5.5 cm diameter, Toyo Roshi Kaisha Ltd., Tokyo, Japan) on callus-inducing medium (CIM) containing B5 salts and vitamins, 3% sucrose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.05 mg/l kinetin and 0.8% agar. After being cultured for 3 days (Seki *et al.*, 1991a) under the conditions described above, they were subjected to particle bombardment.

Plasmid DNA

The 1.8 kb *Hind*III-*Eco*RI fragment of previously reported plasmid pARK5 (Saito *et al.*, 1992) which contains the CaMV 35S promoter-*bar*-NOS terminator (expression cassette of the *bar* gene) was inserted into pTZ18R (Pharmacia Inc., Piscataway, NJ, USA) to give plasmid pARK22 as shown in Figure 1. Plasmids pCaMVNEO (Fromm *et al.*, 1986) and pCH (Matsuki *et al.*, 1989), each of which has basically the same expression cassette as that of the *bar* gene in pARK22 (see Fig. 1) with the *bar* gene being replaced by the *nptII* or *hpt* gene,

respectively, were also used.

Bombardment

The pneumatic particle gun device (Iida *et al.*, 1990) and the methods for gene delivery to the root sections using this device were essentially as reported previously (Seki *et al.*, 1991b). The plasmid DNA, pARK22, pCaMVNEO or pCH was separately coated on gold particles (1.1 μm in diameter, Tokuriki Honten Co., Tokyo, Japan), by co-precipitation in ethanol as reported previously (Morikawa *et al.*, 1989). Bombardment conditions were as follows: accelerating pressure of the projectile, $200\text{kg}/\text{cm}^2$; partial vacuum, 60 mmHg; amount of DNA-coated gold particles, 0.2 mg per projectile; amount of plasmid DNA, 4 $\mu\text{g}/\text{mg}$ of gold particles; and distance between the sample and the stopper, 10 cm. Two bombardments were given to a target tissue sample.

Selection of drug-resistant calli

After being bombarded, the root sections on the filter papers were cultured on drug-free CIM for 2 days, during which each of them became somewhat swollen and formed callus on its surface. The root sections were then divided into small clumps, each of which contained ca. 10 original sections and 20 of them were placed onto three plastic Petri dishes (9 cm internal diameter) containing shoot-inducing medium (SIM). SIM was composed of B5 salts and vitamins, 3% sucrose, 5 mg/l N^6 -(2-isopentenyl)adenine, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES) and 0.8% agar (pH5.7) and supplemented either with 2 mg/l bialaphos (Meiji Seika Kaisha Ltd., Yokohama, Japan), 20 mg/l kanamycin or 20 mg/l hygromycin. They were cultured, by being transferred fortnightly onto a fresh SIM, under the same conditions as described above. Two to four weeks after bombardment, green spots consisting of drug-resistant

callus became clearly visible on callus clumps (see below).

Formation of transgenic plants from bialaphos-resistant calli

Approximately 5 weeks after bombardment bialaphos-resistant shoots were formed from the green spots of bialaphos-resistant callus. The shoots were allowed to grow on the same medium (bialaphos-containing SIM) for another 3 weeks to confirm their bialaphos resistance, after which they were transferred, without being detached from the clump, onto bialaphos-free shoot-elongation medium (SEM) containing MS salts and vitamins (Murashige and Skoog, 1962), 1% sucrose and 0.8% agar in a cylindrical plastic container (ca. 7 cm diameter and 11 cm tall; Agripot, Kirin Co., Tokyo, Japan) to allow their rapid elongation for 2 to 3 weeks. Each of the elongated putative transgenic shoot was then detached from the callus clump and transplanted onto bialaphos-free rooting medium (RM) containing MS salts and vitamins, 1% sucrose, 1 mg/l 4-(3-indolyl)butyric acid (IBA) and 0.6% agarose in the container for root formation. About 2 weeks after the transplantation the shoots were rooted. The plants were kept in the container and allowed to set seed by self-pollination 3 to 4 months after bombardment.

Southern blot analysis

Total DNA was isolated from putative transformants (T_0) and untransformed *A. thaliana* plants by the method of Mettler (1987). Isolated DNA was digested with *Eco* RI or with *Bam* HI, separated by electrophoresis, and blotted to Nytran-N membranes (Schleicher & Schuell Inc., Keene, NH, USA) using standard procedures (Sambrook *et al.*, 1989). The 1.8 kb *Hin* dIII-*Eco* RI fragment from pARK22 containing CaMV 35S promoter-*bar*-NOS terminator (see Fig. 1) was labelled with [γ - 32 P]dCTP using 32 P QuickPrime Kit (Pharmacia Inc.,

Piscataway, NJ, USA) and used as a probe. Hybridization was done under standard conditions (Sambrook *et al.*, 1989). Final washes were performed at 65°C in 0.1 X SSC (1 x SSC contains 0.15 M NaCl and 0.015 M sodium citrate) and 1% SDS. After being washed, the membranes were autoradiographed using X-ray film (Kodak Nagase Medical Co., Osaka, Japan) with an intensifying screen for 4 days.

PAT assay

The PAT assay was carried out according to the method of De Block *et al.* (1987) with slight modification. Leaves (ca. 1 g fresh wt.) of transformants (T₂) and untransformed plants of *A. thaliana* were ground in 1 ml of an extraction buffer containing 5 mM EDTA, 5 mM phenylmethanesulfonyl fluoride, 1 mM iodoacetic acid and 10 μM leupeptin (Ogata and Ida, 1987), and the extract was centrifuged at 30,000 rpm for 30 min at 0°C. A 12.5 μl of the extract (containing 10.5 μg protein) was added to an Eppendorf tube containing 0.75 μl of 1 mM phosphinothricin (PPT) and 1.25 μl of [¹⁴C]AcCoA (59.3 mCi/mmol; NEN Research Products, Boston, MA, USA), and incubated at 37°C for 1h. Six microliters of the reaction mixture was spotted on a silicagel TLC plate (Merck and Co., Inc. Rahway, NJ, USA). Ascending chromatography was carried out in a 3:2 mixture of 1-propanol and NH₄OH (29% NH₃), after which the TLC plate was autoradiographed for 2 days using X-ray film (Kodak Nagase Medical Co., Osaka, Japan).

Progeny analysis

T₁ seeds collected from selfed primary transformants (T₀) were stored at 4°C in a refrigerator for 2 weeks (this cold treatment of the seeds was found to be essential for synchronization of the timing of germination of the seeds), after

which they were sterilized, sown on GM containing 2mg/l bialaphos, and incubated under 16h light/8h dark at 22°C. The seeds germinated 4 days after sowing, and two weeks after germination, bialaphos-resistant seedlings were easily distinguished from sensitive ones by visual inspection; sensitive ones bleached and died without developing primary leaves. One month after germination, these bialaphos-resistant T₁ plants were transferred to pots with rockwool and vermiculite (Tsukaya *et al.*, 1991) moistened with bialaphos-free Hyponex (0.1%, v/v, Hyponex Co. Inc., Marysville, Ohio, USA), and cultured at 22°C as described above. The T₂ seeds were collected from selfed T₁ plants, and from them bialaphos-resistant T₂ plants were cultured, after being tested for bialaphos resistance, in the same way as described above.

For CHAPTER II

Plant materials and DNA extraction

Transgenic *Arabidopsis thaliana* plants were generated by particle bombardment with the double-stranded circular plasmids pARK22 (Sawasaki *et al.*, 1994) or pCH (Takahashi and Morikawa, 1996) that respectively carried the *bar* or *hpt* gene. Three T₂ transgenic *Arabidopsis* plants of ecotype C24 (lines A and B carrying the *bar* gene and line C carrying the *hpt* gene) were used. Total DNA was isolated by the CTAB method (Ausubel *et al.*, 1995) from leaves of aseptically grown T₂ plants.

Cloning of DNA fragments including junction regions using inverse PCR

The isolated total DNA was digested with the appropriate restriction enzyme(s) then extracted and precipitated by the standard procedure (Sambrook *et al.*, 1989). DNA was circularized with T4 DNA ligase (New England Biolabs,

Beverly, MA) according to the manufacturer's instructions. The inverse PCR was performed with circularized genomic DNA as the template using an LA PCR Kit Ver. 2 (Takara Shuzo Co, Ltd., Kyoto, Japan) according to the manufacturer's instructions. Primers a and b were used for the *Xba* I or *Spe* I-*Xba* I digest from line A as a template; c and d for the *Hin* dIII digest from line A; c and d for the *Eco* RI, *Hin* dIII, or *Xba* I digest from line B; h and i for the *Xba* I or *Eco* RI digest from line C; and j and k for the *Eco* RI digest from line C. The reaction mixture was heated to 95°C for 3 min then amplified for 35 cycles at 98°C for 30 sec and at 68°C for 15 min, after which the mixture was kept at 72°C for 5 min in a PROGRAM TEMP CONTROL SYSTEM PC-800 (ASTECCo., Ltd., Fukuoka, Japan). PCR amplification was done with undigested total DNA from line B or C as the template and primers e, f or g and j. The reaction mixture, heated to 95°C for 3 min, was amplified for 35 cycles at 95°C for 1 min, then for 60°C for 2 min and 72°C for 3 min, after which the mixture was kept at 72°C for 5 min. IPCR and PCR products recovered by GENE CLEAN II[®] (BIO101 Inc., La Jolla, CA), were cloned into the pCRII TA cloning vector (Invitrogen, San Diego, USA) then transfected into *Escherichia coli* strain DH5 α according to the manufacturer's instructions. The sequences of the primers were: a, 5'-AAACCCACGTCATGCCAGTTC; b, 5'-GTAAAACGACGGCCAGT; c, 5'-GTCCACTCCTGCGGTTCTG; d, 5'-CGCTCTACACCCACCTGCTG; e, 5'-ATCCAAAGGCTCAAGAAACCTC; f, 5'-TGAAGGGCTTATTTTATGAATG; g, 5'-TTGATTTTATGTTATGTTGTG; h, 5'-AGAAGAAGATGTTGGCGACCTC; i, 5'-TGGACCGATGGCTGTGTAGAAG; j, 5'-TTGATTTTATGTTATGTTGTG; k, 5'-AAGAAACTAGAGAAACCAAACG.

plants

Total DNA was digested with *Sau* 3AI then electrophoresed on 1.8% agarose in 1 x TAE buffer. Fragments of 400 to 700 bp were recovered with GENE CLEAN II[®] (BIO101 Inc., La Jolla, CA), then cloned into the pBluescript II KS+ (Stratagene, La Jolla, CA) digested with *Bam* HI, and transfected into *Escherichia coli* strain DH5 δ . Five randomly white colonies were selected.

Nucleotide sequencing and data analysis

Nucleotide sequences were determined in an automatic sequencer (model 373A; Perkin Elmer Cetus, Norwalk, CT). BLAST search (Altschul et al., 1990) was performed manually against GenBank and plant EST databases using the World Wide Web server at NCBI (<http://www.ncbi.nlm.nih.gov>) and Computational Biology Center (<http://www.cbc.umn.edu>), respectively.

in vitro binding assay

An *in vitro* binding assay was done according to Hall et al. (1991) against nuclear matrices prepared from protoplasts isolated from suspension-cultured cells of tobacco [*Nicotiana tabacum* L. cv Bright Yellow-2 (BY-2)] (Nagata et al., 1990). Aliquots (100 μ l) containing matrices that represented about 3.5×10^5 nuclei were centrifuged at 2000 x g, after which the supernatant was removed, and the pellets resuspended in digestion/binding buffer (D/BB, 70 mM NaCl, 20 mM Tris, pH 8.0, 20 mM KCl, 1% thiodiglycol, 5 mM spermidine, 125 mM spermidine) containing 50 nM PMSF, 5 μ g/ml aprotinin, and 10 mM MgCl₂.

IPCR-amplified clones carrying the junction regions, pYAC4, pUC19, pARK22 and pCH were digested with the appropriate restriction enzyme(s) as described in Table 2. DNA fragments were recovered by the use of GENE CLEAN II[®] or MARMAID[®] (BIO101 Inc., La Jolla, CA). These fragments were labeled

with [γ - 32 P]ATP (Amersham, Buckinghamshire, UK) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) according to Sambrook et al. (1989). For the binding assays, 5 fmol of the 32 P-labeled DNA fragments, 5 fmol of 32 P-labeled restricted pUC19 as the negative control and 10 μ g of sonicated *Escherichia coli* genomic DNA fragments were added to the 100- μ l matrix aliquots. This mixture was incubated at 37°C for 90 min with stirring every 15 min, after which it was centrifuged at 2000 x g for 10 min, which separated the pellet (P) and supernatant (S) fractions. The pellet fraction was washed in 200 μ l of D/BB with 10 mM MgCl₂ then resuspended in 100 μ l of TE buffer containing 0.5% SDS with 0.5 mg/ml proteinase K, and incubated at 22°C overnight. Equal fractions (usually 20 %) of the pellet and supernatant fractions were separated on a 1.8% agarose gel in TAE buffer (Sambrook et al., 1989). After being mixed with 1 fmol of restricted 32 P-labeled pUC19, 1 fmol of each 32 P-labeled DNA fragment, was separated on the gel, which was defined as the total fraction (T). The gel was dried on filter paper then exposed to X-ray film (RX, Kodak Nagase Medical Co., Osaka, Japan). Quantification was done with an image analysis system (BAS3000, Fuji Photo Film, Tokyo, Japan).

CHAPTER I

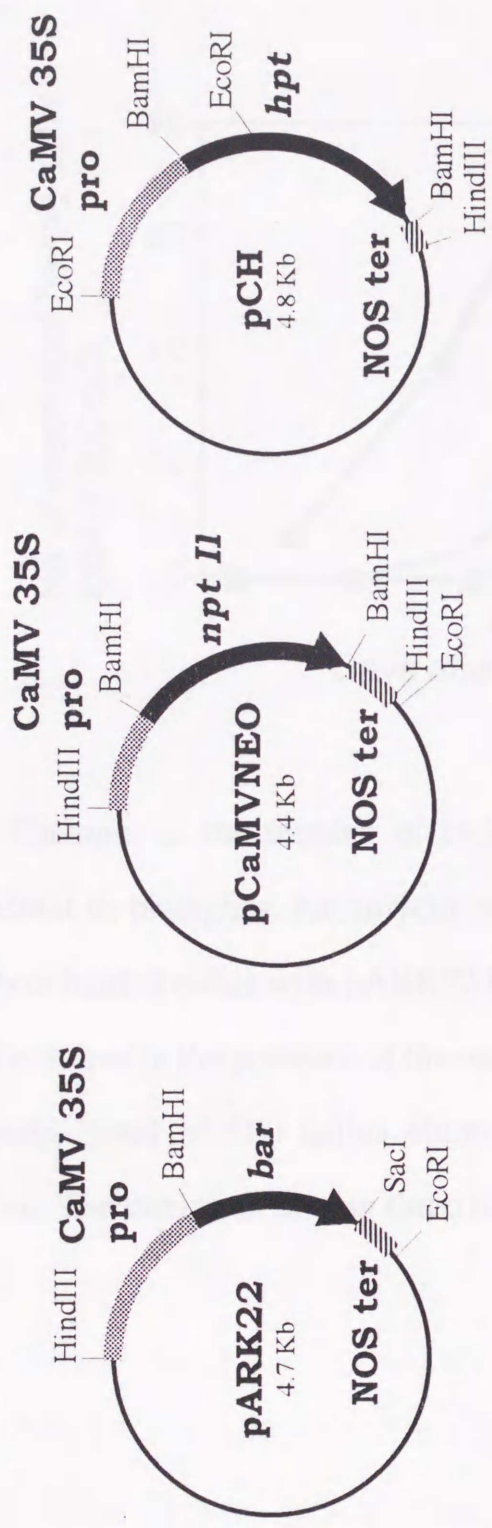
STABLE TRANSFORMATION OF *ARABIDOPSIS* WITH THE *BAR* GENE USING PARTICLE BOMBARDMENT

Section 1. Comparison of 3 selectable markers using changes in the number of drug-resistant calli on *Arabidopsis* root tissues

Seki *et al.*, 1991b in our laboratory have previously reported stable transformation of *Arabidopsis* by introducing the neomycin phosphotransferase II (*nptII*) gene into root sections. Exploitation of novel selectable marker genes for stable transformation of *A. thaliana*, which is one of the most intensively studied model plant species in molecular genetics (Meyerowitz, 1989), will be informative for the development of basic research on transgenic plants.

The *bar* gene encoding phosphinothricin acetyltransferase (PAT) from *Streptomyces hygroscopicus* (Murakami *et al.*, 1986) has been shown to be a useful selectable marker for stable transformation of various monocots (Gordon-Kamm *et al.*, 1990; Toki *et al.*, 1992; Vasil *et al.*, 1992) and dicots (De Block *et al.*, 1987; De Block *et al.*, 1989; Saito *et al.*, 1992). As far as I am aware, however, no reports on stable transformation of *A. thaliana* with this gene have been published. The hygromycin phosphotransferase gene (*hpt*) has also successfully been used for transformation of various plants such as rice (Shimamoto *et al.*, 1989) and *A. thaliana* (Lloyd *et al.*, 1986). These introduced plasmids were summarized in Figure 1.

Figure 2 shows changes in the number of drug-resistant calli, i.e. callus clumps that had green spot(s) consisting of callus resistant to bialaphos, kanamycin



Sawasaki et al. (1994) *Transgenic Res.*, **3**, 279-286.

Fromm et al. (1986) *Nature*, **319**, 791-793.

Figure 1. Schematic maps of pARK22, pCaVNEO and pCH. CaMV 35S pro, cauliflower mosaic virus 35S promoter; *bar*, bialaphos resistance gene encoding phosphinothricin acetyltransferase (PAT); *npt II*, neomycin phosphotransferase II gene; *hpt*, hygromycin phosphotransferase gene; NOS ter, nopaline synthase terminator.

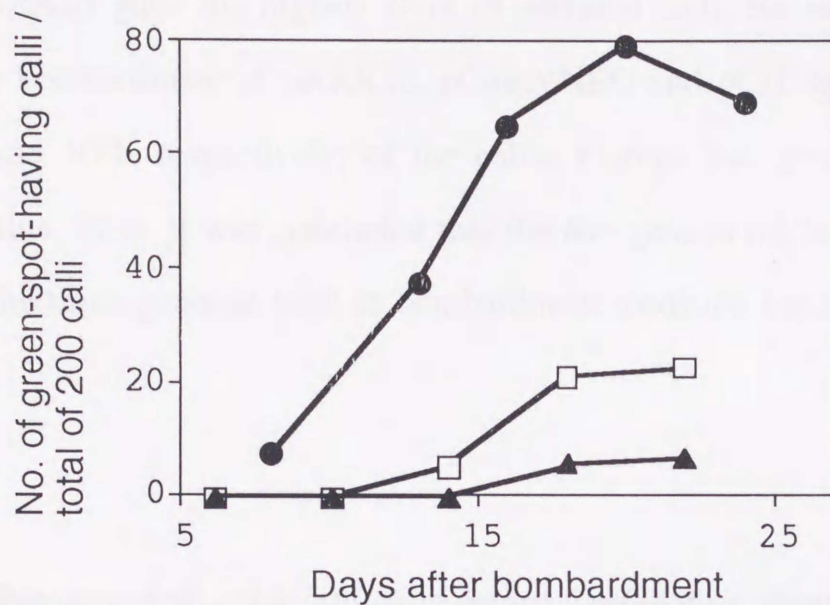


Figure 2. Changes in the number of calli having green spot(s) consisting of callus resistant to bialaphos, kanamycin or hygromycin from root sections that had been bombarded either with pARK22 (●—●), pCaMVNEO (▲—▲) or pCH (□—□), and cultured in the presence of the respective drug as described in Materials and Methods, Total of 200 callus clumps were counted for each selectable marker gene. The deviation among three independent experiments was less than 20%.

or hygromycin. Root sections were bombarded either with pARK22, pCaMVNEO or pCH, and cultured in the presence of 2mg/l bialaphos (Meiji Seika Kaisha Ltd., Yokohama, Japan), 20 mg/l kanamycin or 20 mg/l hygromycin, respectively.

The majority of the resistant calli had a single green spot (see below). Clearly, pARK22 gave the highest yield of resistant calli; for example ca. 4 weeks after bombardment of pARK22, pCaMVNEO and pCH, approximately 40%, 5% and 10%, respectively, of the callus clumps had green spot(s) of resistant callus. Thus, it was concluded that the *bar* gene is the best selectable marker of the three genes at least in bombardment-mediated transformation of *A. thaliana*.

Section 2. Regeneration of bialaphos-resistant *Arabidopsis* plants

Figure 3 shows sequential pictures of formation of bialaphos-resistant shoots of *Arabidopsis* from a single green spot of bialaphos-resistant cells that developed on the root sections bombarded with plasmid pARK22. Four weeks after bombardment, a tiny (1 to 2 mm in diameter) green spot of bialaphos-resistant cells was formed on a callus clump of bombarded root sections (A). In the next week, no shoots were seen on the spot (B) but two weeks later several shoots became clearly visible (C). The size and the number of the shoots were increased to form a bundle during the following two weeks even in the presence of bialaphos (D to E); the number of shoots on the clump was estimated to be ca. 5 in C and >20 in E. When the group of the shoots, without being detached from the callus clump, was transferred onto bialaphos-free SEM in the container and cultured for another 3 weeks as described in the Materials and Methods, the size and the number of shoots were increased greatly; the largest shoot was ca. 9 cm

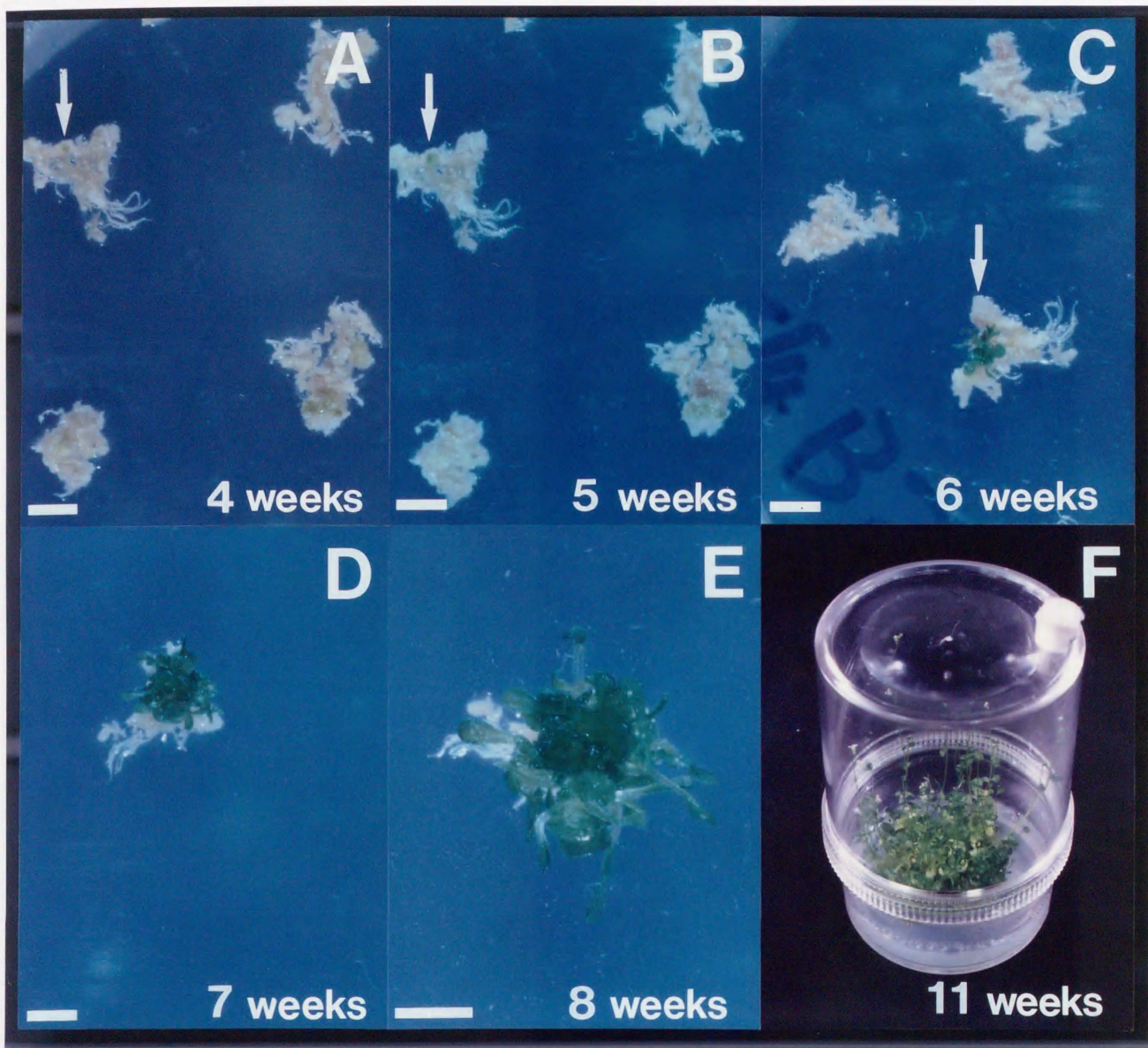


Figure 3. Sequential photographs showing formation of bialaphos-resistant shoots of *A. thaliana* from bombarded root sections. A single green spot of bialaphos-resistant cells (as indicated by an arrow in A) that formed 4 weeks after bombardment increased its size (but formed no visible shoots) during the next one week (see arrow in B), and then developed into a bundle of shoots two weeks later (see arrow in C). The size and the number of the shoots were much increased during prolonged culture (D, E and F). The culture was made in the presence of 2 mg/l bialaphos except for F (see text for details). Bars represent 0.5 mm.

long and the number of shoots was >40 (see F). Each of elongated shoots was then separately detached and transplanted onto bialaphos-free RM in the container. Two weeks after the transplantation the shoots had rooted, and 3 months after bombardment the regenerated plants set seed in the container.

The majority of the bialaphos-resistant green spots (see Fig. 3A) regenerated shoots, and 28 of the shoots that were derived from independent callus clumps were allowed to develop into plants in the containers. Of the 28 T₀ plant lines thus obtained, three lines (lines no. 1, 2 and 26) were randomly chosen and further studied by Southern blot and PAT assay analyses followed by the progeny analysis.

Section 3. Analyses of transgenic *Arabidopsis* plants

Southern blot analysis and enzymatic assay were done to confirm whether introduced plasmid was integrated into the genome of resistant shoots and expressed. Figure 4 shows the results of Southern blot analysis of total DNA from putative primary transformants (T₀) of the lines no. 1, 2 and 26 (lanes 1, 2 and 26) and that of untransformed plant (lane WT). Prior to Southern analysis, total DNA was digested either with *Eco* RI (sublane E) or *Bam* HI (sublane B), both of which have unique sites at different positions in pARK22 (see Fig. 1). The 1.8 kb *Hin* dIII-*Eco* RI fragment of pARK22 which contains CaMV 35S promoter-*bar*-NOS terminator (expression cassette of the *bar* gene) that had been labelled with radioactive ³²P was used as a probe (see Materials and Methods). Clearly, a number of hybridization signals were observed in these three putative transgenic plants (see lanes 1, 2 and 26), while no such signals were detected in the untransformed plant (see lane WT). Thus, these plants

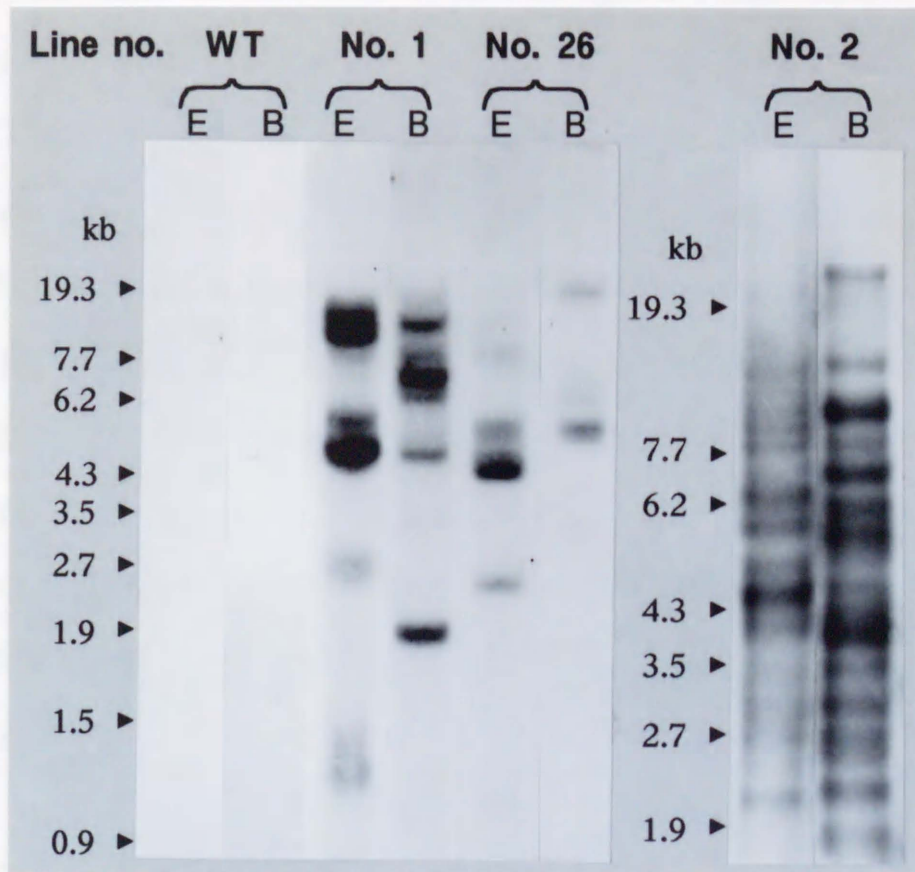


Figure 4. Southern blot analysis of total DNA from putative primary transformed plants (T_0) of the lines no. 1, 2 and 26 and untransformed plant of *A. thaliana*. Lane WT corresponds to untransformed plant and lanes 1, 2 and 26 correspond to the lines 1, 2 and 26, respectively. Sublanes E and B correspond to *Eco* RI and *Bam* HI digests. The amount of DNA loaded was 1 μ g for lane 2 and 2 μ g for lanes WT, 1 and 26. The *Hind*III-*Eco*RI fragment of pARK22 containing CaMV 35S pro-*bar*-NOS ter was used as a probe. The fragment sizes of a lambda DNA marker digested with *Hind*III are indicated.

Number of the bands of the three; >12 bands both in the *Eco* RI and *Bam* HI digests, while line no. 1 (lane 1) had >4 and ca. 6 bands, respectively, in the *Eco* RI and *Bam* HI digests, and line no. 26 (lane 26) had >3 and 2 bands, respectively, in the *Eco* RI and *Bam* HI digests. Based on the Southern blot analysis data, the copy number of the expression cassette integrated in the genome was estimated

from three lines are shown to be true transgenic plants. Similar results were obtained with the remaining 25 T₀ plants (data not shown).

The hybridization pattern and the number of the hybridization bands differed markedly among the three lines. Line no. 2 (lane 2 in Fig. 4) had the highest to be >4, >12 and >2 in the plants of lines no. 1, 2 and 26, respectively.

Figure 5 shows the results of PAT assays with extracts from bialaphos-resistant progeny (T₂ generation) of the three transgenic lines and from an untransformed plant. All of the T₂ plants of the transgenic lines exhibited radioactive signals corresponding to ¹⁴C-labelled acetylated PPT (lanes 1, 2 and 26). Similar results were obtained with T₀ plants of these transgenic lines (data not shown). No such signals were detected with the extract from an untransformed plant (lane WT). Thus, the *bar* gene was expressed in the transgenic plants and this trait was heritable, at least, up to the T₂ generation (see also below).

Note that the PAT activity of the line no. 2 progeny (lane 2) was much weaker than the other two (see Fig. 5). This was also the case with T₀ plants of the lines. Line no. 2 had the highest copy number of the *bar* gene expression cassette (see above) in the genome. Whether or not these results can be accounted for by the inhibitory effect of multiple copies of the introduced gene on its expression (Hobbs *et al.*, 1993) and/or due to position effect (Peach and Velten, 1991) must await further analysis.

Table 1 shows quantitative data on the inheritance of bialaphos resistance in the T₁ and T₂ progeny of the three transgenic lines. Based on χ^2 analysis of the ratio of segregation of the resistance in T₁ plants, the number of loci of the integrated *bar* gene was estimated to be 2, 1 and 1, respectively, in the lines no. 1, 2 and 26. This result of the locus number is somewhat unexpected from the results of the copy number of the expression cassette of the *bar* gene (>4, >12

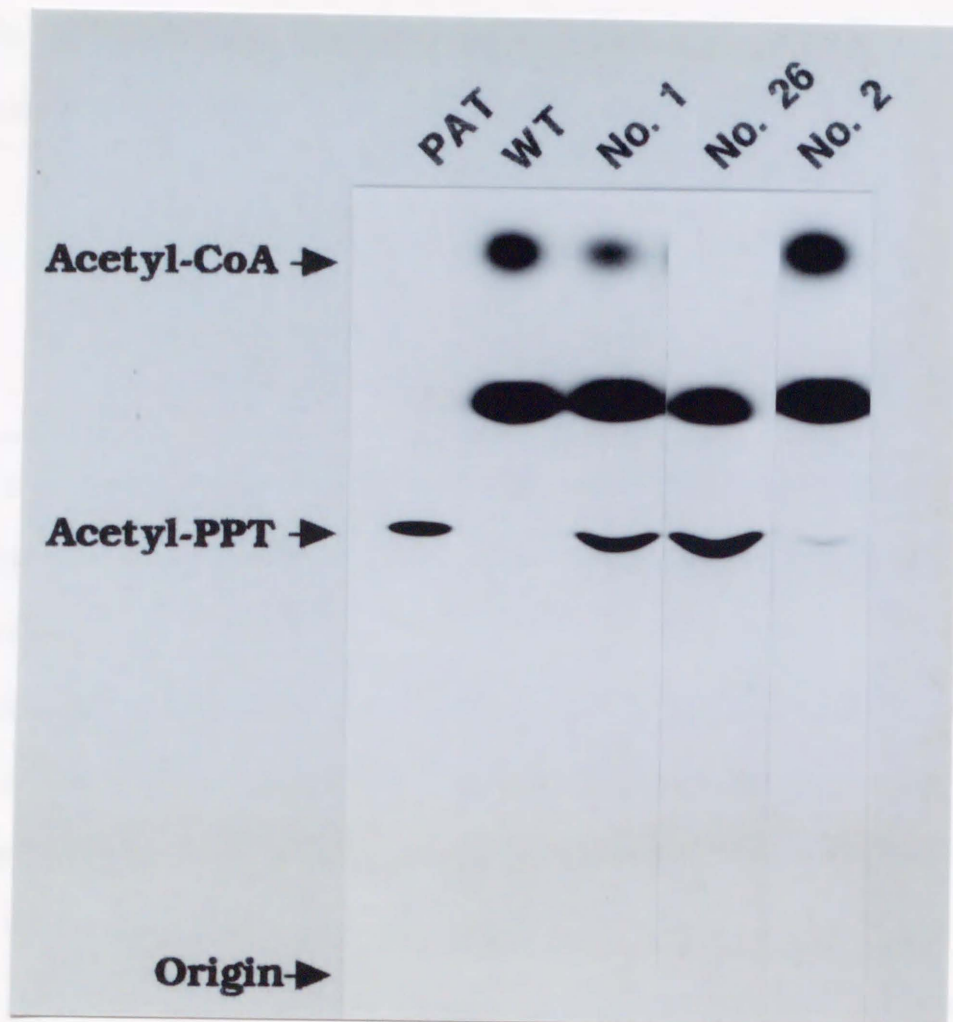


Figure 5. Detection of PAT activity by TLC analysis of leaf extracts from bialaphos-resistant plants of self-pollinated progeny (T_2 generation) of the three lines and untransformed plant of *A. thaliana*. Acetylated product of PPT (acetyl-PPT) indicates PAT activity. Lane PAT corresponds to purified PAT from *Streptomyces hygroscopicus* and the other lane numbers correspond with the numbers in Fig. 3.

Table 1. Inheritance of bialaphos resistance in T_1 and T_2 progenies of three transgenic lines of *A. thaliana*

Line no.	T_1 progeny	T_2 progeny	χ^2
1	10 / 11 ^a	55 / 65 ^a	0.152 ^b
2	7 / 11 6	5 / 74	0.758
26	14 / 20	53 / 55	0.267

^a No. of bialaphos-resistant plants / no. of total germinated plants.

Germination frequencies of the T_1 and T_2 seeds were approximately 92% on average.

^b Calculated by assuming that the ratio of segregation of the resistance in T_1 plants is 15/1, 3/1 and 3/1, respectively, in the lines no. 1, 2 and 26.

and >2 for lines no. 1, 2 and 26, respectively) determined by Southern analysis (see above). In order to clarify this discrepancy, detailed analyses on the progeny plants including in situ hybridization are needed. It is possible that some of the expression cassettes are not functional due to methylation and/or deleted during the meiosis.

Section 4. Analyses of morphology of transgenic *Arabidopsis* plants

The morphology of all of the T_0 plants of the three lines was similar to that of the regenerants of the wild type. However, progeny plants from lines no. 2 and 26 showed morphological abnormalities which differed markedly from that of the wild type as shown in Figure 6.

All of the T_1 plants of the line no. 1 tested (total of 10 plants) appeared to be morphologically normal (see plant 1 in Fig. 6). On the other hand, 2 out of 7 T_1 plants of line no. 2 were ca. one-fifth the height of the wild type at maturity (see plant 2 in Fig. 6); these plants can be considered to be dwarf-mutants (Feldmann *et al.*, 1989). Three of the 14 T_1 plants of line no. 26 had almost two times higher number of rosette leaves than the wild type at the rosette stage, and the timing of flowering was delayed 2 to 4 weeks in comparison with the wild type. At maturity, they were ca. two times taller than the wild type (see plant 26 in Fig. 6). Thus, the plants of line no. 26 can be considered to be late-flowering-type (Koornneef *et al.*, 1991) mutants. These morphological abnormalities were observed to be heritable at least up to T_2 progeny of each line (data not shown). I are currently studying genetic co-segregation of these morphological traits with bialaphos resistance by backcrossing with wild type plants. The results will be published elsewhere.

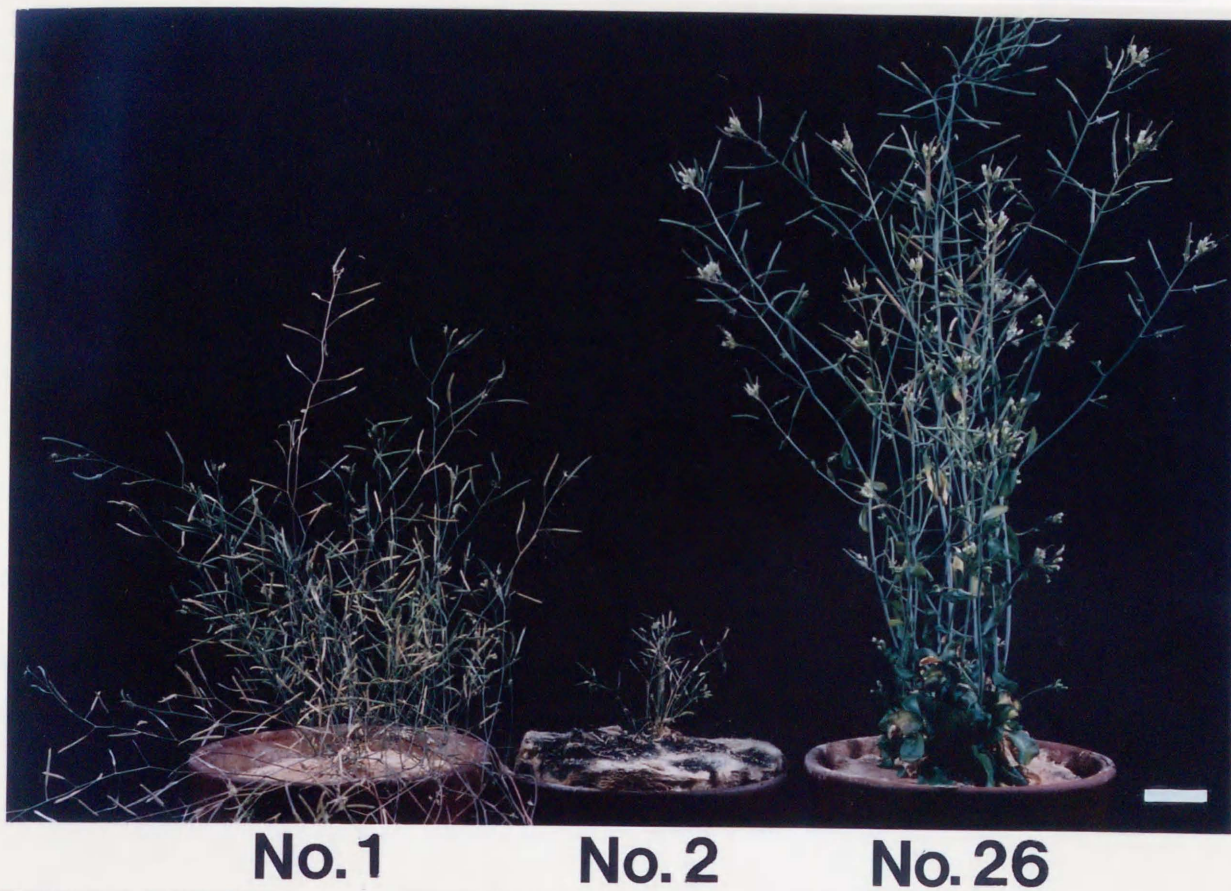


Figure 6. Photographs of bialaphos-resistant transgenic T_1 plants of *A. thaliana* of the lines no. 1, 2 and 26. The plant numbers correspond with their line numbers. All of these plants were 10-week old after germination. Their bialaphos resistance was confirmed by the tolerance against 2 mg/l bialaphos, and integration of the foreign gene in their genome was confirmed by the Southern blot analysis. Note that of the three plants plant 2 is the shortest, and plant 26 is the tallest and still flowering. See text for details. Bar represents 2 cm.

CHAPTER II

CHARACTERIZATION OF JUNCTION REGIONS IN TRANSGENIC *ARABIDOPSIS* PLANTS OBTAINED BY PARTICLE BOMBARDMENT

Section 1. Cloning of transgene and junction regions in transgenic *Arabidopsis*

Three T₂ transgenic *Arabidopsis* plants (lines A, B, C) were used to obtain the DNA sequences of the junction regions and integrated transgenes. Southern blot analysis of the *Xba* I-digested DNA of line B or C, using respectively the *bar* or *hpt* gene as the probe, showed a single hybridization band. In line A, analysis of the *Spe* I-digested DNA with the *bar* gene as the probe gave a single hybridization band (data not shown).

I used the inverse polymerase chain reaction (IPCR) (Ochman et al., 1988) to isolate the junction regions of these three lines. Basically, the total DNAs of the transgenic plants were digested with the appropriate restriction enzyme, circularized by self-ligation, then subjected to IPCR with primers designed within the respective transgene. Sequences between two IPCR primers were amplified by PCR using the appropriate primers (see below). All the amplified DNA fragments were cloned and partially sequenced from physical maps of the amplified fragments and their sequence data. The structure of the transgene locus in each of line was reconstructed (Figure 1).

Fragments of 4.9, 1.9, and 3.3 kb were amplified respectively with the *Xba* I, *Spe* I-*Xba* I, and *Hin* dIII digests of the total DNA from line A as the template. In line B, the 4.0-, 4.5-, and 4.0-kb fragments were amplified respectively with the *Eco* RI, *Hin* dIII, and *Xba* I digests. In line C, the 5.4- and 1.8-kb

fragments respectively were amplified with *Xba* I and the *Eco* RI digests. The lengths of these amplified fragments corresponded to the length of the hybridization band produced by Southern blot analysis of these transformant DNAs with the *bar* or *hpt* gene as the probe (data not shown), evidence that these amplified fragments were not artifacts.

The nucleotide sequences of 4992, 4475 and 5984 bp (thick white and black lines in Fig. 1) were determined in the transgene loci of lines A, B and C, part of which sequences have been deposited in the DDBJ. The 4992 bp sequence of line A covers 1695 bp upstream of primer a (accession no. AB003139) and 2256 bp downstream of primer d (no. AB003140). The sequence between primers a and b (shown by the dotted line in Fig. 1), estimated length about 2.7 kb by Southern analysis, was not sequenced because no well defined bands were obtained by PCR amplification. Presumably this is because of the presence of multiple fragments of the *bar* gene in this region. The sequence between primers c and d was cloned but not determined. The 4475 bp sequence of line B (accession no. AB003141) covers parts of the two IPCR bands described above. The sequence between primers c and d was determined after amplification by PCR with primers e and f. The sequence between primers h and i in line C was determined after PCR amplification with primers g and j. A 2.0-kb fragment was amplified by IPCR with primers j and k and the *Eco* RI digest of the total DNA from line C as the template, then sequenced. The 5984 bp sequence of line C (accession no. AB003142) covers parts of all the fragments amplified by IPCR and PCR, including the two IPCR fragments described.

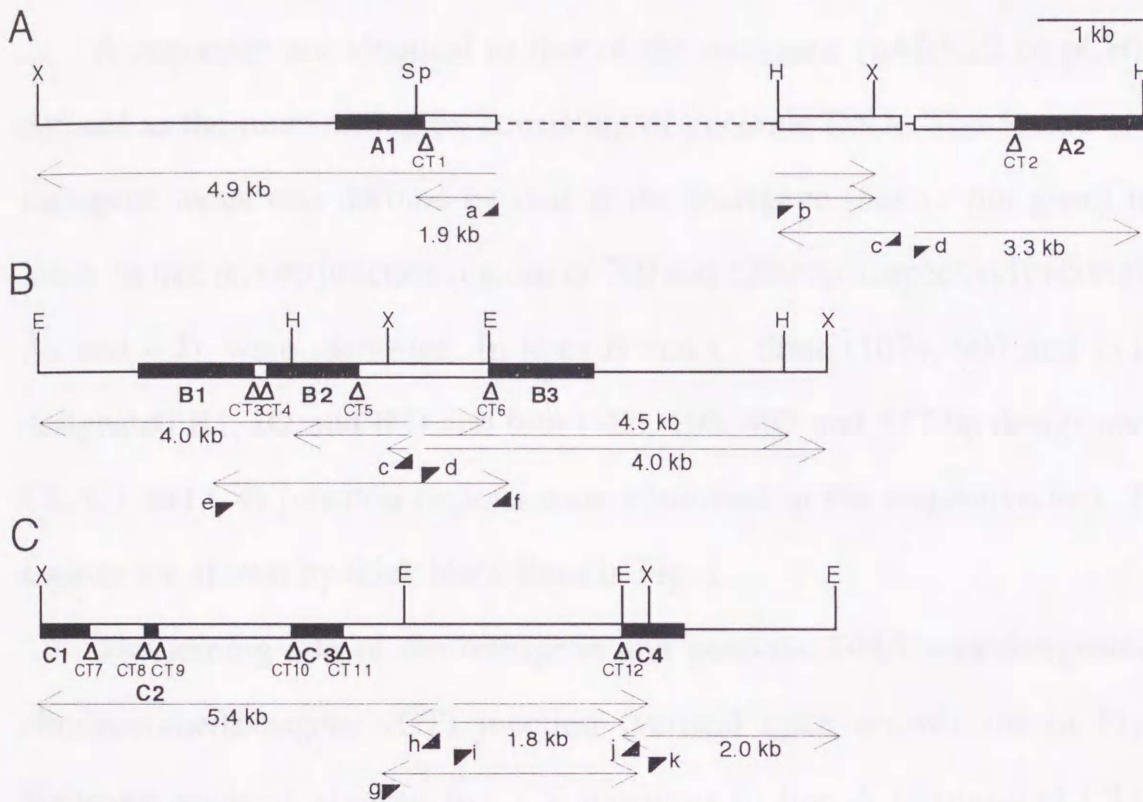


Figure 1 Structure of transgenic loci in transformant lines A, B and C of *Arabidopsis thaliana*. Locations of primers used for IPCR or PCR are shown by arrowheads (a to k), fragments amplified by IPCR by arrows and by PCR by double arrows. Thick white lines indicate transgenes, black ones junction regions. Dotted lines indicate the region not cloned. Thin lines indicate the cloned sequence that was not determined. Vertical open arrowheads (CT1 to CT12) show chromosome/transgene (CT) junction positions. X, *Xba* I; Sp, *Spe* I; H, *Hin* dIII; E, *Eco* RI.

In the locus of line A, totally 2940 bp of pARK22 (full length 4648 bp) were integrated in the genome. In the locus of line B, two fragments of pARK22 (137 and 1243 bp) were integrated separately. In the locus of line C, three (497, 1224, and 2668 bp) fragments of pCH (full length 4790 bp) were integrated. These transgene fragments are shown by thick white lines in Fig. 1.

A sequence not identical to that of the transgene (pARK22 or pCH) was defined as the junction region consisting of genomic DNA. The 5' side of each transgene locus was defined by that of the transgene (*bar* or *hpt* gene) in the locus. In line A, two junction regions of 789 and 1259 bp, respectively (designated A1 and A2), were identified. In lines B and C, three (1074, 907 and 1111 bp designated B1, B2 and B3) and four (480, 106, 482 and 527 bp designated C1, C2, C3 and C4) junction regions were identified in the respective loci. These regions are shown by thick black lines in Fig. 1.

The joining site of the transgene and genomic DNA was designated the chromosome/transgene (CT) junction (vertical open arrowheads in Fig. 1). Sequence analysis showed two CT junctions in line A (designated CT1 and CT2). Four CT junctions were found in line B (CT3 to CT6) and six in line C (CT7 to -CT12). The respective transgenes in lines B or C were divided by one and two junction regions.

Section 2. Characterization of sequences of junction regions

To eliminate the effect of sequence length on the analysis of the characteristics, sequences of approximately 400 bp upstream or downstream of the junction point (if available) in each junction region were analyzed. Results are shown in Table 1. For comparison, five sequences of similar length cloned randomly from the genome of non-transformed *Arabidopsis thaliana* plants (see footnote to Table 1) were analyzed, and the results (NT-1 to -11) included

in the table. Because junction region B2 was flanked by transgenes at both ends (see Fig. 1), it was analyzed in two parts, B2a and B2b. Although C3 also had junction points at both ends, its entire length was 482 bp, therefore the entire region was analyzed. Results for C2, even though its length was much shorter than 400 bp, are included in the table.

All the junction regions except C2 and C4 had high AT contents (65 to 76%, see Table 1). In contrast, those of three of the five “non-junction-region” sequences from the genome of non-transformed plants were 57 to 59%, and those of the other two 67%. In addition, all the junction regions except C2 and C4 were rich in scaffold/matrix-attachment region (S/MAR) motifs, including the A and T boxes (Gasser and Laemmli, 1986) and *Drosophila* topoisomerase II cleavage consensus sites (Topo-II box; Sander and Hsieh, 1985). Of the five genomic sequences from non-transformed plants, only the two AT-rich sequences had many S/MAR motifs. Five 400-bp fragment DNAs of sequences randomly selected from the GenBank database of the *Arabidopsis* genome (position 1-400 in accession nos. AC002130, AC002329, AC002354, AC002534, and ATAC002340) gave similar results. Furthermore the 1.9-megabase of a contiguous sequence from chromosome 4 of *Arabidopsis thaliana* indicated an average AT content of 64.11% (Bevan et al., 1998). AT-rich sequences carrying S/MAR motifs therefore appear to be characteristic of junction regions. These findings suggest that the integration of transgenes by particle bombardment tends to occur at AT-rich regions carrying S/MAR motifs rather than at random regions in the genome.

The T-DNA in transgenic tobacco (Gheysen et al., 1990) and the junction region found in transgenic rice by the calcium phosphate method (Takano et al., 1997) also suggest that AT-rich sequences are preferred target sites for integration. AT-rich regions on plant DNA seem to be hot spots for transgene integration.

Table 1

Characteristics of sequences of junction regions compared with sequences randomly cloned from the genome of non-transformed *Arabidopsis thaliana* plants

Fragment	Length analyzed (bp)	AT cont. (%)	S/MAR motif			Curvature propensity ^d	
			A box ^a	T box ^b	Topo-II ^c	Ave. (SD)	Max. ^e
A1	400	70	7	4	1	0.19 (0.31)	1.99
A2	400	71	10	7	1	0.77 (1.86)	13.38
B1	400	65	3	5	0	0.40 (0.61)	3.41
B2a ^f	400	66	5	2	0	0.25 (0.44)	2.84
B2b ^g	400	76	13	5	0	0.56 (0.94)	6.53
B3	400	66	3	2	1	0.50 (1.10)	7.82
C1	400	65	4	0	1	0.45 (0.72)	4.11
C2	106	58	0	0	1	0.89 (1.68)	8.88
C3	482	66	9	12	1	0.35 (0.48)	2.73
C4	400	60	1	0	0	0.26 (0.44)	3.32
NT -1 ^h	498	57	0	0	0	0.15 (0.22)	1.61
-2	311	58	1	1	0	0.15 (0.19)	1.01
-3	402	67	10	6	0	0.58 (1.06)	5.65
-4	438	59	0	0	0	0.12 (0.16)	0.82
-11	376	67	6	1	2	0.32 (0.53)	3.68

^a A box score of eight for a 10 or better match with the consensus sequence AATAAAYAAA. Y is pyrimidine.

^b T box score of nine for a 10 or better match with the consensus TTWTWTTWTT. W is A or T.

^c Topoisomerase II cleavage sites score as 13 of a 15 or better match with the consensus GTNWAYATTNATNNG.

^d Curvature propensity was calculated using the bend.it server on the world wide web (http://www.icgeb.trieste.it/dna/bend_it.html), with the DNase I based bendability parameters of Brukner et al. (1995) and the consensus bendability scale (Gabrielian and Pongor, 1996).

^e Highest curvature propensity values.

^h 400 bp downstream from the CT4 junction.

^g 400 bp upstream from the CT5 junction.

^h Genome DNA from non-transformed plants of *Arabidopsis thaliana*.

S/MAR harbors intrinsically curved DNA (Sykes et al., 1988). Furthermore, Milot et al. (1992) suggest that bent DNA is associated with illegitimate chromosomal recombination. The propensity of these junction regions for curvature and values for the five randomly chosen non-transformed genomic fragments therefore were calculated using a computer program on the world wide web (Gabrielian and Pongor, 1996, see footnote to Table 1). On the basis of the values obtained, 7 of the 10 junction regions should have a propensity for curvature (A2, B1, B2b, B3, C1, C2 and C3 in Table 1). The highest value (13.38) for the A2 junction region was similar to that of yeast SAR (13.24) which is reported to have strongly curved DNA (Snyder et al., 1986). Three of the ten junctions showed no potential for curvature [i.e., values below 0.3 were very close to the genomic average value for this parameter (Gabrielian et al., 1996)]. In contrast, two of the five non-transformed genomic fragments appeared to have the propensity for curvature (fragments 3 and 11 of NT in Table 1). Seven of the ten junction regions had the potential for curvature, whereas three of the five non-transformed genomic fragments did not. The propensity for curvature therefore appears to be characteristic of junction regions. These findings suggest that transgene integration by particle bombardment tends to occur near regions that have the potential for curvature.

Section 3. *In vitro* binding assay of junction regions against tobacco matrices

Using the method of Hall et al. (1991), I made an *in vitro* binding assay of the junction regions against nuclear matrices isolated from tobacco. DNA fragments carrying about a 350 to 700 bp part of each junction region were cut with the appropriate restriction enzyme(s) from the respective IPCR-amplified fragments (Table 2). The C2 junction region of 106 bp was excluded for <350 bp. Eight junction region fragments had solely genomic DNA, except for A2-457,

B1-682, B3-480 and C3-357. These four had the pARK22- or pCH-derived transgene sequence, respectively about 20%, 13%, <1% and 44% of the total (see Table 2). Yeast SAR, carrying ARS1 and CEN4 and cut from pYAC4 (Amati et al., 1990), as the positive control linearized pUC19 the negative control. After being linearized, the two input DNAs (pARK22 and pCH) also were used in the binding assay.

Figure 2 shows a typical autoradiogram of an *in vitro* binding assay of the DNA fragments. All the junction region fragments, as well as yeast SAR, show distinct bands in the pellet fraction (P), whereas there are no bands with pUC19, pARK22 and pCH. This indicates that all the junction region fragments contain the scaffold/matrix attachment region (S/MAR). Of the eight junction region fragments, A2-457, B1-682, B3-480 and C3-357 have, to some extent, the respective input DNA (see above). Because pUC19, pARK22 and pCH do not bind to the nuclear matrices of tobacco, the binding ability of the four DNA fragments is owing to their genomic DNA. In fragments A1-447, A2-457, B1-682 and B2-600 the signal intensity was higher in P than in the supernatant fraction (S), whereas in B3-480, C1-437 and C3-357 the intensity was similar in both fractions. In C4-395 and yeast SAR, the intensity was higher in S than in P.

Binding efficiency was quantified by $\% \text{binding} = P_{\text{RA}} / (S_{\text{RA}} + P_{\text{RA}}) \times 100$, where P_{RA} and S_{RA} are the respective radioactivities of the fragments in P and S (see footnote to Table 2). Results are given in Table 2. Junction region fragments had a binding efficiency of 32 ± 7 to $87 \pm 8\%$, higher than the value for yeast SAR ($24 \pm 2\%$). These quantitative results confirm my conclusion that all these junction regions carry S/MAR. pUC19, which is reported not to bind to nuclear matrices (Hall et al., 1991), had a value of $5 \pm 1\%$, and both input DNAs (pARK22 and pCH) had a very similar value ($4 \pm 1\%$), confirming that these input DNAs do not bind to the nuclear matrices. Probably that the bombarded transgenes first

are delivered to the S/MARs then integrated.

S/MAR has been found close to a T-DNA end in a transgenic petunia plant (Dietz et al., 1994) and within the target sites of retroviral vectors (Mielke et al., 1996). SAR-like structures also have been found in the integration sites of transgenic rice by the calcium phosphate method (Takano et al., 1997). Furthermore, Sperry et al. (1989) found an S/MAR next to a long interspersed repetitive element (LINE) within the recombination junction of human ring chromosome 21. The S/MAR on the genome may have an important role in the integration of transgenes or in recombination.

DNA elements binding to the nuclear matrices of HeLa cells have been calculated experimentally as approximately 2% of the entire genome (Jackson et al., 1996). Assuming that the ratio of S/MAR sequences in the genome is the same as that of HeLa cells and that the length is 1 kb, the number of S/MARs in the *Arabidopsis* genome (10^8 bp/haploid genome) is be approximately 2000. The length of the loop domain therefore is thought to approximately 49 kb. In maize, the locations of matrix attachment sites along a contiguous region of 280 kb on chromosome 1 show nine potential loops that vary in length from 6 to >75 kb (Avramova et al., 1995). Approximately 4000 sites on the diploid genome

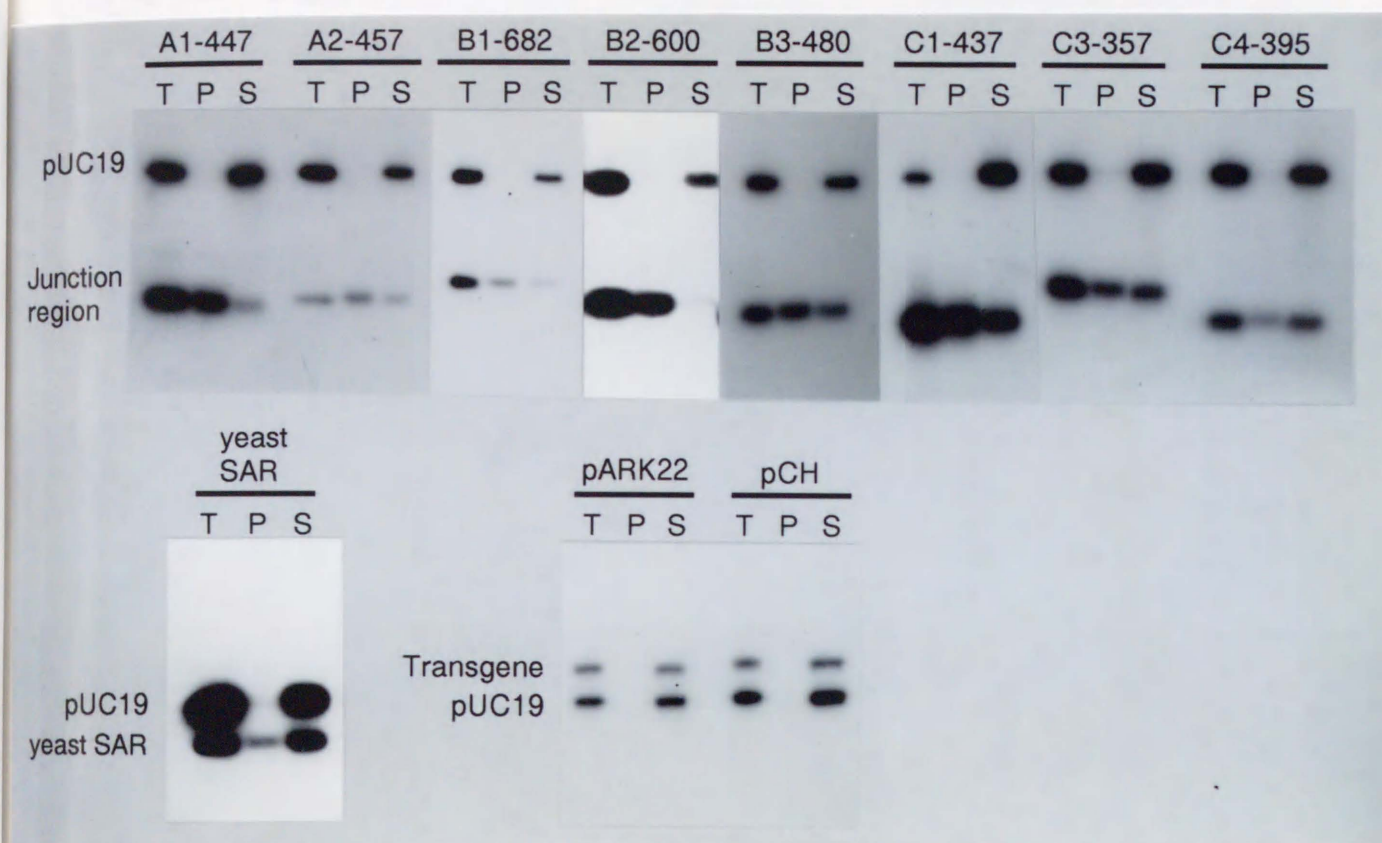


Figure 2. Typical results of the *in vitro* binding of DNA fragments carrying a part of the junction region and of the binding of several control DNA fragments to nuclear matrices isolated from tobacco cells. Characteristics of these DNA fragments are listed in Table 2. The positions of the fragments are shown at the left of the blots. T, P and S correspond to the total, pellet and supernatant fractions (see below).

Table 2

Binding efficiency of DNA fragments of junction regions to nuclear matrices of tobacco based on the *in vitro* binding assay

Fragment no.	Origin ^a	Total length of the fragment (bp)	Genome DNA in the fragment	Binding efficiency (%) ^b
A1-447	<i>Ase</i> I- <i>Hin</i> dIII digest of 4.9-kb fragment carrying A1	447	447 bp of A1	85± 4
A2-457	<i>Eco</i> RI digest of 3.3-kb fragment carrying A2	573	457 bp of A2	76± 8
B1-682	<i>Spe</i> I- <i>Pst</i> I digest of 4.0-kb fragment carrying B1	779	682 bp of B1	64± 4
B2-600	<i>Hin</i> dIII digest of 4.0-kb fragment carrying B2	600	600 bp of B2	87± 8
B3-480	<i>Eco</i> RI digest of 4.0-kb fragment carrying B3	484	480 bp of B3	69± 8
C1-437	<i>Xba</i> I- <i>Aat</i> II digest of 5.4-kb fragment carrying C1	437	437 bp of C1	67± 3
C3-357	<i>Sal</i> I- <i>Eco</i> RV digest of 5.4-kb fragment carrying C3	634	357 bp of C3	57± 6
C4-395	<i>Eco</i> RI- <i>Xho</i> I digest of a clone carrying C4	395	395 bp of C4	32± 7
Yeast SAR	<i>Hin</i> dIII- <i>Pvu</i> II digest of pYAC4	1811	-	24± 2 ^c
pUC19	<i>Hin</i> dIII digest	2682	-	5± 1 ^d
pARK22	<i>Hin</i> dIII digest	4644	-	4± 1
pCH	<i>Hin</i> dIII digest	4786	-	4± 1

^a IPCR-amplified fragments (Fig. 1) with junction region, A1, A2, B1, B2, B3, C1 or C3 were digested with the respective restriction enzyme(s). The clone with C4 was reconstructed by joining the 5.4- and 2.0-kb fragments at the *Xba* I site (Fig. 1).

^b $P_{RA} / (P_{RA} + S_{RA}) \times 100$, where P_{RA} and S_{RA} are the respective imaging data for that fragment in the pellet and supernatant fractions. The number is the mean ± standard deviation (SD) of results of 3 independent experiments, except for yeast SAR and pUC19.

^c results of 5 experiments.

^d results of 35 experiments.

Section 4. Homology of junction regions

The junction region sequences were compared with those from GenBank and Plant EST databases using BLAST program (Altschul et al., 1990) to identify possible homologies. The entire A1 (position 1-789) and part of the A2 respectively (33, 52 and 98 bp in positions 3058-3091, 3169-3221 and 3317-3415), sequences were similar to those of the *Arabidopsis* genomic clone of chromosome 1 (96%, position 111455-112243, GenBank accession No. AC002130) and to an EST clone of *Arabidopsis* (98%, positions 1-185 in 198A4T7 MSU.Ath EST). Sequence analysis showed the 486 bp sequence of the B2 (position 1633-2118) to be homologous to the sequence of the *Arabidopsis* genome DNA of chromosome 1 (80%, position 38391-38892, GenBank accession No. AC000104). The 310 bp of the C1 (position 1-310), 139 bp of the C3 (position 2592-2730), and 219 bp of the C4 (position 5766-5984) showed respective homologies to the *Arabidopsis* chloroplast *ndhG* gene (100%, position 668-977, EMBL accession No. X99278), tobacco chloroplast gene for tRNA (94%, position 399-536, EMBL accession No. X01016) and an EST clone of *Arabidopsis* (89%, position 203-430, GenBank accession No. N96239).

Although the C1 and C3 junction regions carry sequences highly homologous to chloroplast DNA, the C4 junction region was similar to that of an EST clone and unlike chloroplast DNA. Furthermore, hygromycin-resistant plants of line C segregated 37 to 9 (resistant to sensitive plants). The transgenes of line C therefore are thought to be integrated in the nuclear genome. Previously Kikuchi et al. (1987) showed copy number changes of nuclear DNAs between the embryo and callus and that they contain chloroplast DNA. Thorsness and Fox (1990) showed that the mitochondrial DNA in yeast escapes from the mitochondria to

the nucleus. Moreover, 10% of yeast transformants are reported to have mitochondrial DNA insertions at their junctions (Schiestl et al., 1993). Chloroplast DNA which has escaped from the chloroplast to the nucleus therefore may be integrated within the junction region.

Section 5. Characteristics of sequences of the CT junctions

Figure 3 shows the characteristics of sequences at the 12 CT junctions and terminals of the transgene. The cleavage sites for topoisomerase I (5'-G/C-A/T-T or 5'-A-A/T-G/C; Been et al., 1984) are in the sequences of the CT junctions (boxes on junction in Fig. 3). In 10 of the 12 CT junctions they are located near the junction point between the transgene and genomic DNA. The other 2 CT junctions have these sites within 6 bp of the junction point. The presence of cleavage sites near the junction points suggests that the integration of a transgene into a chromosome may be catalyzed by topoisomerase I. In yeast and mammalian cells, cleavage sites are present at the junctions of insertions of transforming DNA (Wilkie and Palmiter, 1987; Tanizawa et al., 1993; Hamada et al., 1993; Zhu and Schiestl, 1996). Topoisomerase I activity has been found in the nuclear matrix (Nishizawa et al., 1984), but it is thought to bind facultatively to nuclear matrices, whereas topoisomerase II binds constitutively.

There was a pair of short direct repeats, GAAAG, ATAGTG, GAAG, GAT(C/A)AG and AT(A/G)CAGT, within 10 bp of the junction point on a transgene sequence (horizontal arrows in Fig. 9). This suggests that short direct repeats are integration sites for transgenes introduced by particle bombardment. Short direct repeats were present in the T-DNA region of the Ti plasmid that is

INTERACTION MODELS FOR THE TRANSDUCED INTERACTORS IN
PARTICLE-BINDING TARGETS

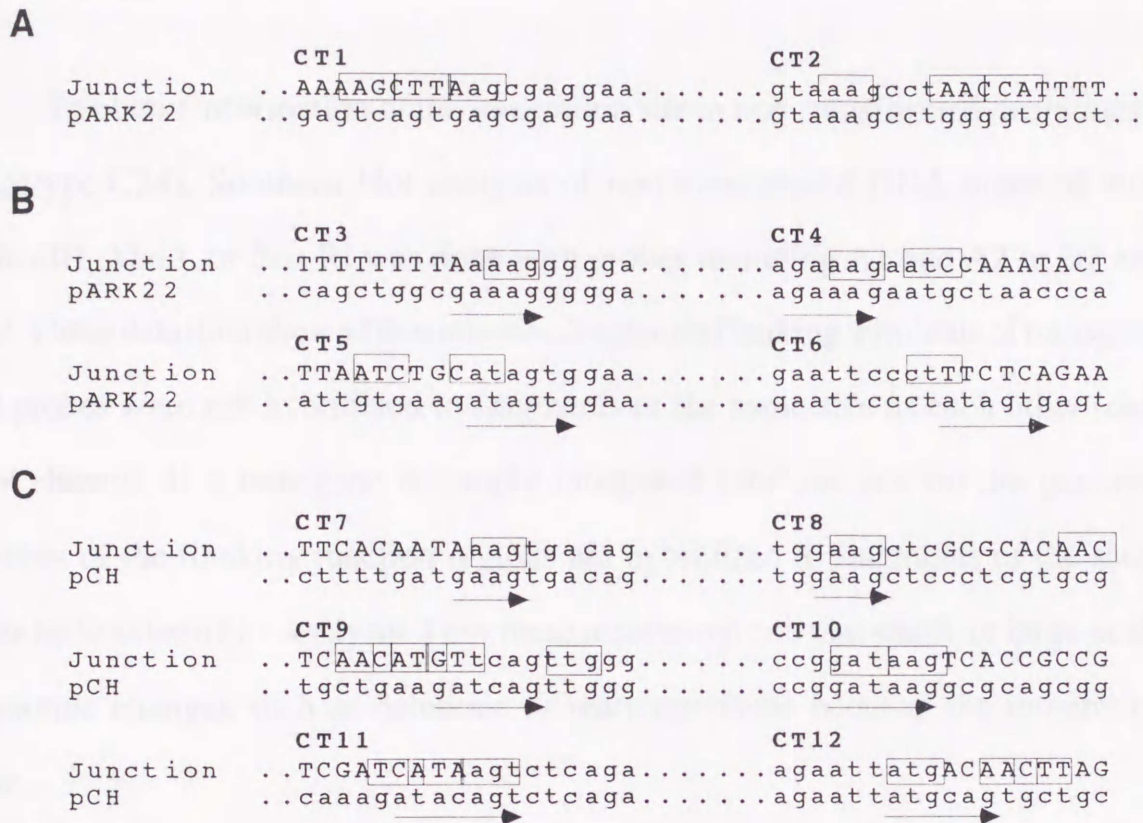


Figure 3. Characteristics of the sequences of chromosome/transgene junctions.

Sequences of 12 chromosome/transgene junctions (Junction) are shown. pARK22 and pCH denote transgenes. Upper case letters denote genomic DNA, and lower case ones the transgenes. Boxes in the sequence of the 'Junction' show topoisomerase I cleavage sites (5'-G/C-A/T-T or 5'-A-A/T-G/C). Horizontal arrows indicate a short direct repeat.

CHAPTER III

INTEGRATION MODEL FOR THE TRANSGENE INTRODUCED BY PARTICLE BOMBARDMENT.

To obtain information of the integration site in non-transformed *Arabidopsis* (ecotype C24), Southern blot analysis of non-transformed DNA digested with *Hin* dIII, *Xba* I, or *Eco* RI was done with probes including A1 and A2 or B2 and B3. These data then showed that junction fragments flanking terminals of transgene as probes were not hybridized to fragments of the same size as each other (data not shown). If a transgene is simply integrated into one site on the genome, probes of the flanking junction regions are hybridized to fragments of the same size by Southern blot analysis. Thus these results indicate that small- or large-scale genomic changes such as deletions or rearrangements occur at the integration site.

Figure 2 and Table 2 in chapter II indicate that the junction regions are S/MAR with the ability to bind to nuclear matrices. Based on these observations, Figure 1 shows two putative integration models including S/MAR as the junction regions. When the transgene including short direct repeats (horizontal arrows in Fig. 3 in chapter II) is introduced by particle bombardment into nuclear, it is integrated within one S/MAR (left in Figure 1). At this point, small-scale genomic change may occur. As the other integration model, the transgene is integrated into two independent S/MARs followed by the large deletion or rearrangement of a loop domain (right in Figure 1). Then the integration of the transgene may be catalyzed by topoisomerase I (Fig. 3 in chapter II). A chromosomal domain released from S/MAR by the integration of the transgene may function as 'a

novel transgene'.

Large-scale genomic changes such as a duplication, deletion, or rearrangement of DNA have been reported in mammalian (Wilkie and Palmiter, 1987; Hamada et al., 1993) and plant cells (Gheysen et al., 1991; Takano et al., 1997). A loop domain defined by S/MAR may function as 'a recombination unit' for large-scale genomic change

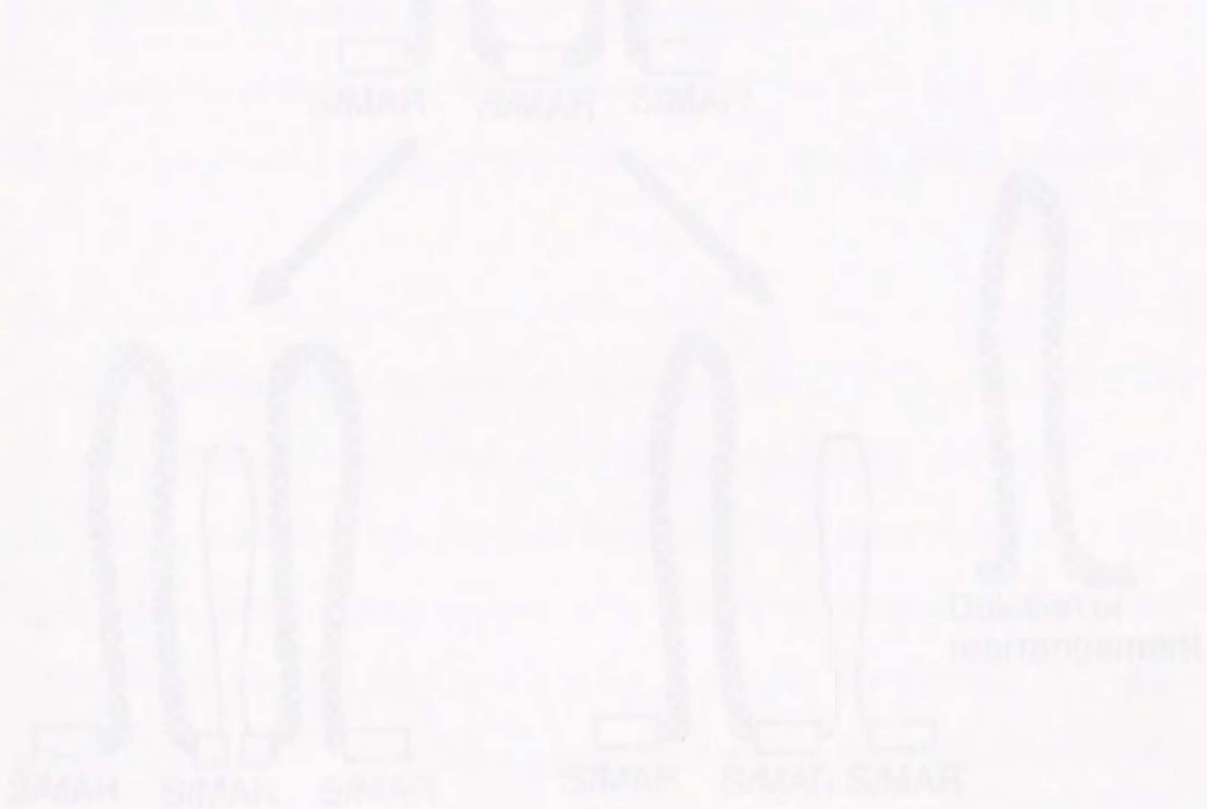


Figure 1. Putative recombination models of the transgenes initiated by particle bombardment. Arrows on the molecules indicate a short direct repeat. Boxes show S/MAR, and the regions bounded by S/MAR indicate inverted and duplications.

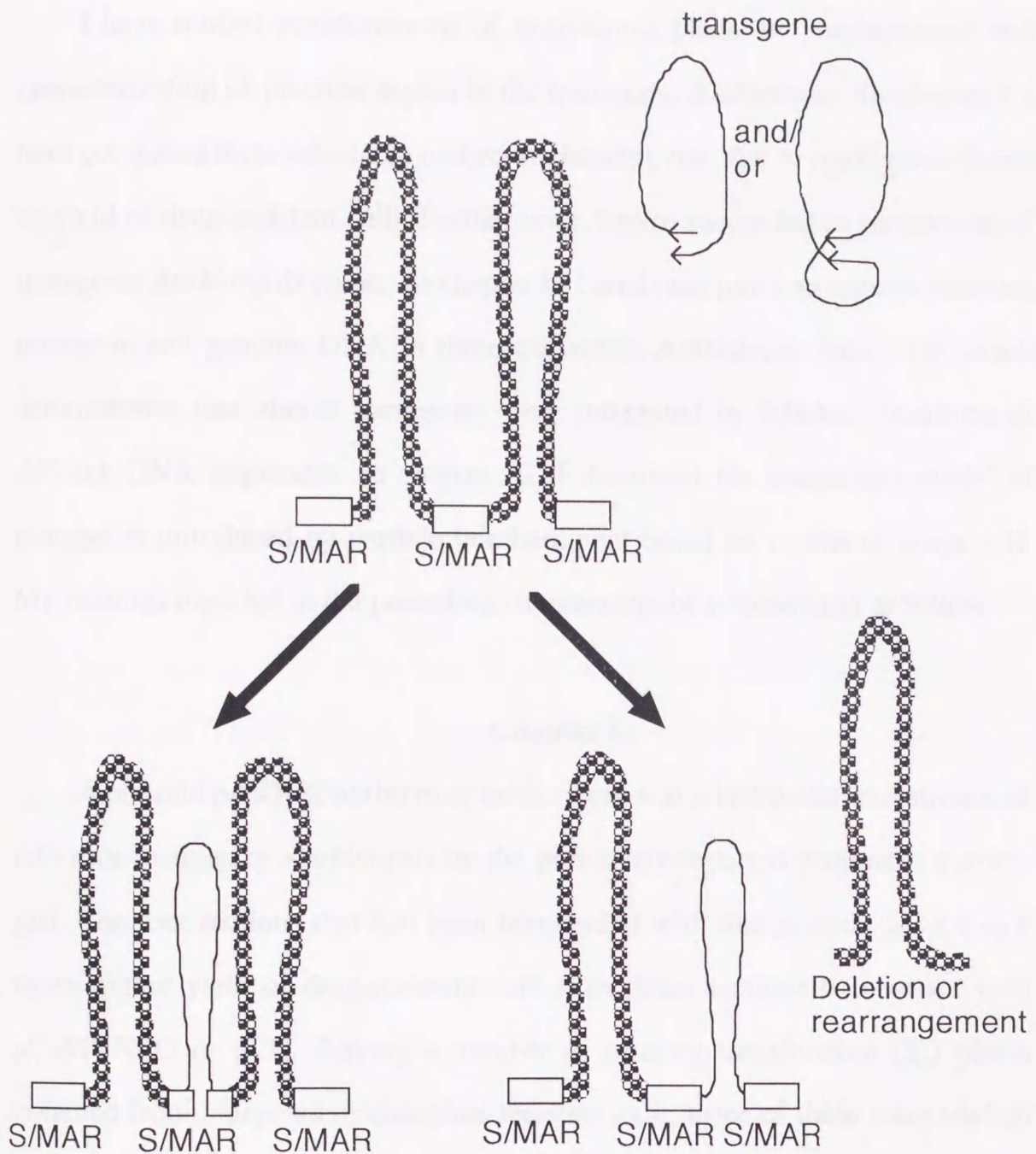


Figure 1. Putative integration models of the transgene introduced by particle bombardment. Arrows on the transgene indicate a short direct repeat. Boxes show S/MAR, and the regions bordered by S/MAR indicate chromosomal loop domains.

CONCLUSIONS

I have studied transformation of *Arabidopsis* plants by bombardment and characterization of junction region in the transgenic *Arabidopsis*. In chapter I, I have compared three selectable markers harbouring *bar*, *hpt* or *nptII* genes based on yield of drug-resistant calli. Furthermore, I have succeeded in production of transgenic *Arabidopsis* plants. In chapter II, I analyzed junction regions between transgene and genome DNA in three transgenic *Arabidopsis* lines. The result demonstrates that almost transgenes were integrated in S/MAR consisting of AT-rich DNA sequences. In chapter III, I discussed the integration model of transgenes introduced by particle bombardment based on results of chapter II. My findings reported in the preceding chapters can be summarized as follow:

Chapter I

A plasmid pARK22 harbouring the *bar* gene was constructed and introduced into root sections of *Arabidopsis* by the previously reported pneumatic particle gun. The root sections that had been bombarded with this plasmid gave 4 to 8 times higher yield of drug-resistant calli than those sections bombarded with pCaMVNEO or pCH. Among a number of primary transformant (T_0) plants obtained from independent bialaphos-resistant calli, three of them were studied by Southern blot hybridization and PAT enzyme activity analyses, confirming the stable integration of the foreign gene into *Arabidopsis* genome and its expression in the plants. The progeny analysis showed transmission of the foreign gene and its expression in up to the T_2 generation. Some of the T_1 progeny showed morphological abnormalities. Thus, the *bar* gene can be used to produce transgenic *Arabidopsis* plants effectively.

Chapter II

(1) Characteristics of the DNA sequences of ten junction regions were investigated. All but two regions were AT-rich and carried motifs characteristic of a scaffold/matrix-attachment region (S/MAR). Furthermore, seven regions were calculated to have a propensity for curvature. These findings suggest that in the genome AT-rich regions with S/MAR motifs and a propensity for curvature are hot spots for the integration of transgenes delivered by particle bombardment.

(2) An *in vitro* binding assay against nuclear matrices isolated from tobacco showed that all the junction regions could bind to the nuclear matrices, evidence that junction regions have S/MARs.

(3) Cleavage sites for topoisomerase I were present at 10 of 12 CT junctions near the junction point. The other 2 junctions had sites within 6 bp of the junction point. These findings suggest that transgene integration is catalyzed by topoisomerase I.

(4) A pair of a short direct repeats of 4 to 6 bp were present within 10 bp of the junction point on a sequence of the transgene, indicative that integration requires short direct repeats on the terminal of the transgene.

Chapter III

From Southern blot analysis, the junction regions flanking transgenes were not sequential regions on non-transformed *Arabidopsis* plants. Thus the genomic change may occur in the integration site on the non-transformed plants. In addition to binding of the junction regions to nuclear matrices, I proposed two integration models into genomic DNA for transgene introduced by particle bombardment. One is that transgene were integrated within the same S/MAR, the other is done between different S/MAR followed by deletion of genome.

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論文リスト

主論文

Studies on genomic DNA flanking with transgene in transgenic *Arabidopsis* plants

公表論文

Sawasaki, T., Seki, M., Anzai, H., Irifune, K. and Morikawa, H. (1994) Stable transformation of *Arabidopsis* with the *bar* gene using particle bombardment. *Transgenic Res.* 3, 279-286.

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