Analysis of transcripts of aminoglycoside phosphotransferase II and nopaline synthase in *Nicotiana glauca* transformants mediated by recombinant Ti plasmid

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ABSTRACT

The vector possessing two marker genes encoding for, aminoglycoside phosphotransferase II (APH(3')II) and nopaline synthase (NOS) was inserted into the octopine-type Ti plasmid through homologous recombination. Following the confirmation of foreign gene expression in *Nicotiana glauca* tumor cells with respect to kanamycin resistance and nopaline synthesis, the analysis was made on the transcripts. There was a quantitative difference in the amount of mRNA between APH(3')II and NOS. The size of mRNA for APH(3')II and NOS were 1250 bases and 1550 bases, respectively.

1. INTRODUCTION

The Ti plasmid of *Agrobacterium tumefaciens* has been employed as a natural vector for transferring foreign DNA to host plants. It has been demonstrated that the T-region of the Ti plasmid (T-DNA) is integrated into the plant genome (Chilton *et al.* 1977). Regarding markers readily detectable in recipient cells, dominant selectable genes such as chimeric DNA consisting of a structural gene from a bacterial transposon resistant to specific antibiotics and the 5' promoter—3' terminator region of nopaline synthase have been developed in some laboratories (Bevan *et al.* 1983a, 1984; Fraley *et al.* 1983; Herrera-Estrella *et al.* 1983a, b). We obtained *Nicotiana glauca* transformants by the infection of *A. tumefaciens* harboring a recombinant Ti plasmid which possesses two marker genes responsible for kanamycin resistance and nopaline synthesis. In this communication, the outcome of the analysis of transcripts of both genes is presented.

2. MATERIALS AND METHODS

Bacterial strains and plasmids: The following bacterial strains were em-

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ployed: *Escherichia coli* C600 (F^- , thi-1, thr-1, leuB6, lacY1, tonA21, supE44, λ^-), GJ23 carrying helper plasmids (R64drd11, pGJ28) (Van Haute *et al.* 1983), *A. tumefaciens* C58ClCm^R (pTiB6S3tra^C) (Petit *et al.* 1978) or C58ClRif^R (pGV 2215) (Leemans *et al.* 1982). Several plasmids including pKC7 (Rao and Roger 1979), pUC9-nopneo Δ (Bevan 1984) and pGVO415 containing HindIII fragment 23 of a nopaline type Ti plasmid (Depicker *et al.* 1980) were used. The pBR322 containing BamHI fragment 19 and HindIII fragment 1 of the octopine type Ti plasmid, pTiB6-806 (Chilton *et al.* 1977), were served for the vector construction.

E. coli transformation: Plasmid DNA was supplied to Ca²⁺-treated *E. coli* according to the method of Cohen *et al.* (1972). Colonies resistant to 20 μ g/ml ampicillin were selected.

Intermediate vectors (pNK1): The pNK1 was constructed as following. A DNA fragment (Knop) containing the promoter and the terminator regions of nopaline synthase and APH(3')II-coding sequence was isolated from pUC9-nopneo Δ (Bevan 1984) and inserted into the HindIII/EcoRI sites of pKC7 (Rao and Roger 1979). The resulting plasmid, pKC7KN was fused with the HindIII fragment 23 of a nopaline type Ti plasmid (pTiC58) which contained an intact nopaline synthase gene and the right border region (Depicker *et al.* 1980). To the BglII site of the new plasmid pKC7KNH23, the BamHI fragment 19 (4.5 Kd) of an octopine type Ti plasmid (pTiB6-806) (McPherson *et al.* 1980) was inserted. The final product was named as pNK1. Direction of Knop and nopaline synthase gene was determined on the basis of molecular size

Fig. 1. The intermediate vector pNK1. H, HindIII; E, EcoRI; Bg, BglII; B, BamHI; Amp^R, ampicillin resistance gene; Knop, a chimeric gene containing the promoter and terminator region of the nopaline synthase and APH(3')II coding sequences; H23, a HindIII fragment 23 obtained from EcoRI fragment 1 of pTiC58 cloned in pGV0415 (Depicker *et al.* 1980). NOS; nopaline synthase gene; B19, a BamHI fragment 19 of an octopine type Ti plasmid (pTiB6-806) containing genes coding for part of octopine synthase (ocs) and a *tml* product (Garfinkel *et al.* 1981); wavy line, the right border region of pTiC58.

generated by the restriction endonuclease digestion.

Conjugation of bacterial cells: Equal volumes of overnight culture of *E. coli* GJ23 and *E. coli* C600 harboring the intermediate vector (pNK1) were mixed and plated on solid LB medium containing 20 µg/ml ampicillin and 10 µg/ml tetracycline. After incubation at 37°C overnight, *E. coli* colonies resistant to antibiotics were selected. An equal volume of overnight cultures of *E. coli* containing R64drd11, pGJ28 and pNK1, and *A. tumefaciens* possessing Ti plasmid were also mixed and plated on solid LB medium with 100 µg/ml carbenicillin and 25 µg/ml chloramphenicol. Finally, colonies of *A. tumefaciens* carrying the Ti plasmid co-integrated with the intermediate vectors were selected.

Tumor induction on tobacco seedlings: Induction and culture of crown gall tumor followed the method described by Leemans *et al.* (1981). Surface-sterilized tobacco seeds (*N. glauca*) were sown in a test tube containing Murashige and Skoog medium (1962) solidified with 0.8% agar but lacking phytohormones (MS-H). After 4–6 weeks, tobacco plants were decapitated and inoculated with *A. tumefaciens* containing the recombinant Ti plasmid. After 2–3 weeks, tumors were excised and transferred to MS-H containing cefotaximum (Claforan, Hoechst) at 500 µg/ml for disinfection. Bacteria-free transformants were subsequently obtained by the serial subculture of tumors in the same medium.

Detection of opines: A sample of tumor tissue (approximately 10 mg) was incubated in a liquid MS-H containing 0.1 M arginine overnight at 28°C. The tissue was placed on Whatman 3 MM paper and squeezed with a spatula. After drying the Whatman paper, electrophoresis was carried out according to the method of Otten and Schilperoort (1978).

Bacterial DNA: Plasmid DNA from *E. coli* was prepared from overnight cultures by the rapid alkaline procedure of Birnboim and Doly (1979). Total DNA from *A. tumefaciens* was isolated according to Kado and Liu (1981).

Plant DNA: Tissues were frozen in a liquid nitrogen and ground in a coffee mill. Preparation of plant DNA followed the method described by Paskowski *et al.* (1984).

Southern hybridization: DNA preparations digested with restriction endonuclease(s) were applied to a gel containing 0.7% agarose, 40 mM Tris-HCl (pH 7.8), 20 mM Na-acetate and 2 mM EDTA. Following electrophoresis at 25 V for 16 hr, the gel slab was stained with ethidium bromide and photo-

graphed. The DNA in the gel slab was transferred to a nitrocellulose filter (Southern 1975). The filter was baked at 80°C for 2 hr, and the DNA was hybridized with a ³²P-labelled DNA probe prepared by nick-translation (Wahl *et al.* 1979). Finally, an autoradiographic image of the blot hybridization was obtained.

Preparation of RNA: Total RNA and poly(A⁺)RNA was prepared according to the modified method of Chirgwin *et al.* (1979), Glisin *et al.* (1974) and Aviv *et al.* (1972). Tissues were frozen in a liquid nitrogen and ground with a mortar and pestle. Frozen powder was rapidly mixed with an extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroyl sarcosine, 0.1 M 2-mercaptoethanol, 0.1% Sigma antiform A). After removing cell debris by centrifugation, cleared extracts were gently laid on CsCl cushion (5.7 M CsCl, 0.1 M EDTA, pH 7.0) in a centrifuge tube. After 22 hr at 25,000 rpm with SW 28.1 Ti, the pellet was suspended in a buffer containing 7.5 M guanidinium hydrochloride, 25 mM sodium citrate (pH 7.0), 5 mM dithiothreitol. The total RNA was precipitated with cold ethanol and washed twice with 70% ethanol. To prepare poly(A⁺)RNA, total RNA was redissolved in a buffer (0.5 M NaCl, 0.01 M Tris-HCl pH 7.6), and passed through an oligo (dT)-cellulose column. Poly(A⁺)RNA was thus eluted with a buffer containing 0.01 M Tris-HCl (pH 7.6) and stored in liquid nitrogen.

Northern hybridization: RNA was applied to a gel containing 1.5% agarose, 2.2 M formaldehyde, 0.04 M morpholinopropanesulfonic acid, 10 mM sodium acetate, and 1 mM EDTA (pH 7.0) (Maniatis *et al.* 1982). Following electrophoresis at 25 V for 13 hr, the RNA in the gel slab was transferred to Gene Screen (NEN) paper. The filter was baked at 80°C for 2 hr, and the RNA was hybridized with a ³²P-labelled DNA probe prepared by nick-translation (Wahl *et al.* 1979).

Enzymes: Enzymes were products of Takara Shuzo Company (Kyoto, Japan), and used according to methods outlined on the data sheets provided by the supplier.

3. RESULTS

General observation of transformants: Overnight cultured *A. tumefaciens* was inoculated onto the decapitated stem of plants. After 2-4 weeks, the gall was excised and cultured in a medium containing cefotaximum. Analysis of the production of opines in 22 transformants obtained by pTiB6S3::pNK1 indicated that 13 of them produced both octopine and nopaline, while the others produced nopaline only. The transformants induced by pTiB6S3::pNK1

were subjected to kanamycin resistance tests. Transformants were able to grow in the medium containing 400 $\mu\text{g/ml}$ kanamycin, whereas wild-type transformants were not.

DNA analysis: In order to confirm the integration of foreign DNA into

Fig. 2. An autoradiogram of Southern blot hybridization of DNA prepared from callus transformed by *A. tumefaciens* harboring (a) pGV2215::pNK1 and (b) pGV2215. The DNA was digested with a mixture of *EcoRI* and *HindIII* (a, b). The *EcoRI*-*HindIII* digested DNA (pKC7KNH23) was used as one (c) and ten (d) copy reconstruction. The pKC7KNH23 also served as a probe. Numerals indicate location of *HindIII* fragment 23 (3.4 Kb), a sequence containing *APH(3')II* (1.6 Kb) and pKC7 (5.8 Kb) deduced from the physical map of the pGV2215::pNK1 plasmid. In the bottom of this figure, possible manner of foreign DNA integration into plant genome is presented. Numerals indicate size of each fragment in Kb. *Knop*; a chimeric gene containing *APH(3')II*, *NOS*; nopaline synthase gene, *OCS*; octopine synthase gene. Waved lines (A and B) indicate right and left border of T-DNA of pGV2215, respectively. Waved line C is the border sequence of T-DNA derived from nopaline type Ti plasmid, pTiT37. Dotted line is possible genomic DNA of host plant.

the Ti plasmid and plant cells, DNAs isolated from transformants were digested with restriction endonuclease(s), and subjected to Southern hybridization analysis. The recombinant Ti plasmid in *A. tumefaciens* contained co-integrated foreign DNA (data not presented). In the case of the transformants obtained by pGV2215::pNK1, EcoRI-HindIII digested DNA resulted in the production of HindIII fragment 23 (3.4 Kb) and the sequence containing Knop (1.6 Kb) (Fig. 2). The number of foreign molecules in this transformant was estimated to be about 5 copies per genome. Similar results were also obtained in the transformant containing pTiB6S3::pNK1 (data not shown).

Analysis of transcripts—Nopaline synthase (NOS) and APH(3')II: The level of foreign gene expression was analysed by the dot blot hybridization of poly(A)⁺RNA using specific DNA probe. As shown in Fig. 3, both NOS and APH(3')II mRNA were expressed in callus transformed by pGV2215::pNK1. However, the level of expression was higher in NOS than APH(3')II. Control samples did not show any hybridization. Northern blot analysis indicated that the size of transcripts for NOS and APH(3')II was 1550 and 1250 bases, respectively (Fig. 4). Roots derived from transformed callus by pGV2215::pNK1 did not produce foreign gene transcripts.

Fig. 3. Autoradiograms of dot hybridization of poly(A)⁺RNA prepared from cells transformed by *A. tumefaciens* harboring (a) pGV2215::pNK1 and (b) pGV2215. Yeast tRNA was used to determine nonspecific hybridization (c). (A) HindIII fragment 23 containing nopaline synthase gene and (B) BglII-SmaI fragment from pKC7 containing APH(3')II coding sequence were used as probe.

Fig. 4. Autoradiograms of Northern blot hybridization of poly(A)+RNA prepared from (a) callus transformed by *A. tumefaciens* harboring pGV2215::pNK1 and (b) roots originated from transformed callus (pGV2215::pNK1), and (c) roots of untransformed *N. glauca*. (A) HindIII fragment 23 containing nopaline synthase gene, and (B) BglII-SmaI fragment pKC7 containing *APH(3')II* coding sequence were used as probe. Numerals indicate bases.

T-DNA genes: Using a probe DNA containing major transcripts of T-region of the Ti plasmid, a similar northern analysis was carried out. The result presented in Fig. 5 indicated that, callus (pGV2215::pNK1 transformants) expressed transcripts 3, 4, 6a and 6b (Willmitzer *et al.* 1983). No such transcripts were found in roots originated from callus containing pGV2215::pNK1.

4. DISCUSSION

There have been several reported instances where integration and expression of foreign DNA in higher plants have been demonstrated with the aid of the Ti plasmid (Bevan *et al.* 1983a; Broglie *et al.* 1984; Fraley *et al.* 1983; Herrera-Estrella *et al.* 1983a, b, 1984; Horsch *et al.* 1983; Murai *et al.* 1983). The expression of chimeric genes such as *APH(3')II* would be the most useful marker for the selection of putative transformants (Bevan *et al.* 1983a, 1984; Fraley *et al.* 1983; Herrera-Estrella *et al.* 1983b). The objective of this study was to confirm the expression of two marker genes, nopaline synthase and a chimeric gene consisting of the promoter and the terminator regions of nopaline synthase and the *APH(3')II* coding region in *N. glauca* transformants. There-

Fig. 5. An autoradiogram of Northern blot hybridization of poly(A)⁺RNA prepared from (a) callus transformed by *A. tumefaciens* harboring pGV2215::pNK1 and (b) roots originated from transformed callus (pGV2215::pNK1), and (c) roots of untransformed *N. glauca*. A HindIII-EcoRI fragment (6.2 Kb) derived from octopine type Ti plasmid pTiB6-806 was used as probe. Numerals indicate number of transcripts (Willmitzer *et al.* 1983).

fore, several galls were obtained from decapitated stem by infecting *A. tumefaciens* harboring recombinant Ti plasmid. Nopaline was detected in every galls induced. These transformants expressed kanamycin resistance and produced APH(3')II enzyme identical to bacterial one. In transformants obtained by pGV2215 (Ti plasmid)::pNK1, about 5 copies of foreign genes were detected. On the basis of these observations, we were prompted to analyse the expression of foreign DNA at the transcriptional level.

Regarding the size of transcripts, Bevan *et al.* (1983b) reported that NOS possessed mRNA of 1550-1600 bases. Nevertheless, the precise size of a chimeric APH(3')II transcript has not been determined yet. As we demonstrated here, the size for APH(3')II transcript should be 1250 bases, which presumably covers from the transcription initiation site to the polyadenylation signal of a chimeric gene (Bevan 1984).

The results of dot hybridization may suggest that NOS gene was expressed higher (about 50 fold) than APH(3')II gene. This conclusion was based on the calculation of specific radioactivity of probes and the exposure length for autoradiography. Assuming the similar copy number of both genes, this difference may be attributed to the difference in the structure of two genes. We used intact NOS gene possessing 1.4 kb upstream from initiation codon

including right border region of nopaline type Ti plasmid (Bevan *et al.* 1983b; Depicker *et al.* 1982), whereas chimeric kanamycin resistant gene contained promoter region (~ 310 bp) of nopaline synthase. In this respect, Koncz *et al.* (1983) and Shaw *et al.* (1984) found promoter activity within ~ 310 bp region upstream from initiation codon. However, there had not been presented any evidence concerning the enhancer like property of the upstream region (1.1 kb) from the right border sequence of nopaline type Ti plasmid. Our result suggests that this region might be used for the transcriptional activation of a neighboring gene. Another reason for the different level of gene expression would be due to the chimeric construction of kanamycin resistant gene. In this respect, Jones *et al.* (1985) pointed out that the level of transcripts in the cnimeric gene obtained by transcriptional fusion was lower than that of endogenous gene. Further study would be needed to confirm these conclusions.

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REFERENCES

- AVIV, H. and LEDER, P. (1972) Purification of biologically active globin mRNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. (USA)* **69**, 1408-1412.
- BEVAN, M. W., FRAVELL, R. B. and CHILTON, M.-D. (1983a) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304**, 184-187.
- BEVAN, M., BARNES, W. M. and CHILTON, M.-D. (1983b) Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res.* **11**, 263-385.
- BEVAN, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* **12**, 8711-8721.
- BIRNBOIM, H. C. and DOLY, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513-1523.
- BROGLIE, R., CORUZZI, G., FRALEY, R. T., ROGERS, S. G., HORSCH, R. B., NIEDERMEYER, J. G., FINK, C. L., FLICK, J. S. and CHUA, N.-H. (1984) Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plant cells. *Science* **224**, 838-843.
- CHILTON, M.-D., DRUMMOND, M. H., MERLO, D. J., SCIACKY, D., MONTAYA, A. L., GORDON, M. P. and NESTER, E. W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**, 263-271.
- CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, J. and RUTTER, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. **18**, 5294-5299.
- COHEN, S. N., CHAN, A. C. Y. and HSU, I. (1972) Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. (USA)* **69**, 2110-2114.
- DEPICKER, A., DE WILDE, M., DE VOS, G., DE VOS, R., VAN, MONTAGU, M. and SCHELL, J. (1980) Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. *Plasmid* **3**, 193-211.
- DEPICKER, A., STACHEL, S., DHAESE, P., ZAMBRYSKI, P. and GOODMAN, H. M. (1982) Nopaline

- synthase: Transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* **1**, 561-573.
- FRALEY, R. T., ROGERS, S. G., HORSCH, R. B., SANDERS, P. R., FLICK, J. S., ADAMS, S. P., BITTNER, M. L., BRAND, L. A., FINK, C. L., FLY, J. S., GALLUPPI, G. R., GOLDBERG, S. B., HOFFMANN, N. L. and WOO, S. C. (1983) Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. (USA)* **80**, 4803-4807.
- GARFINKEL, D. J., SIMPSON, R. B., REAM, L. W., WHITE, F. F., GORDON, M. P. and NESTER, E. W. (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. *Cell* **27**, 143-153.
- GLISIN, V., CRKVENJAKOV, R. and BYUS, C. (1974) Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**, 2633-2637.
- HERRERA-ESTRELLA, L., DEPICKER, A., VAN MONTAGU, M. and SCHELL, J. (1983a) Expression on chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* **303**, 209-213.
- HERRERA-ESTRELLA, L., DE BLOCK, M., MESSENS, E., HERNALSTEENS, J. P., VAN MONTAGU, M. and SCHELL, J. (1983b) Chimeric genes as dominant selectable markers in plant cells. *The EMBO J.* **2**, 987-995.
- HERRERA-ESTRELLA, L., VAN DEN BROECK, G., MAENHAUT, R., VAN MONTAGU, M., SCHELL, J., TIMKO, M. and CASHMORE, A. (1984) Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. *Nature* **310**, 115-120.
- HORSCH, R. B., FRALEY, R. T., ROGERS, S. G., SANDERS, P. R., LLOYD, A. and HOFFMANN, N. (1983) Inheritance of functional foreign genes in plants. *Science* **223**, 496-498.
- JONES, J. D. G., DUNSMUIR, P. and BEDBROOK, J. (1985) High level expression of introduced chimaeric genes in regenerated transformed plants. *The EMBO J.* **10**, 2411-2418.
- KADO, C. I. and LIU, S.-T. (1981) Rapid procedure for detection and isolation of large and small plasmids. *J. of Bacteriol.* **145**, 1365-1373.
- KONCZ, C., DE GREVE, H., ANDRE, D., DEBOECK, F., VAN MONTAGU, M. and SCHELL, J. (1983) The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants. *The EMBO J.* **2**, 1597-1603.
- LEEMANS, J., SHAW, C., DEBLAERE, R., DE GREVE, H., HERNALSTEENS, J. P., MAES, M., VAN MONTAGU, M. and SCHELL, J. (1981) Site-specific mutagenesis of *Agrobacterium* Ti plasmids and transfer of genes to plant cells. *J. Mol. Appl. Genet.* **1**, 149-164.
- LEEMANS, J., DEBLAERE, R., WILLMITZER, L., DE GREVE, H., HERNALSTEENS, J. P., VAN MONTAGU, M. and SCHELL, J. (1982) Genetic identification of function of TL-DNA transcripts in octopine crown galls. *The EMBO J.* **1**, 147-152.
- MANIATIS, T., FRITSH, E. F. and SAMBROOK, J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- MCPHERSON, J. C., NESTER, E. W. and GORDON, M. P. (1980) Protein encoded by *Agrobacterium tumefaciens* Ti plasmid DNA (T-DNA) in crown gall tumors. *Proc. Natl. Acad. Sci. (USA)* **77**, 2666-2670.
- MURAI, N., SUTTON, D. W., MURRAY, M. G., SLIGHTOM, J. L., MERLO, D. J., REICHERT, N. A., SENGUPTA-GOPALAN, C., STOCK, C. A., BARKER, R. F., KEMP, J. D. and HALL, T. C. (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. *Science* **222**, 476-482.
- MURASHIGE, T. and SKOOG, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.
- OTTEN, L. A. B. M. and SCHILPERROOT, R. A. (1978) A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochim. Biophys. Acta* **527**, 497-500.
- PASZKOWSKI, J., SHILLITO, R. D., SAUL, M., MANDAK, V., HOHN, T., HOHN, B. and POTRYKUS, I. (1984) Direct gene transfer to plants. *The EMBO J.* **3**, 2717-2722.
- PETIT, A., TEMPE, J., KERR, A., HOLSTERS, M., VAN MONTAGU, M. and SHELL, J. (1978) Substrate induction of conjugative activity of *Agrobacterium tumefaciens*. *Nature* **271**, 570-572.
- RAO, R. N. and ROGER, S. G. (1979) Plasmid pKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. *Gene* **7**, 79-82.

- SHAW, C. H., CARTER, G. H., WATSON, M. D. and SHAW, C. H. (1984) A functional map of the nopaline synthase promotor. *Nucleic Acids Res.* **12**, 7831-7846.
- SOUTHERN, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.
- VAN HAUTE, E., JOOS, H., MAES, M., WARREN, G., VAN MONTAGU, M. and SCHELL, J. (1983) Intergenetic transfer and exchange recombination of restriction fragments cloned in pBR 322: a novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. *The EMBO J.* **2**, 411-417.
- WAHL, G. M., STEEN, M. and STARK, G. R. (1979) Efficient transter of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. (USA)* **76**, 3683-3687.
- WILLMITZER, L., DHAESE, P., SCHREIER, P. H., SCHMALENBACH, W., VAN MONTAGU, M. and SCHELL, J. (1983) Size, location and polarity of T-DNA-encoded transcripts in nopaline crown gall tumors: common transcripts in octopine and nopaline tumors. *Cell* **32**, 1045-1056.