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Cloning of *Petunia hybrida* chloroplast DNA sequences capable of autonomous replication in yeast

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Summary

Sequences from *Petunia hybrida* chloroplast DNA which have the property to promote autonomous replication in *Saccharomyces cerevisiae* were cloned in vector YIp5. Seven cloned chloroplast DNA fragments are localized at one of two different sites on the chloroplast genome. One site, *arsA* was mapped on a 1.8 Kb fragment at position 27.0–28.8 Kb on the *P. hybrida* chloroplast genome. The plasmids containing this *arsA* are stable both in yeast and *E. coli*. The other site, *arsB*, was shown to be very unstable and is located either in the small single copy region close to the inverted repeat or just in the inverted repeat. The functioning of these sequences as a possible origin of replication *in vivo* is discussed.

Introduction

Eukaryotic DNA segments capable of autonomous replication in *Saccharomyces cerevisiae* have been isolated from a variety of nuclear chromosomal DNA (1, 7, 13, 27), extrachromosomal DNA (11, 14, 15, 33) and very recently from Tobacco chloroplast DNA (29). Vectors such as YIp5 have been used for this purpose. This vector is a hybrid between pBR322 and the yeast *ura*3 gene and cannot replicate autonomously in *S. cerevisiae* (28).

For various *a*utonomously *r*eplicating *s*equences (*ars*'s) isolated from yeast it has been shown that they can function as an origin of replication *in vivo* (6, 8, 12). However, due to the lack of appropriate methods, it is difficult to obtain indications about the *in vivo* functioning of *ars*'s from other eukaryotic organisms. Nevertheless, there is evidence that for *Xenopus leavis* mitochondrial DNA and *Tetrahymena thermophila* rDNA the *ars*'s do function as an origin of replication *in vivo* (15, 33).

In the context of our studies on the mechanism and regulation of the replication of the *P. hybrida* chloroplast (cp)DNA and the identification of the cpDNA replication origin we describe in this paper the isolation and analysis of *P. hybrida* cpDNA sequences that are capable of autonomous replication in yeast and their localization on the chloroplast genome.

Material and methods

Strains, plasmids and media. The *E. coli* strain used was JA221 *(recA, hsdR leuB*6, *trpE*5, Δ *lacY)* and the *S. cerevisiae* strains used were YNN27 (α *trp*1–289, *ura*3–52, *gal*2; (27) and DL1 (α *leu*2–3, *leu*2–112, *his*3–11, *his*3–15, *ura*3–251, *ura*3–372, *ura*3–328; (31)).

The plasmids YIp5 (a hybrid between pBR322 and the yeast *ura*3 gene, (28) and YRp12 (vector YIp5 containing a yeast *ars;* (23) were kindly provided by R. Davis. Plasmid pFL1 is a hybrid between pBR322, the yeast *ura*3 gene and the yeast 2 μ plasmid (9). The hybrid plasmids constructed in this study between YIp5 and *P. hybrida* cpDNA are named pPCY.

E. coli cells were grown at 37 °C in BHI (3.7% Brain Heart Infusion, Difco) medium, supplemented with the appropriate antibiotics when the cells harboured a plasmid. *S. cerevisiae* strains were grown at 30 °C with aeration on rich YPDA medium (2% glucose; 2% Bacto-peptone, Difco; 1% yeast extract, Difco) or on YMM medium (0.67%

yeast nitrogen base without amino acids, Difco; 2% glucose; appropriate amino acids at 20 μg/ml).

DNA isolation. Plasmid DNA was isolated from *E. coli* by the cleared lysate procedure (10) followed by CsCl-ethidium bromide isopycnic centrifugation.

S. cerevisiae total DNA preparations enriched for plasmids were isolated from 100 ml cultures grown under selective conditions. Cells were converted to spheroplasts with zymolyase 60 000 (0.1 mg/ml; Seicagacu, Tokyo) and lysed in 50 mM EDTA pH 8.0, 0.1% SDS, 0.3% diethyl pyrocarbonate. After incubation at 65 °C for 30 min the proteins were precipitated in 1 M potassium acetate on ice and centrifuged for 20 min at 3 000 **g**. Nucleic acids in the soluble fraction were precipitated with isopropanol and NaCl at −20 °C. The pellet was dissolved in 200 μl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA, extracted with phenol, precipitated again and finally dissolved in 50 μl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA, extracted with phenol, precipitated again and finally dissolved in 50 μl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

P. hybrida chloroplast DNA was isolated as described by Bovenberg *et al.* (5) with some minor modifications. Briefly, chloroplasts were isolated from 200 g of leaves of 7 week old *P. hybrida* plants grown in the green house. The final chloroplast pellet was gently resuspended in 25 ml 50 mM Tris-HCl pH 8.0, 20 mM EDTA and lysed by addition of an equal volume 50 m Tris-HCl pH 8.0, 20 mM EDTA, 4% sodium sarkosyl. After an incubation for 30 min at 20 °C the lysate was centrifuged for 10 min at 3 000 **g** to remove starch grains and chloroplast debris. CsCl was added (1.1 gr/ml lysate) and very gently dissolved. After addition of ethidium bromide (120 μg/ml) the lysate was centrifuged for 22 h at 40 000 rpm at 15 °C in a vertical rotor (TV 805, Sorvall). The lower band was carefully collected and recentrifuged in a Beckmann 65 H rotor at 35 000 rpm for 60 h at 15 °C. The lower DNA band was again carefully collected and the ethidium bromide removed by extraction with CsCl saturated isopropanol. Finally, the DNA preparation was dialyzed extensively against 10 mM Tris-HCl pH 8.0, 1 mM EDTA and stored in small aliquots at −20 °C.

Transformation procedures. The $CaCl_2$/RbCl method described by Kushner (17) was used to transform *E. coli* strain JA221 at high frequency (10^7 transformants/μg purified plasmid DNA). Transformation of *S. cerevisiae* was performed by the method of Beggs (2) with the modification that cells were converted to spheroplasts by incubation with 0.1 mg/ml zymolyase 60 000.

Analysis of DNA. DNA preparations were analyzed by electrophoresis on horizontal 1% agarose gels. Digestion of DNA with the various restriction enzymes (Boehringer, Mannheim) was performed as prescribed by the supplier. For hybridization analysis, electrophoretically separated DNA was transferred to nitrocellulose (type BA85, Schleicher and Schull) by bidirectional capillary blotting (24). Hybridization was performed as described by Wahl *et al.* (32) with DNA probes which were ^{32}P-labelled by nick-translation (22).

Results

Cloning and analysis of P. hybrida ars*'s in yeast*

In order to isolate *ars*'s from the cpDNA of *P. hybrida* we used the vector YIp5. This plasmid, which is a hybrid between pBR322 and the yeast *ura3* gene, has no yeast replication origin. Therefore, it can not transform *ura*-yeast strains YNN27 and DL1, which have deletions and/or rearrangements in the *ura3* gene, to a *ura*$^+$ phenotype (28). This vector YIp5 was linearized with one of the restriction endonucleases *Eco*RI, *Hin*dIII or *Bam*HI and ligated with appropriate digested cpDNA preparations. The resulting hybrid molecules were used to transform *S. cerevisiae* strain DL1 and *ura*$^+$ transformants were selected after 1 to 2 weeks of growth.

Yeast strains harbouring *ars*-containing plasmids have the property that they grow only slowly on selective medium without uracil and that they rapidly loose the *ura*$^+$ phenotype after growth on non-selective medium with uracil (27). We analyzed the obtained *ura*$^+$ transformants for these properties to establish their *ars* character. Determination of growth rates of 40 isolated transformants showed generation times between 5 and 20 h on selective medium. Less than 1% of the cells maintained their *ura*$^+$ phenotype after growth for 10 generations on non-selective medium with uracil. In both these

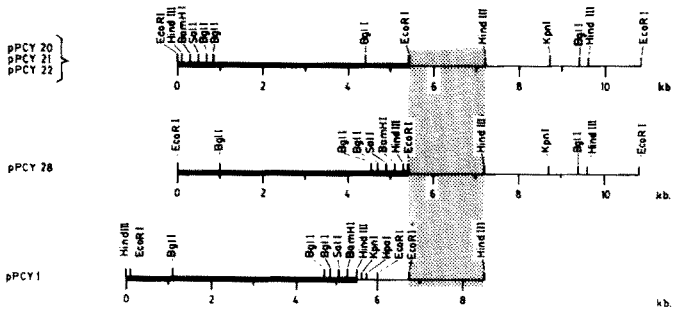

Fig. 1. Physical maps of the plasmids pPCY20, 21, 22, pPCY28 and pPCY1. The restriction sites for the endonucleases *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Kpn*I and *Sal*I were determined by analysis of the DNA fragment patterns of digests and appropriate double digests after electrophoresis on 1% agarose gels. The vector YIp5 is presented by the thick line and the insert by the thin line. The dotted area indicates the part of the plasmids which they all have in common as was analyzed by hybridization experiments (see text).

properties the transformants resemble cells with the plasmid YRp12 (a hybrid between YIp5 and a yeast *ars*). However, they differ from yeast cells harbouring plasmid pF11, a hybrid between pBR322, the yeast *ura*3 gene and the yeast 2 μ plasmid replication origin, which have a generation time of only 3 h while 98% of the cells maintained the *ura*$^+$ phenotype after growth for 10 generations under non selective conditions. These results strongly suggest that the isolated yeast *ura*$^+$ cells are the result of transformation by *ars* type plasmids.

Analysis of the yeast ars *plasmids in* E. coli

In order to establish that the yeast transformants contain plasmids with chloroplast *ars*'s and to characterize these plasmids in detail, plasmid enriched DNA preparations were isolated from the transformed yeast cells grown on 100 ml selective medium and used to transform cells of *E. coli* strain JA221 to ampicilline resistance. Plasmid DNA was isolated from the *E. coli* transformants obtained with DNA preparations from independently isolated yeast clones. Five plasmids were isolated which were larger than the vector while two had about the same size.

Physical maps were constructed for the five plasmids that were larger than YIp5 as a result of a distinct insert. Three plasmids pPCY20, pPCY21 and pPCY22 have a 5.4 Kb *Eco*RI insert (Fig. 1, top row). Plasmid pPCY28 has the same 5.4 Kb *Eco*RI insert but in the reversed orientation (Fig. 1, middle row). Plasmid pPCY1 has a 3.0 Kb *Hin*dIII insert (Fig. 1, bottom row). Hybridization of ^{32}P-labelled DNA of plasmids pPCY1 and pPCY20 with

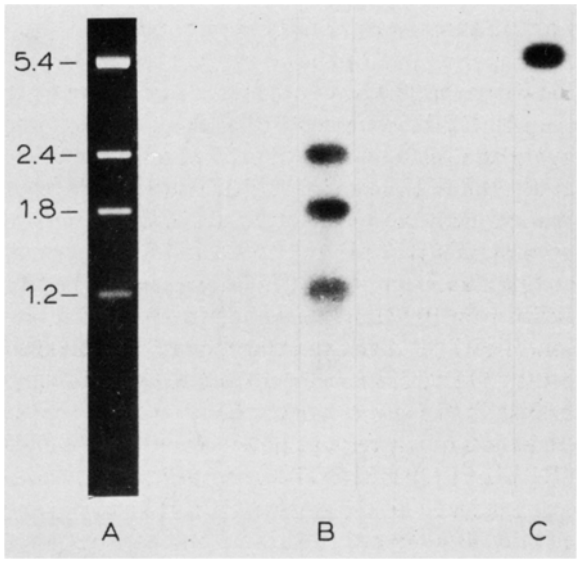

Fig. 2. Hybridization of pPCY20 *Eco*RI/*Hin*dIII restriction fragments stained with ethidium bromide after separation by electrophoresis on a 1% agarose gel (A) with ^{32}P-labelled cpDNA (B) and ^{32}P-labelled pBR322 DNA (C).

*Eco*RI/*Hin*dIII double digests of these plasmids showed that they have in addition to the 5.4 Kb vector fragment a 1.8 Kb *Eco*RI/*Hin*dIII insert fragment in common (dotted area in Fig. 1).

The plasmids pPCY3 and pPCY4 which have about the same size as the vector YIp5, resulted from a ligation of *Bam*HI cpDNA fragments in the *Bam*HI site of the vector. This site is located in the tetracycline resistance gene of pBR322. The plasmids pPCY3 and pPCY4 were sensitive to tetracycline suggesting the presence of an insert, but were converted with endonuclease *Bam*HI appar-

ently only into the linear form. The most likely explanation is that these plasmids contain a *Bam*HI insert which is too small to be detected by agarose gel electrophoresis. To test this possibility, the plasmids were digested with *Bam*HI and 5′ labelled with ^{32}P as described by Maxam & Gilbert (18). Subsequent electrophoresis on a 5% acrylamide gel followed by autoradiography indeed revealed a small fragment of about 50 basepairs in addition to the large vector fragment of 5.4 Kb (results not shown). As these plasmids did not hybridize with the insert of the plasmids pPCY1 and pPCY20 we conclude that they originate from different sequences.

Characterization of the pPCY plasmids

To determine whether the inserts originate from the chloroplast genome ^{32}P-labelled cpDNA was hybridized with nitrocellulose blots of plasmid digests. Figure 2 shows that ^{32}P-labelled cpDNA hybridizes with the pPCY20 *Eco*RI/*Hin*dIII restriction fragments of 2.4 Kb, 1.8 Kb and 1.2 Kb, which all originate from the 5.4 Kb insert (see Fig. 1). The fragment of 5.4 Kb representing the linearized vector YIp5 hybridizes specifically with ^{32}P-labelled pBR322 DNA. Similar results showing specific hybridization of ^{32}P-labelled cpDNA with the insert fragments only were also obtained for the plasmids pPCY1, pPCY21, pPCY22 and pPCY28. The insert fragments of the plasmids pPCY3 and pPCY4 can not be analyzed by this method as DNA fragments, smaller than about 0.25 Kb do not bind to nitrocellulose. However, because these ^{32}P-labelled plasmids hybridized with cpDNA (see next section) we can conclude that the *ars* insert of all seven plasmids analyzed originates most likely from the cpDNA.

Hybridization of ^{32}P-labelled DNA of plasmids pPCY20, 21, 22 and 28 with an *Eco*RI digest of cpDNA showed a specific binding with a 5.4 Kb fragment, while pPCY1 hybridized with a 3.0 Kb *Hin*dIII cpDNA fragment. These results show that authentic cpDNA fragments have been cloned and that no detectable deletions have occurred during the cloning in yeast and the subsequent transfer into *E. coli*. This is confirmed by the fact that all these plasmids after isolation from *E. coli* can transform yeast strains YNN27 or DL1 to a *ura*$^+$ phenotype at high frequency (>10^3 transformants/μg DNA). The resulting yeast transformants have exactly the same phenotypic properties with respect to growth rate and instability as the yeast transformants from which the DNA was originally isolated for the transformation into *E. coli*. Plasmids pPCY3 and pPCY4 can also transform yeast cells, but the phenotype of the transformed cells has changed into a decreased growth rate and an increased instability. From these results we conclude that the 0.05 Kb fragment present in the plasmids isolated from *E. coli* has most likely resulted from a partial deletion in the *ars* originally present in the transformed yeast cells.

Localization of the ars *sequences on the chloroplast genome*

A physical map has been constructed for the *P. hybrida* chloroplast genome (5). Hybridization of ^{32}P-labelled pPCY plasmids with various digests of cpDNA will therefore provide information about the localization of the *ars*'s on the chloroplast genome. Digests of cpDNA with the enzymes *Sal*I, *Bam*HI, *Pst*I and *Hpa*I were separated by electrophoresis on agarose gels (Fig. 3B, D, F, H), transferred to nitrocellulose and hybridized with ^{32}P-labelled plasmid pPCY20 DNA. Analysis of the autoradiogram (Fig. 3A, C, E, G) showed hybridization with specific fragments of the cpDNA digests: *Sal*I fragment 5, *Bam*HI fragment 3, *Pst*I fragment 6 or 7 (these fragments were not separated on this agarose gel) and *Hpa*I fragment 2. Furthermore hybridization of pPCY20 was also observed with *Kpn*I fragments 1 and 8 and *Bgl*I fragments 1 and 4. The position of these fragments on the chloroplast physical map are indicated in Fig. 4 by dots. The part of the chloroplast genome which all these fragments have in common and on which therefore the *ars* is located, can be defined between the *Hpa*I site at 26 Kb and the *Sal*I site at 32 Kb. By comparison of the physical maps of the insert of pPCY20 and the chloroplast genome, the insert can be localized exactly as indicated in Fig. 4. Identical results were obtained with the plasmids pPCY21, 22 and 28. The insert of plasmid pPCY1 hybridized with the *Sal*I fragment 5, *Bam*HI fragment 3, *Hpa*I fragments 2 and 3, *Pst*I fragment 6 or 7 and *Kpn*I fragments 8 and 9. Based on these results and the physical maps of plasmid pPCY1 and the chloroplast genome the insert is localized overlapping

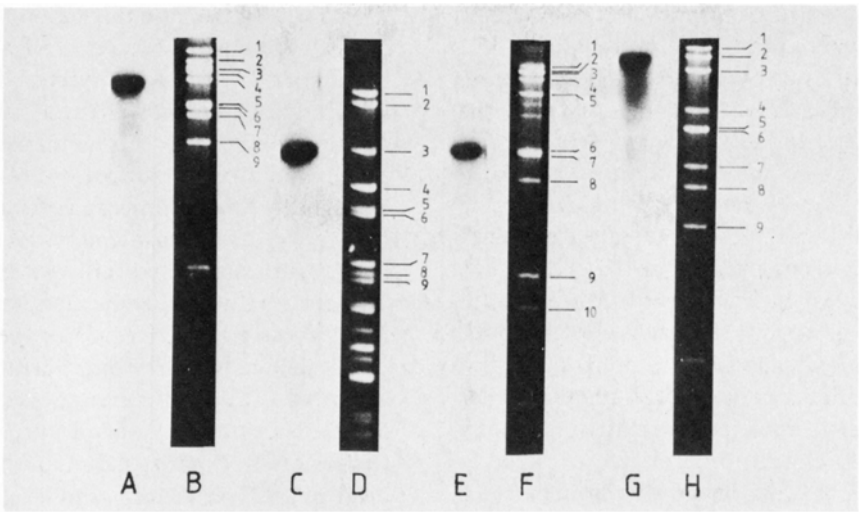

Fig. 3. Hybridization of ^{32}P-labelled pPCY20 plasmid DNA with various cpDNA digests. The cpDNA was cleaved with *Sal*I (A, B), *Bam*HI (C, D), *Pst*I (E, F) and *Hpa*I (G, H). The digests were separated by electrophoresis on a 0.7% agarose gel and the DNA fragments were visualized by ethidium bromide staining (B, D, F, H). The DNA fragments are numbered according to their size (Bovenberg *et al.* 1981). The DNA fragments were subsequently transferred to nitrocellulose, hybridized with ^{32}P-labelled pPCY20 DNA and the hybridized fragments were detected by autoradiography (A, C, E, G).

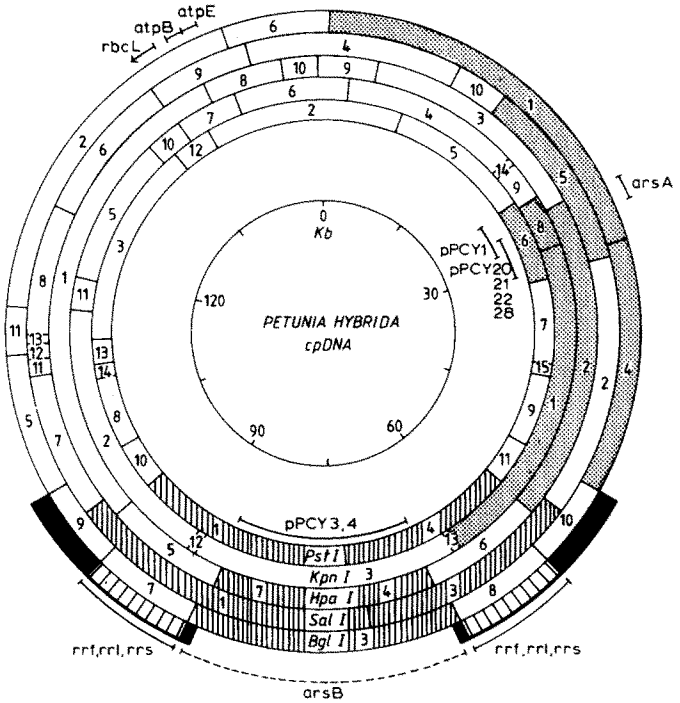

Fig. 4. Schematic presentation of the hybridization pattern of ^{32}P-labelled pPCY20 (dots) and pPCY3 (bars) DNA with the various cpDNA restriction fragments present on the physical map of *P. hybrida* cpDNA (Bovenberg *et al.* 1981). The exact localization of the inserts of the plasmids pPCY20, 21, 22, 28 and pPCY1 based on the hybridization patterns (see text) and physical maps (see Fig. 1) are indicated. The region where the inserts of plasmids pPCY3 and pPCY4 are localized is also indicated. The location of the respective *ars*A and *ars*B are indicated on the outer circle. The inverted repeats of the cpDNA as well as the position of several structural genes are indicated: the genes coding for 16S, 23S and 5S rRNA *(rrs, rrl, rrf)* for the large subunit of RuBPCase *(rbcL)* and for chloroplast encoded components of the ATP-ase system *(atpB, atpE)*. Data from Bovenberg *et al.* (manuscript submitted).

with the insert of pPCY20. The *ars* is most likely confined to the overlapping 1.8 Kb *Eco*RI/*Hin*dIII fragment that all these plasmids have in common. This region is indicated as *ars*A (Fig. 4).

Plasmids pPCY3 and pPCY4 hybridized with the *Sal*I fragments 1 and 3, *Pst*I fragments 1 and 4, HpaI fragments 4 and 7, *Bgl*I fragment 3 (indicated with bars in Fig. 4). All these fragments are located in the small single copy region of the chloroplast genome and contain at least a part of the inverted repeat. As these plasmids are most likely deleted for a part of the *ars*B because their *ars* phenotype has changed we can not conclude at this point whether the *ars*B is located once in the small single copy region (probably close to one of the inverted repeats) or twice, just in the inverted repeat (see Fig. 4).

Discussion

From the results described above we conclude that at least two different sites of the P. hybrida chloroplast genome named arsA and arsB, are able to replicate autonomously in yeast. As very recently cpDNA *ars*'s have been isolated from Tobacco, (29) their localization and that of cpDNA *ars*'s from other higher plants will learn whether these sequences are localized at identical map positions like is known for structural genes of the chloroplast genome (4).

Although there is evidence that the *ars*'s cloned from *S. cerevisiae, X. laevis* and *T. thermophila* do function as an origin of replication *in vivo* (6, 8, 12, 15, 33), the biological meaning of *ars*'s cloned from most eukaryotic organisms remains an intriguing question. Cloning of chloroplast *ars*'s has been reported for *Chlamydomonas reinhardii* (30), *Petunia hybrida* (this study) and *Nicotiana tabacum* (29), while electron microscopic localization of *in vivo* active chloroplast replication origins has only been established in *Euglena gracilis* (16, 21). A combination of both methods could answer the question whether these *ars* sequences can function as a replication origin *in vivo*. However, this has not been performed so far. For *Euglena* it should be possible to determine whether the replication origin can function as an *ars* in yeast. For higher plants, however, it will be very difficult to analyse the chloroplast replication origin by electron microscopic studies because this requires the isolation of cpDNA from a very active replicating chloroplast population which has only been achieved for the green algae by specific synchronisation procedures. A useful alternative can be the analysis of *ars* sequences in an *in vitro* DNA synthesizing systems because the functioning of a yeast *ars* as an origin of replication has been demonstrated in an *in vitro* DNA synthesizing system isolated from yeast (6). Therefore we will study the replication of the cloned *P. hybrida* cpDNA *ars*A in an organelle-free DNA-synthesizing system from purified chloroplasts, which can, after elimination of the endogenous DNA, use externally added purified cpDNA as a template (20). For the analysis of the *ars*B sequence, however, this sequence has to be cloned by another approach to avoid the occurrence of deletions.

The most direct evidence that the *ars*'s are sequences that can function as an origin of replication *in vivo* will be the reintroduction of the *ars* in the chloroplast, followed by the demonstration that the sequence is stably maintained as a result of autonomous replication. This will require the combination of such an *ars* with a selectable marker. A good candidate for this purpose is the *neo* gene of transposon Tn5 conferring resistance to kanamycin and G418 (3, 25). Also chloroplast encoded selection markers can be considered like for instance the cpDNA encoded streptomycin resistance of *N. tabacum* (19) or the atrazine resistance which is determined by an alteration in the so-called 32 Kd gene of the chloroplast DNA (26).

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