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Effect of repeated DNA sequences on direct gene transfer in protoplasts of *Nicotiana plumbaginifolia*

M.L. Marchesi [1], S. Castiglione [1] and F. Sala [1, 2, *]

[1] Department of Genetics and Microbiology, University of Pavia, Via S. Epifanio 14, I-27100 Pavia, Italy
[2] Institute of Botany, University of Parma, Italy

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Summary. Highly repeated nuclear DNA sequences from leaves of *Nicotiana plumbaginifolia* were cloned in pBR322 and tested for their effect on direct gene transfer in protoplasts of the same organism. Protoplasts were prepared from suspension cultures and were incubated in the presence of the plasmid pHP23 carrying the kanamycin resistance gene *APH(3')II* and in the presence of the plasmids carrying the cloned sequence. DNA uptake was induced by a polyethyleneglycol (PEG) treatment. Out of the 22 tested clones, 3 significantly stimulated the frequency of appearance of transformed colonies. DNA was extracted from some of the kanamycin-resistant calli obtained by co-transformations. Dot-blots have shown that the stimulatory effect on transformation frequency is often accompanied by a consistent increase in integrated genes sequences.

Key words: *Nicotiana plumbaginifolia* – Protoplasts – Gene transfer – Repeated DNA sequences

Introduction

Techniques for direct gene transfer from bacterial plasmids to protoplasts have already been described for several species (Paszkowski et al. 1984; Lörz et al. 1985; Potrykus et al. 1985). In these cases it has been shown that, following appropriate treatments to the protoplasts, one or more copies of the functional gene, as well as gene fragments, are integrated at different sites of the genome. Integration is apparently at random and is transmitted to the cell progeny and to the regenerated plants.

The transformation protocols have recently been simplified to a mere treatment with PEG (Negrutiu et al. 1987; Sala et al. 1989). Co-transformation has also been shown to occur at high frequency when treating protoplasts with a mixture of two plasmids carrying different genes (Schoecher et al. 1986), thus suggesting that an interaction may occur between the two DNA molecules before or after their uptake by the protoplast. On the other hand, we still have no information on the mechanism of gene integration and on the integration site: this, as well as the number of inserted gene copies or gene fragments, appears to occur at random. Recently, Paszkowsky et al. (1988) have shown that homologous recombination may be the clue to gene targeting.

In this paper, we report the results of experiments in which repeated DNA sequences cloned from genomic DNA of *N. plumbaginifolia* were tested for their effect on direct gene transfer in protoplasts of the same organism when used in co-transformation with a selectable gene. The rationale was that these DNA sequences, which are homologous to large portions of the DNA of *N. plumbaginifolia*, may positively affect the extent of integration of the foreign gene and, perhaps, direct it to specific sites.

Materials and methods

Materials

Calf thymus DNA and kanamycin were from Sigma. BamHI, EcoRV, NdeI were from BRL. EcoRI and ligase were from Boehringer. Cellulase RS and Macerozyme R10 were from Yakult Biochemicals. Polyethyleneglycol (PEG) 6000 was from Merck. Plasmid pHP23, containing a gene coding for neomycin phosphotransferase *(APH(3')II)* under the control of the CaMV 35S-promoter, was kindly supplied by I. Paszkowsky (ETH-Zentrum, Zurich). Plasmid pBG35, containing a flax ribosomal DNA sequence (Goldsbrough and Cullis 1981), was kindly supplied by V. Walbot (Stanford University USA).

* To whom correspondence should be addressed

Protoplast preparation

Protoplasts of *N. plumbaginifolia* were prepared either from sterile shoot cultures (Negrutiu et al. 1986) or from suspension-cultured DOBA cells harvested in the exponential phase of growth (Sala et al. 1989). DOBA cells were obtained from R. Shields (Unilever Research Laboratories, Bedford) and subcultured as described by Barfield et al. (1985). Protoplasts were prepared overnight at 26 °C in the dark in the presence of 1% cellulase RS and 1% macerozyme R10 dissolved in K3 medium containing 0.4 *M* sucrose (for leaves) or in MS medium containing 0.4 *M* mannitol and 1.0 m*M* MES (for cultured cells) (Sala et al. 1989), filtered with a 63 μ mesh and floated (leaf protoplasts) or sedimented (cultured cells). After two washes in W5 (154 m*M* NaCl, 125 m*M* $CaCl_2 \cdot 2H_2O$, 5 m*M* KCl, 5 m*M* glucose, pH 5.6), protoplasts were used for cloning of repeated sequences (leaf protoplasts) or for transformation experiments (DOBA protoplasts).

DNA transformation of protoplasts

Protoplasts were resuspended at 1.5×10^6/ml in the W5 salt solution containing 15 m*M* $MgCl_2$. Aliquotes of 0.2 ml were supplemented with, in order, calf thymus DNA (50 μg/ml) and pHP23 (10 μg/ml) linearized with NdeI. When indicated, plasmids carrying the cloned *N. plumbaginifolia* sequences (linearized with BamHI) were added at 20 μg/ml. Then, 72% PEG was added to a final concentration of 24% and the mixture was incubated for 20 min at 22 °C. Incubation was stopped by adding 12 volumes of the W5 salt solution and pelleting at low speed.

Protoplasts were resuspended at 3×10^4/ml in K3 liquid medium (Sala et al. 1989) containing 0.4 *M* glucose and transferred to petri dishes. Incubation was at 26 °C in the dark. After 8 days, colonies were resuspended at 3×10^3 colonies/ml in K3 medium containing 0.4 *M* glucose + 0.8% agar and transferred, at 3 ml aliquotes, to the center of petri dishes. After agar had solidified, 12 ml of K3 liquid medium containing 0.4 *M* glucose and 50 μg/ml kanamycin were added.

Incubation was at 26 °C in the dark. Liquid medium was replaced after 10 days with K3 medium containing 0.2 *M* glucose and 50 μg/ml kanamycin. Visible colonies were scored after an additional 10-day incubation.

Cloning of repeated DNA sequences

Isolation of nuclei from leaf protoplasts, lyses, DNA extraction and purification from RNA and proteins was essentially as described (Castiglione et al. 1985; Zheng et al. 1987). The DNA preparation was digested with BamHI and ligated to BamHI-digested pBR322 in a 1:1 molar ratio, as described by Evans et al. (1983). The recombinant plasmid mixture was used to transform *Escherichia coli* (strain HB101) as described by Cohen et al. (1972).

Extraction and purification of plasmid DNA

Rapid isolation of plasmid DNA from *E. coli* was performed with the rapid alkaline extraction procedure described by Birnbaim and Doly (1979). DNA concentration in purified preparations was determined by measuring the optical density at 260 nm (1.0 optical density unity corresponding to 50 $\mu g/ml^{-1}$).

Quantification of gene copy number

The quantity of base pairs homologous to gene sequences integrated into genomic DNA has been calculated by counting radioactivity fixed in the dot-hybridizations of Fig. 3. Serial dilutions of the EcoRI 1.7-kb fragment of pHP23, spotted and hybridized with the homologous fragment, were used as standard.

Restriction endonuclease digestion and agarose gel electrophoresis

Restriction endonuclease digestions were performed at 37 °C for 2–3 h with 2.5 units of enzyme per μg DNA in the buffer recommended by the supplier. Proteins were then eliminated by a phenol-chloroform (1:1 by vol.) extraction, followed by a chloroform extraction.

Horizontal agarose gels were run in TBE (89 m*M* TRIS-borate, 89 m*M* boric acid and 2 m*M* EDTA) with agarose at 0.7% or 0.8% (w/v). Electrophoresis was at 70 V for 1–2 h.

Labelling of DNA, colony hybridization, dot-blot hybridization and Southern blotting

These were performed with standard techniques as previously described (Zheng et al. 1987).

Results

Cloning in pBR322 of highly repeated nuclear DNA sequences from the genome of N. plumbaginifolia

DNA was isolated from nuclei of aseptically grown young leaves of *N. plumbaginifolia*, digested with BamHI and ligated to BamHI-digested pBR322. The recombinant plasmid mixture was then used to transform *E. coli*, and 384 bacterial clones containing plasmids with an insert were randomly selected. Out of these, 22 clones harboring plasmids with highly repeated plant DNA sequences were identified following colony hybridization with ^{32}P-labelled genomic DNA from *N. plumbaginifolia*.

The dot-blot hybridizations of the cloned DNA molecules with ^{32}P-labelled genomic DNA from *N. plumbaginifolia* shown in Fig. 1 A give an appreciation of the extent of their repetition in the plant genome. Although quantitative data were not produced, the intensity of the radioactive signal shows that the majority of the tested sequences is considerably more abundant in the plant genome than a ribosomal DNA sequence used as control. The inserts range in dimension from 1.5–2.0 kbp to 6.0–7.0 kbp. Plasmid rearrangements have occurred in some cases.

The Southern blots of Fig. 2 show the distribution of the selected repeated sequences on the DNA of *N. plumbaginifolia* after electrophoresis of a BamHI digest. The results suggest that most sequences are interspersed in the genome.

Effect of the cloned sequences on the frequency of protoplast transformation

The cloned repeated DNA sequences were tested for their effect on direct gene transfer in protoplasts of *N. plumbaginifolia*.

To this purpose, protoplasts prepared from suspension cultures were supplemented with the linearized plas-

mids and incubated in the presence of 24% PEG as described in 'Materials and methods'. Co-transformations were with pHP23, carrying the selectable gene, and with each of the 22 cloned plasmids. pHP23 bears the *APH(3')II* gene coding for neomycin phosphotransferase, under the control of the 35S promoter of gene VI of cauliflower mosaic virus. This gene confers resistance to kanamycin and has no sites of homology on the plant genome (Paszkowsky et al. 1988). The results of typical co-transformation experiments in the presence of the 22 cloned repeated sequences are shown in Table 1: some of the cloned plasmids positively affected the frequency of transformation, some were ineffective while others considerably depressed this value. Controls showed that these effects were not due to pBR322 itself or to the presence of calf thymus DNA.

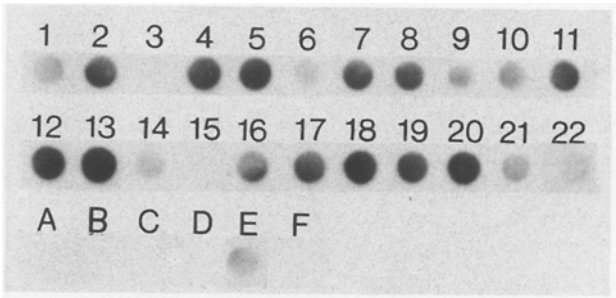

Fig. 1 A–F. Dot-blot hybridization of the cloned DNA molecules with ^{32}P-labelled nuclear DNA from *N. plumbaginifolia*. 1.0 µg of each plasmid preparation was bound to the nitrocellulose filter and hybridized with labelled DNA (1.0×10^7 dpm of a probe at 1.6×10^9 dpm µg^{-1}). Plasmids (containing *N. plumbaginifolia* repeated DNA sequences) were: *1* pAA7; *2* pAB11; *3* pAD3; *4* pAD5; *5* pAE5; *6* pBA12; *7* pBB3; *8* pBBd10; *9* pC12; *10* pBD2; *11* pBD5; *12* pBF2; *13* pBF11; *14* pBG6; *15* pBH6; *16* pBH7; *17* pCF1; *18* pCG1; *19* pDA3;k *20* pDC2; *21* pDC9; *22* pDE9. Controls containing moderately repeated or single copy sequences were: **A** pAH9; **B** pAH10; **C** pAH12; **D** pAH11; **E** pBG35 (containing a flax ribosomal DNA sequence); **F** pBR322

The peculiar effect of each insert in pBR322 turned out to be reproducible in three repetitions of the experiments of Table 1, although quantitative variations occurred in independent experiments. Also the efficiency of transformation in the controls was found to change with different protoplast preparations.

In the case of pBF2, the most effective plasmid, five independent experiments, with different protoplast preparations, gave stimulations of 3.4, 3.7, 5.2, 19.7 and 20.1 times over the control without pBF2. In all repetitions plasmids pBB3 and pBD5, besides pBF2, turned out to be the most effective enhancers of frequency of transformation.

The three plasmids are very different constructs. An insert of about 5.5 kbp is present in pBB3, plasmid pBD5 is a rearranged molecule with an insert of about 2.5 kbp while two inserts of about 3.0 and 4.0 kbp are present in pBF2.

Integration of the APH(3')II gene sequences into the DNA of transgenic cells

Plasmids pBF2, pBD5 and pBB3, which increase the number of transformed colonies, may also affect the frequency of integration of the foreign sequence. To verify this, some of the colonies transformed with pHP23 in the presence of pBF2, pBD5 or pBB3 were grown to produce calli. DNA was extracted and probed, with an EcoRI 1.7-kbp fragment of pHP23 containing the complete *APH(3')II* gene sequence.

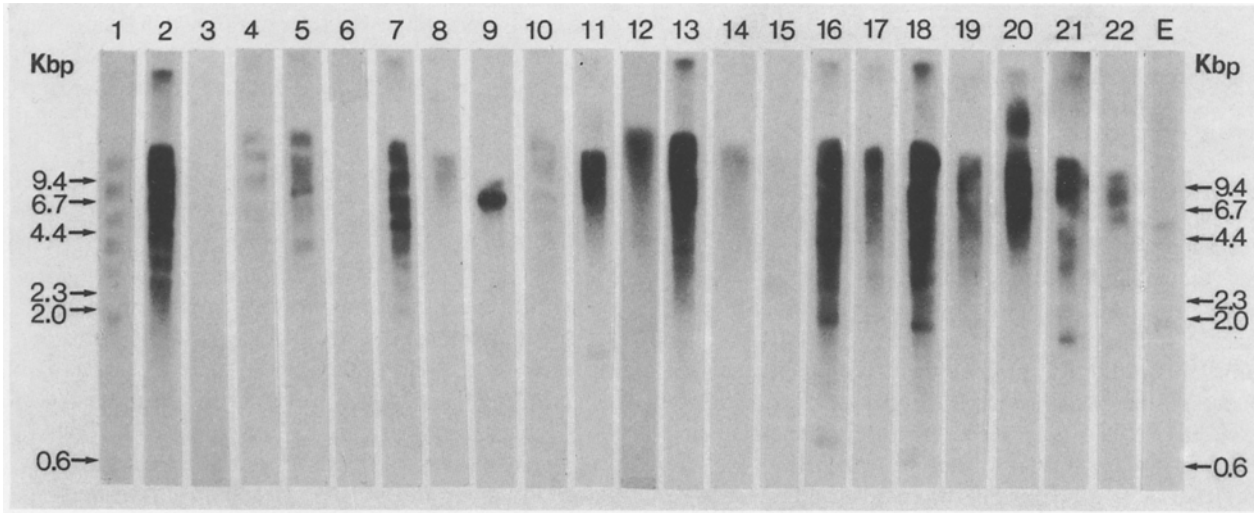

Fig. 2. Southern blots of total DNA of *N. plumbaginifolia* (2.0 µg per lane) after digestion with BamHI and electrophoresis on a 0.7% agarose gel. Blots were hybridized with the ^{32}P-labelled plasmid DNAs. From *lanes 1–22*, plasmids were as listed in Fig. 1. *E*–pBG35 (containing a flax ribosomal DNA sequence). Markers were produced by HindIII digestion of lambda phage DNA

Table 1. Frequency of transformation of *N. plumbaginifolia* protoplasts upon addition of cloned repeated DNA sequences from the same organism. Transformation conditions were as described in 'Materials and methods'. The complete transformation mixture (0.2 ml) contained the W5 salts, supplemented with 15 m*M* $MgCl_2$, 3.10^5 protoplasts, 50 μg/ml calf thymus DNA, 10 μg/ml linearized pHP23 and 24% PEG-6000. Additions or deletions were as described in the table. Cloned repeated DNA sequences were linearized with BamHI and added at 20 μg/ml. pHP23 was linearized with NdeI. Colonies transformed for kanamycin resistance were scored after 4 weeks

Exp. no.	Additions (or deletions) to the complete mixture	Transformants	
		Total no.	% of the complete mixture
	Co-transformations		
1	+pAA7	15	53
2	+pAB11	10	36
3	+pAD3	31	111
4	+pAD5	21	75
5	+pAE5	26	93
6	+pBA12	43	153
7	+pBB3	73	260
8	+pBB10	26	93
9	+pBC12	28	100
10	+pBD2	24	86
11	+pBD5	95	340
12	+pBF2	148	521
13	+pBF11	60	214
14	+pBG6	62	221
15	+pBH6	18	64
16	+pBH7	12	43
17	+pCF1	31	111
18	+pCG1	32	114
19	+pDA3	12	43
20	+pDC2	22	78
21	+pDC9	14	50
22	+pDE9	56	200
	Controls		
23	(complete mixture)	28	100
24	−pHP23	0	0
25	−calf thymus DNA	2	7
26	+pBR322 (10 μg/ml)	20	71
27	+calf thymus DNA (20 μg/ml)	24	86

The dot-blot hybridizations of Fig. 3 A show that, at least in some cases, the integration of sequences of the selectable genes is considerably higher than that in the controls (calli transformed in the presence of pHP23 alone). Numbers under the spots are an estimation of copies of the foreign gene (or, more precisely, of gene equivalents, totalling complete and incomplete gene sequences) integrated into the host genome. For instance, 52.8 gene copies were found integrated in callus 1 obtained following co-transformation with pBB3.

The DNA preparations were also digested with EcoRI or with EcoRV, electrophoresed on agarose gel, blotted and hybridized with the same radioactive probe. The autoradiographs of Fig. 3 B show that the integration of gene sequences is at a number of discrete sites with a pattern that is specific for each transformant.

Lanes with DNA digested with EcoRI (left) show hybridization with the 1.7-kbp EcoRI fragment in regions of high molecular weight, thus suggesting rearrangements of the sites that produced the probe. From 3 to 6 radioactive bands are observed in the three calli transformed with pHP23, while the number of discrete bands increases with the increased gene sequence integration observed in some co-transformants: from 10 to 15 bands can be counted in lanes h, l and p. A similar situation, but with far better resolution, is found in a parallel experiment with cellular DNA digested with EcoRV (right in Fig. 3 B).

Discussion

Strategies to increase the relatively low transformation frequency of direct gene transfer to *N. plumbaginifolia* by using optimized Mg^{++} and PEG concentrations have already been described (Negrutiu et al. 1987; Sala et al. 1989). We now present evidence that the addition to the transformation mixture of selected highly repeated DNA sequences cloned from *N. plumbaginifolia* may further increase this frequency.

In a screening for their capacity to affect stable genetic transformation of protoplasts, we have in fact noticed that some of the cloned repeated sequences have strong stimulatory effect, others are neutral or inhibitory.

We have also demonstrated that the stimulatory effect may be accompanied by a significant increase in the amount of integrated sequences. This was the case in some but not all transgenic cell lines. In fact, there was considerable variability in the frequency of integration of *APH(3')II* gene sequences in the different transformants obtained from a single transformation experiment. This was not unexpected, since the transformation of each protoplast is an independent event and, furthermore, co-transformation may not have occurred at the same extent and in all cases.

The mechanism by which the selected repeated sequences affect protoplast transformation remains to be verified. It appears to be different from that described by Meyer et al. (1988) who found that a 2-kbp fragment isolated from petunia improves the transformation frequency of petunia and tobacco protoplasts when inserted upstream of the selectable marker gene. In this case, a super-coiled plasmid DNA was used and a single copy of the foreign gene was preferentially integrated. Thus, it was postulated that the insert either might guide the plasmid to a chromosomal location convenient for transcription of the neighboring gene or that it may confer an open chromatin structure which would facilitate transcriptional activity.

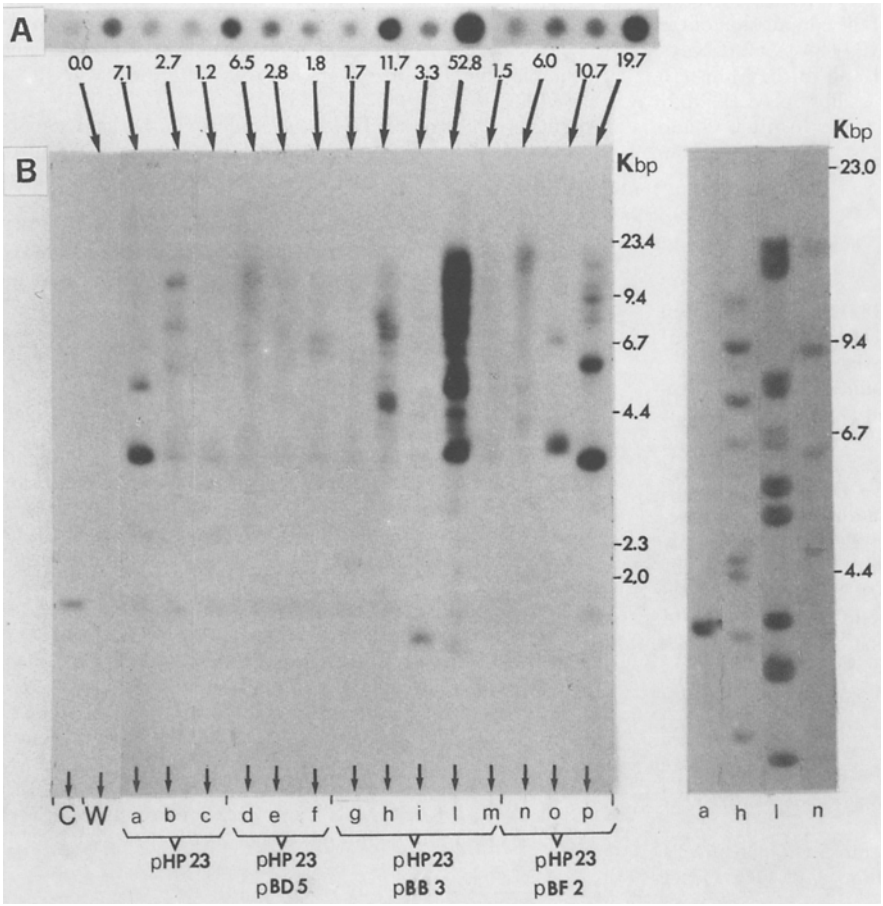

Fig. 3A–C. Quantitative **A** and qualitative **B** evaluation of the integration of *APH(3')II* gene sequences into the DNA of transgenic calli by using a ^{32}P-labelled EcoRI 1.7-kb fragment of pHP23 containing the resistance gene. In **A**: dot-blot hybridization of DNA from different callus cultures of *N. plumbaginifolia* with the ^{32}P-labelled EcoRI 1.7-kbp fragment of pHP23. DNA preparations (0.5 µg/dot) were from: parental cells (*W*), three transgenic lines transformed with pHP23 (*a, b, c*) three co-transformants with pHP23 and pBD5 (*d, e, f*), five co-transformants with pHP23 and pBB3 (*g, h, i, l, m*) and three co-transformants with pHP23 and pBF2 (*n, o, p*). After autoradiography, spots were utilized for radioactivity counts. *Numbers* under the spots refer to the number of gene equivalents homologous to the probe. In **B**: Southern blots of total DNA prepared from the different callus cultures of transgenic *N. plumbaginifolia* (from a to p) and from the non-transformed cells (W). **C** is a Southern blot of the 1.7-kbp fragments of pHP23. DNA preparation were digested with EcoRI (left) or EcoRV (right). 2.0 µg were electrophoresed on a 0.7% agarose gel and blots were hybridized with the ^{32}P-labelled EcoRI 1.7-kbp fragment of pHP23

In the case of *N. plumbaginifolia*, linear plasmid DNA was used in transformation. This is known to lead to more complex integration patterns (Shillito et al. 1985) and this complexity is increased when co-transforming with our selected repeat sequences. Preliminary results (M. L. Marchesi, S. Castiglione and F. Sala, unpublished results) have shown overlaps in Southern blots when comparing, in the genome of co-transformed *N. plumbaginifolia*, the distribution of the integrated gene sequences with that of the sequence cloned in pBB3, thus suggesting that, at least in some cases, co-transformation may have resulted in the preferential integration of the gene in sites adjacent to the repeated sequence itself. Ligation of the two plasmids followed by events of recombination with sites of homology within the repeated DNA sequence in the genome of the recipient cell may be a plausible working hypothesis. By favouring the integration of the foreign gene, this mechanism would account both for the increase in transformation frequency and for the higher extent of gene insertion. Further molecular and genetic data are now necessary to verify this.

Recently Paszkowsky et al. (1988) demonstrated that homologous recombination may actually occur during the PEG-induced protoplast transformation. In fact, by using protoplast prepared from leaves of *Nicotiana tabacum* which carried a copy of a partial, non-functional *APH(3')II* gene in the nuclear DNA and transforming with plasmid constructs carrying overlapping parts of the gene, an active gene in the host chromosome was obtained.

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