Marker rescue from the *Nicotiana tabacum* plastid genome using a plastid/*Escherichia coli* shuttle vector

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**Abstract** We recently reported an 868-bp plastid DNA minicircle, NICE1, that formed during transformation in a transplastomic *Nicotiana tabacum* line. Shuttle plasmids containing NICE1 sequences were maintained extrachromosomally in plastids and shown to undergo recombination with NICE1 sequences on the plastid genome. To prove the general utility of the shuttle plasmids, we tested whether plastid genes outside the NICE1 region could be rescued in *Escherichia coli*. The NICE1-based rescue plasmid, pNICER1, carries NICE1 sequences for maintenance in plastids, the CoIE1 ori for maintenance in *E. coli* and a spectinomycin resistance gene (*aadA*) for selection in both systems. In addition, pNICER1 carries a defective kanamycin resistance gene, *kan* *,* to target the rescue of a functional kanamycin resistance gene, *kan*, from the recipient plastid genome. pNICER1 was introduced into plastids where recombination could occur between the homologous *kan/kan* *sequences*, and subsequently rescued in *E. coli* to recover the products of recombination. Based on the expression of kanamycin resistance in *E. coli* and the analysis of three restriction fragment polymorphisms, recombinant *kan* genes were recovered at a high frequency. Efficient rescue of *kan* from the plastid genome in *E. coli* indicates that NICE1-based plasmids are suitable for rescuing mutations from any part of the plastid genome, expanding the repertoire of genetic tools available for plastid biology.

**Key words** Extrachromosomal element · *Nicotiana tabacum* · Plastid transformation · Recombination · Shuttle vector

**Introduction**

Stable transformation of higher plant plastids has only recently been achieved, using *Nicotiana tabacum* as the model species (Svab et al. 1990). Since all characterized recombination events involve homologous DNA sequences, the transformation vectors target the integration of foreign DNA by means of flanking plastid DNA sequences. Replication of the transplastome and sorting of the transformed genome copies under selection pressure yields homoplasmic lines with all of the 1000–10000 identical plastid genome (ptDNA) copies uniformly altered, while the transforming plasmid DNA is rapidly lost (Zoubenko et al. 1994; for reviews see Maliga 1993; Maliga et al. 1993).

We have identified an 868-bp ptDNA minicircle, NICE1 (*Nicotiana plastid extrachromosomal element*), that formed by homologous recombination via a 16-bp imperfect direct repeat in the transforming ptDNA (Staub and Maliga 1994a). The endogenous NICE1 ptDNA sequence is located in the repeated region of the plastid genome. Formation of the NICE1 extrachromosomal element was apparently linked to transformation, since ptDNA minicircles are normally absent in higher plant plastids (Palmer 1991).

However, *Escherichia coli* plasmids containing NICE1 sequences and a plastid selectable marker gene were maintained as extrachromosomal elements when reintroduced into plastids by the particle bombardment process. Marker exchange between NICE1 sequences on the plasmid and the cognate sequences on the plastid genome was also shown (Staub and Maliga 1994a). However, it was not tested if plastid genes outside the NICE1 region could be rescued by NICE1-based shuttle plasmids.

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To demonstrate the general utility of NICE1-based shuttle plasmids, we attempted to rescue a gene located in the large unique region of the plastid genome. We took advantage of the availability of a transplastomic tobacco line, Nt-P70A, which contains a chimeric kanamycin resistance gene (kan) stably integrated in the large single-copy region of the plastid genome. This kan gene is approximately 50 kb and 75 kb away from the endogenous NICE1 loci in the repeated region of the 156-kb N. tabacum plastid genome (Carrer et al. 1993). A NICE1-based rescue plasmid, pNICER1, that carries a mutant (sensitive) kan* gene, was constructed to rescue the functional kan gene from the plastid genome. Efficient recombination between kan* on pNICER1 and the plastidic kan copy facilitated the rescue of kan markers in E. coli at a high frequency. The data presented here indicate that any target sequence on the plastid genome is accessible for marker rescue using NICE1-based plasmids.

Materials and methods

Transformation vectors

The pNICER1 vector is based on a pUC18 plasmid (Vieira and Messing 1987) which carries a mutant kan* gene, NICE1 ptDNA sequences, and a selectable spectinomycin resistance (aadA) gene. The kan* gene was obtained by cloning a chimeric kan gene into the pUC18 KpnI-PstI cloning sites. The chimeric kan gene is the progenitor of kan in plastid pTTH32 (Carrer et al. 1993), but lacks the five N-terminal amino acids of the ribulose-1,5-bisphosphate carboxylase large subunit. For expression in plastsids, the kan coding region was inserted into the Prrn/TpsbA cassette as an Ncol-Xbal DNA fragment, in which the Ncol restriction site includes the translational initiation codon (Carrer et al. 1993). Since kan does not have this Ncol site, this restriction fragment length polymorphism (RFLP) is suitable for distinguishing kan from kan*. The two additional kan* RFLP markers were created through the following steps.

1. The SpfI site in the kan coding region was blunt-ended with the Klenow fragment of DNA polymerase I, and converted to a BglII site by linker ligation (5'-pCAGATCTG-3'). The linker ligation alters the kan reading frame, yielding a defective kan* allele.

2. The SpfI site in the psbA 3' end was converted to a SalI site by the same approach using the oligonucleotide 5'-pGGTCGACC-3'.

NICE1 and aadA were cloned into BspHI-digested pUC18, which resulted in the replacement of the ampicillin resistance gene sequences between the BspHI restriction sites at nucleotides 1526 and 2639 with the linked NICE1 and aadA sequences. The NICE1 segment was excised from plasmid pJS75 (Staub and Maliga 1992) as an 0.8-kb Sall-Xbal plastid DNA fragment (nucleotides 136330-137154 of the ptDNA; originally a HindIII-Sphl ptDNA fragment; Shinozaki et al. 1986) from a derivative of plasmid pJS75 (Staub and Maliga 1993); the aadA gene was obtained by cloning a chimeric cassette as an 0.8-kb Sall-Xbal DNA fragment containing the kan coding region; P2, the kan marker probe which is the 1.4-kb SacI-BamHI DNA fragment (restriction sites in the ptDNA at nucleotides 57750 and 59286; Shinozaki et al. 1986) from a derivative of plasmid pJS197 (Shinozaki et al. 1993); P3, the NICE1 flank probe is the 1.2-kb SmaI-SacI DNA fragment (at nucleotides 137112 and 138452 in ptDNA; Shinozaki et al. 1986) from plasmid pJS75 (Staub and Maliga 1992).

Plant DNA analysis

Total cellular DNA was isolated from leaf (Mettler 1987) and digested with the appropriate restriction enzymes. Digested and undigested DNA was electrophoresed in 0.8% agarose gels, and the DNA was transferred to nylon membrane (Amersham) using the PosiBlot transfer apparatus (Stratagene) according to the manufacturer's protocol. Blots were probed using Rapid Hybridization buffer (Amersham) with 32P-labeled probes generated by random priming (Boehringer-Mannheim). The probes used for hybridization were: P1, the 0.8-kb Ncol-Xbal DNA fragment containing the kan coding region; P2, the kan marker probe which is the 1.4-kb SacI-BamHI DNA fragment (restriction sites in the ptDNA at nucleotides 57750 and 59286; Shinozaki et al. 1986) from a derivative of plasmid pJS197 (Shinozaki et al. 1993); P3, the NICE1 flank probe is the 1.2-kb SmaI-SacI DNA fragment (at nucleotides 137112 and 138452 in ptDNA; Shinozaki et al. 1986) from plasmid pJS75 (Staub and Maliga 1992).

E. coli transformation and plasmid analysis

Competent E. coli JM83 or DH5x cells were prepared and transformed with total cellular plant DNA (ca 0.5 µg) using the Hanahan (1983) procedure. Kanamycin resistance of E. coli was tested on LB plates (Sambrook et al. 1989) containing 50 µg/ml of kanamycin sulfate. Plasmid DNA was recovered from E. coli transformants using a modified DNA minipreparation method (Zhou et al. 1990). Plasmid RFLPs were tested by digestion with restriction enzymes following the manufacturer's recommendations.

Results

Plasmids for testing marker rescue

The pNICER1 plasmid carries NICE1 ptDNA sequences and the ColE1 ori for extrachromosomal maintenance in plastids and E. coli, respectively, and a spectinomycin resistance gene, aadA, which is selectable in both systems. To facilitate the study of marker rescue, the shuttle plasmid contains a mutant (sensitive) kanamycin resistance gene (kan*) which differs by three RFLPs from the kan gene in the plastid genome of the kanamycin resistant recipient Nt-P70A N. tabacum line (Fig. 1A, B; Carrer et al. 1993). In pNICER1, a BglII linker was inserted into the wild-type SpfI site in the