Functional analysis of plastid DNA replication origins in tobacco by targeted inactivation

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Received 16 May 2002; accepted 11 June 2002.
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Summary
Sequences described as chloroplast DNA replication origins were analysed in vivo by creating deletion and insertion mutants via plastid transformation in tobacco. Deletion of the described oriA sequence, which is located within the intron of the trnI gene, resulted in heteroplastomic transformants, when the selection marker was inserted within the intron. Removal of the complete intron sequence together with the oriA sequence, however, yielded homoplastomic transformants of normal phenotype, in which wild-type signals were no longer detectable through Southern analysis, thus bringing the role of the described oriA sequence for plastome replication into question. Similarly, deletion of sequence elements upstream of trnI, which have a possible ori function in Oenothera, did not show any effect in tobacco. The two copies of oriB, which are located at the very end of the plastome Inverted Repeats, were targeted with two different transformation vectors in a cotransformation approach. While in initial transformants integration of the selection marker could be detected at both sites, the transgene was found exclusively at one site or the other after additional rounds of regeneration. Whereas the copy of oriB in Inverted Repeat B could be completely deleted, targeting of the copy in Inverted Repeat A resulted in heteroplastomic lines, as the essential ycf1 gene was also affected. Due to the strong selection against cotransformants we conclude that at least one copy of the oriB sequence is essential for plastome replication, whereas replication appears possible without oriA elements.

Keywords: replication, chloroplast, transformation, oriA, oriB, plastome.

Introduction
Replication origins of plastid DNA have been studied in several plant species by electron microscopy, primer extension mapping, and in vitro replication in a partially purified chloroplast protein fraction (Kunnimalaiyaan and Nielsen, 1997a). For most organisms examined, two different replication origins, named oriA and oriB, have been proposed, supporting the model of double D-loop replication, which proposes that each origin promotes unidirectional replication of only one strand of DNA (Kolodner and Tewari, 1975a). In addition, a rolling circle replication mechanism was proposed (Kolodner and Tewari, 1975a). Although different positions of the two replication origins within chloroplast DNA have been described in different organisms, they were localised at the same sites in pea and tobacco (Kunnimalaiyaan and Nielsen, 1997b; Meeker et al., 1988; Nielsen et al., 1993). As these are both within the Inverted Repeat (IR) regions of the tobacco plastome, tobacco plastid DNA contains four ori sequences, while in pea only two have been proposed due to the lack of an Inverted Repeat. oriA was localised within the rRNA operon, oriB downstream of the trnN gene. As the rRNA operon shows a high degree of conservation between different species, it appears possible that oriA is also located at this site in other species (Kunnimalaiyaan and Nielsen, 1997a). Both replication origins are found within transcribed regions of the plastome, which supports findings made in Chlamydomonas reinhardtii that transcription and RNA processing may play a role in the initiation of plastid DNA replication (Chang and Wu, 2000).

In tobacco, the sequence described as the minimal oriA is a 82-bp element (plastome positions 104769–104850 and 137776–137857; GenBank accession number Z00044) within the intron of the trnI gene; it contains two 8 bp direct repeats and is capable of forming a relatively stable stem-loop...
structure (Kunnimalaiyaan and Nielsen, 1997b). Interestingly, there are two elements in the near surrounding of this sequence which could also be of importance in plastid DNA replication:

(a) The region upstream of the trnL gene shows striking length and sequence differences in the plastid DNA of different species of the genus *Oenothera* (Hornung et al., 1996; Sears et al., 1996). As the different plastid types show different replication rates when transferred to the same nuclear background, the region showing these sequence differences is assumed to be involved in the regulation of DNA replication. This is corroborated by the fact that a replication origin (confusingly named *oriB* in *Oenothera*) was mapped in this region by electron microscopic analysis of displacement loops (Chiu and Sears, 1992). The sequence differences are caused by repetition and variation of short DNA elements. In tobacco, the corresponding region consists of non-repeated elements A, B, C, D, F, E and G; no participation in DNA replication has been ascribed to them up to now.

(b) 322 bp downstream of *oriA* is a 852-bp DNA element termed NICE1, which has been found in extrachromosomal DNA circles in transformed tobacco chloroplasts, also in multimeric form (Staub and Maliga, 1994). It is bordered by 16 bp non-identical direct repeats and contains part of the trnL intron, the second trnL exon, a spacer, the first trnA exon, and part of the trnA intron. If these extrachromosomal elements are replicated like bacterial plasmids, the sequence must contain an origin of replication. However, they could also be generated by excision from the plastid DNA by recombination events.

*oriB* in tobacco has been described as a 243-bp element, also capable of forming a stem-loop and containing repeated elements (Kunnimalaiyaan and Nielsen, 1997b). It is located downstream of the trnN gene (111778–112020 and 130606–130848), close to the border of the IR regions at the small single copy region (Kunnimalaiyaan et al., 1997). Therefore, the two copies of *oriB* in the plastome have a different surrounding: In IR*_, oriB* is located within the coding sequence of the ycf1 gene, which starts within the Inverted Repeat. The function of this gene is not yet known, but it appears to be essential, as homoplasmic *ycf1* deletion mutants of *Chlamydomonas* and tobacco could not be achieved (Boudreau et al., 1997; Drescher et al., 2000). In IR*, *oriB* is found within a truncated version of the *ycf1* gene, termed *orf350*, whose reading frame stops 52 bp after the end of the IR region. Up to now, no function has been ascribed to this open reading frame.

Fine mapping of the sequences of both tobacco replication origins has been made in *in vitro* replication experiments (Kunnimalaiyaan and Nielsen, 1997b), which do not necessarily reproduce the situation in the plastid. *In vivo* analysis of replication origins has been restricted to the analysis of displacement loops up to now, which does not allow precise localisation (Kunnimalaiyaan et al., 1997; Lu et al., 1996). In an earlier study with proplastids of cultured tobacco cells, displacement loops were mapped to the ends of the Inverted Repeat near to, but not identical to the described *oriB* locus, and to the large single copy region of the plastome, but not to the region of *oriA* (Takeda et al., 1992). In this study, we used for the first time the technique of plastid transformation, which allows targeted and precise modification of the plastome, in order to investigate the significance of defined sequences for plastid DNA replication *in vivo*. Furthermore, using this technique, we were able to address the question of whether both *oriA* and *oriB* are essential, and if two copies of these sequences are needed.

Results

For *in vivo* analysis of the elements described above, tobacco plastome mutants showing deletions or disruptions of these sequences were generated by plastid transformation. Targeted modification of the plastome can be achieved by using transformation vectors containing plastid DNA sequences, which allow homologous recombination with the plastome (Svab and Maliga, 1993). If two plastid sequences flank a sequence on the transformation vector, recombination events in both flanks lead to insertion of the flanked sequence into the plastome at a targeted site (Koop et al., 1996; Svab and Maliga, 1993). If the flanking sequences are not adjacent in the plastome, sequences located in between the two flanks are replaced by the sequence on the transformation vector; this allows targeted deletion or modification of plastid sequences.

In order to enable selection of plastid transformants, the *E. coli* aadA coding sequence, which confers resistance to aminoglycoside antibiotics spectinomycin and streptomycin (Goldschmidt-Clermont, 1991; Svab and Maliga, 1993), was included in the transformation vectors. Plastid transformation experiments in tobacco were carried out using the biolistic method. Potential transformants were selected on medium containing spectinomycin. In order to promote segregation of plastomes, which should result in homoplastic lines, plants were subjected to repeated cycles of regeneration *in vitro* from leaf explants on spectinomycin-containing medium.

Analysis of *oriA*

Several plasmids were created for analysis of *oriA* and its surrounding region, all of them containing a stretch of tobacco plastid DNA sequence corresponding to position 103415–106944. The *aadA* selection marker was inserted at different positions in the various constructs. As the insertion site is within the rrn16 operon in all plasmids, the *aadA* coding sequence was only provided with an artificial

Plasmid A1 (Figure 1) was constructed for disruption of the oriA sequence in the tobacco plastome. The selection marker was inserted into the singular BamHI restriction site (104805), which divides the oriA sequence in two halves. In order to investigate the effects of insertion of a foreign gene into the rm16 operon, two control constructs, AK1 and AK2 (Figure 1), were made. Both contain the same plastid DNA sequence stretch as A1. In AK1, the aadA coding sequence is inserted between the trnI and trnA genes, utilizing the singular NsiI restriction site at position 105376. In plasmid AK2, the aadA coding sequence was inserted into the plastid DNA after restriction with XhoI (104847 and 104891); therefore, the selection marker is located within the trnl intron, replacing 40 bp immediately downstream of the oriA sequence. Spectinomycin-resistant regenerates were obtained with each of these plasmids after bombardment of tobacco leaves. Plastome insertion of the aadA sequence at the targeted position was verified by PCR (data not shown). Transplastomic lines were subjected to repeated cycles of vegetative regeneration before Southern analysis. AK1 transformants showed the expected signals for transformed plastome and did not contain detectable amounts of untransformed plastome (Figure 2a,b). In contrast to this, in A1 and AK2 transformants strong wild-type signals were detected even after 16 rounds of regeneration (Figure 2a,b). The ratio of transformed plastome to wild-type plastome of about 1:1 did not significantly change over time and was comparable in independent lines (data not shown). Restriction fragments were chosen which allow differentiation between the IR\textsubscript{A} and IR\textsubscript{B} copy of the targeted sequences. In all cases, transformant or wild-type signals were comparable for both sites.

Plasmids A2 (Figure 1), A2a, and A2b were constructed for the deletion of the tobacco sequence corresponding to the variable elements of Oenothera. In A2, the sequence from 104429 to 104543, corresponding to elements A, B, C, D and F, was replaced by the aadA coding sequence; elements E and G were not changed, in order to avoid deletion of the first trnl exon, which starts 8 bp from the deletion site. Vector A2a deletes only elements A, B, and C (104429–104487), and A2b deletes only element A and part of B (104429–104465). Transformation with all of these vectors resulted in transplastomic lines, as shown by PCR analysis. As with AK1 transformants, no untransformed plastome could be detected in these lines by Southern analysis after several cycles of regeneration (Figure 2a,b and data not shown).

Plasmid A3 (Figure 1) was constructed for deletion of the complete described oriA sequence; here, the aadA sequence replaces plastome sequence 104747–104891. Transplastomic lines produced with this vector showed a substantial amount of untransformed plastome even after nine cycles of regeneration (Figure 2a,b); however, in contrast to A1 and AK2 transformants, the wild-type signal was significantly weaker than the transformant signal. The ratio of wild-type and transformed plastome was about the same in several independent lines, which may indicate a balanced selection.

As transformation with vectors A1, AK2, and A3 could also affect splicing of the trnl transcript due to insertion of the aadA sequence within the intron, a further construct, A4 (Figure 1), was made, where the complete intron sequence

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**Figure 1.** Plastid transformation vectors for analysis of the oriA region. The uppermost panel shows the region of tobacco plastid DNA containing the described oriA sequence. Arrows mark the direction of transcription; all of the genes shown are transcribed from the promoter upstream of the rm16 gene as an operon. e1 and e2 denote the exons of the trnl and trnA genes. The other panels show schematic maps of the plastid transformation vectors used for analysis of the oriA region. In these vectors, the aadA sequence is inserted into oriA (A1), immediately downstream of oriA (AK2), between trnl and trnA (AK1), upstream of trnl, deleting the sequence corresponding to the variable elements of Oenothera plastomes (A2), within the trnl intron, deleting the complete oriA-sequence (A3), and between trnl and trnA, whereby the two exons of trnl are immediately joined, deleting the trnl intron including oriA (A4). Black box: oriA; white arrows: trnl exons; horizontally striped arrows: trnA exons; checked arrow: aadA; dotted arrow: 3'-part of rm23; vertically striped arrow: 5'-part of rm23. Drawing is not to scale.
is removed together with the oriA sequence, and the aadA sequence is inserted behind an intron-less trnI gene at the same position as in AK1. Also with this vector several transplastomic lines were obtained. Correct integration into both Inverted Repeats was shown by Southern analysis (Figure 2a,b). After six cycles of regeneration, no signal for untransformed plastome could be detected; therefore, in these transplastomic lines, deletion of the oriA sequence could be achieved completely, as judged by Southern analysis.

Deletion of oriB

While both copies of oriA could be targeted with the same transformation vector, the location of oriB close to the end of the Inverted Repeat region requires two different transformation vectors for deletion of oriB in both positions. Plasmid B1 (Figure 3) was constructed for deletion of oriB in IR

Figure 2. Southern analysis of plastid transformants in the oriA region. (a) Total plant DNA was restricted with PstI and XbaI and hybridised with a fragment corresponding to tobacco plastid DNA 105828–106503 and 136123–136798. Roman numerals give the number of regeneration cycles before analysis. wt: wild-type tobacco; M: Lambda Mix Marker 19, MBI Fermentas, Vilnius, Lithuania, the length of relevant fragments in kilobase-pairs (kb) is indicated. IR_A wt indicates the 19.6 kb fragment of wild-type plastome containing the IR_A copy of oriA; IR_B wt indicates the 12.5 kb wild-type fragment from IR_B, IR_A TF and IR_B TF indicate the corresponding transformant bands, respectively. The size of these fragments is slightly different, depending on the transformation vector (A1: 15.1 kb for the IR_A fragment and 7.9 kb for the IR_B fragment; AK1 and AK2: 15.1 kb and 7.9 kb; AK2 and AK3: 15.1 kb and 7.9 kb; A2 and A2b: 15.3 kb and 8.2 kb). Filters were also hybridised with a probe derived from Lambda DNA in order to detect size marker bands. (b) Schematic depiction of the relative positions of the probe used in 2(a) and detected fragments. Drawing is not to scale.

Figure 3. Plastid transformation vectors for analysis of oriB. In the circles, a schematic depiction of the integration cassette of transformation vectors B1 and B2 is given. The targeted region of the plastid genome, containing the oriB sequence at the end of Inverted Repeat A or B, respectively, is shown on top of the plasmids; regions of homology are indicated by dotted lines. Genes are indicated as open arrows marking the direction of transcription. JSA and JSB denote the border between Inverted Repeat A or B, respectively, and the small single copy region of the plastome. Black arrows indicate the binding sites of PCR primers used for detection of plastome integration of aadA as shown in Figure 4 (a). Black box: oriB; vertically striped arrow: tmR; horizontally striped arrow: trnN; dotted arrow: ycf1 or orf350; diagonally striped arrow: ndhF; checkered arrow: aadA cassette. Note that the drawings are not to scale.

Plastid transformation was performed with a co-bombardment approach, using a mixture of both plasmids for bombardment of leaves. Primary regenerates were analysed for integration of the \textit{aadA} sequence into the plastome with PCR using primer combinations specific for each of the targeted positions. Thirty-five independent lines were found to contain the \textit{aadA} sequence in the plastome; in 22 of these integration could be detected at both positions, while in the residual lines integration of either B1 only (four lines) or B2 only (nine lines) could be shown (Figure 4a).

After three cycles of vegetative regeneration, the transplastomic lines were subjected to Southern analysis in order to determine the ratio of transformed to untransformed plastomes. Surprisingly, the same lines that had initially given positive PCR signals for transgene integration at both targeted positions were now found to show exclusively one of the integrations (Figure 4b,c). In lines showing \textit{aadA} integration at the position targeted by B2, untransformed plastome was barely or not detectable (Figure 4b,c). In contrast to this, in all of the lines showing \textit{aadA} integration resulting from B1 a strong wild-type signal of the targeted region could be detected in addition to the transformant signal (Figure 4b,c). Lines that had shown integration at only one site in the primary regenerate did not differ

**Figure 4.** Molecular analysis of \textit{oriB} transformants.

(a) PCR analysis of individual primary regenerates resulting from co-bombardment with vectors B1 and B2. Upper panel: PCR analysis showing plastome integration of \textit{aadA} at the site targeted by B1; the expected product of 1455 basepairs is indicated. Lower panel: PCR analysis of the same lines, showing plastome integration of \textit{aadA} at the site targeted by B2; the expected product of 1287 basepairs is indicated. One primer binds within the \textit{aadA} sequence, the second primer binds in the small single copy region of the plastome, outside of the sequence used in the transformation vector; primer binding sites are depicted schematically in Figure 3. M: DNA size marker (Mass Ruler\textsuperscript{TM} DNA Ladder Mix, MBI Fermentas, Vilnius, Lithuania), the length of selected fragments in basepairs is indicated on the left.

(b) Southern analysis of \textit{oriB} transformants. Plant lines that had given positive PCR signals for plastome integration of B2 (273–45, left), B1 (273–15, right), or both plasmids (all other lines) were analysed after three cycles of regeneration. Tobacco wild-type DNA (wt) was included as control. Total plant DNA was restriced with Ncol and Xbal and hybridised with two different probes. The upper panel shows hybridisation with probe \textit{“23S”}, corresponding to tobacco plastome sequence 108325 to 109255 and 133371 to 134301. IR\textsubscript{R} wt indicates the 7.1 kb fragment of wild-type plastome containing the IR A copy of \textit{oriB}; IR\textsubscript{L} wt indicates the 6.0 kb wild-type fragment from IR\textsubscript{L}. The IR\textsubscript{R} copy of \textit{oriB} is targeted by B1, the IR\textsubscript{L} copy of B2. In transformed molecules, a 4.8 kb fragment (TF) is detected instead of the corresponding wild-type fragment, independent of the targeted copy. The lower panel shows hybridisation with a probe specific to the \textit{aadA} sequence in order to differentiate between the two transgene integration sites. TF B1 indicates the 1.6 kb fragment resulting from \textit{aadA} integration at the site targeted by B1; TF B2 indicates the 2.7 kb fragment resulting from \textit{aadA} integration at the site targeted by B2. M: Lambda DNA Eco130I/Mul, MBI Fermentas, visible fragments in the upper panel are (from top): 26.3 kb + 19.3 kb (not separated), 9.8 kb, 7.7 kb, 6.2 kb, 5.1 kb, 4.3 kb, 3.5 kb; in the lower panel (from top): 3.5 kb, 2.7 kb, 2.4 kb, 2.2 kb, 1.9 kb, 1.5 kb. Filters were also hybridised with a probe derived from Lambda DNA in order to detect size marker bands.

(c) Schematic depiction of the binding sites of the probes used in 4(b) and the detected fragments. Drawing is not to scale.
from lines that had lost one of the integrations with respect to the ratio of transformed and untransformed plastome.

Discussion

Duplication of ori sequences is not essential

To our knowledge, this is the first study where replication origins of plastid DNA were analysed by targeted modification using the technique of plastid transformation. A difficulty of this approach is that modifications that would completely abolish plastid DNA replication activity would not lead to transformant lines, as tobacco plants are not able to grow without plastids even in tissue culture. Also, selection marker gene would not be maintained if transformed plastomes did not replicate. Furthermore, inactivation of essential genes such as ycf1 might contribute to this problem. However, in all of the experiments described here, we obtained phenotypically normal transplastomic lines, even if integration of the transplastome caused deletion of ori sequences. This allows us to conclude that at least one copy of each of the described ori sequences is dispensable. The most likely explanation for this is that duplication of the ori sequences on the Inverted Repeats of the tobacco plastome is not essential for plastome replication, which is confirmed by the fact that this duplication is not found in the plastome of pea (Kunnimailaayen and Nielsen, 1997a). Moreover, we conclude that duplication of the ori sequences in tobacco is not important for plastome replication, as all of our mutants showed normal regeneration and growth kinetics in comparison with other neutral plastome transformants in our laboratory.

Plastid transformation of higher plants is more complex in comparison with nuclear transformation due to the high copy number of the plastid genome: a single plastid can contain more than 100 plastome copies, and a single leaf cell contains up to 100 plastids (Bendich, 1987; Svab et al., 1990). As during the process of transformation a single plastid is usually targeted, the primary transformed cell is heteroplasmatic. A homoplasmatic state of the transformed plastome can usually be obtained by applying selection during repeated cycles of regeneration, whereby segregation of transformed and non-transformed plastids can take place. Heteroplasmy can also occur within single plastids, as shown in experiments with the unicellular alga Chlamydomonas reinhardtii, which contains only a single chloroplast per cell (Fischer et al., 1996). The plastid transformants obtained in our experiments showed substantial differences with respect to their degree of homoplasmy: some transformants reached homoplasmy after few cycles of regeneration, whereby there was a strong tendency towards homoplasmy from the beginning (AK1, A2, A2a, A2b, A4, B2); plastome modifications with these plasmids obviously do not disturb plastid DNA replication or other essential functions. Other transformants, however, still contained a high percentage of untransformed plastome molecules even after prolonged regeneration (A1, AK2, B1).

OriA transformants

One explanation for the heteroplasmatic state of A1 and AK2 transformants, where the transgene was inserted within or close to the oriA sequence, could be that the function of oriA was disrupted by the modifications, but at least one copy of oriA per plastome molecule is essential for replication; therefore, plastome molecules would contain the selection marker in one IR and the wild-type sequence in the other IR. However, with our constructs generated for complete deletion of the described oriA sequence (A3, A4) we obtained homoplasmatic transformants or transformants with a high transplastome content. A transplastome content above 50% means that fewer copies of oriA than plastome molecules are present, so that there must be molecules not containing oriA, which replicate in plastids. Therefore, we consider it much more probable that the reason for heteroplasmy in A1 and AK2 lines is disruption of an essential gene function, namely trnl. Insertion into the intron of this gene could prevent or decelerate correct splicing of the RNA, which is dependent on the secondary structure of the group II intron (Holländer and Kück, 1999; Michel et al., 1989). As deprivation of the trnl gene product would prevent plastid translation, and therefore also expression of the essential plastid genes ycf1 and ycf2, the transformed plants could not become homoplasmatic (Boudreau et al., 1997; Drescher et al., 2000). The lower content of wild-type plastome in A3 transformants compared with A1 or AK2 transformants could be explained by a weaker effect on trnl splicing, which still requires some wild-type copies in order to sustain a sufficient level of trnl gene product. In A4 transformants, however, where the complete trnl intron was removed together with the oriA sequence in order to exclude effects on splicing, the oriA sequence could be deleted completely without obvious effects. This allows the conclusion that the described oriA is dispensable for plastome replication in vivo. Interestingly, in an earlier study with proplastids of cultured tobacco cells, no displacement loops were detected in the region of oriA (Takeda et al., 1992). If one follows the model of double-D-loop-replication, which requires two reverse replication origins (Kolodner and Tewari, 1975a), the two reverse copies of oriB might serve this role, or replace oriA in case of its deletion; alternatively, a further sequence not described so far could have ori activity in addition to oriB. A further explanation could be the activation of a different mode of replication in oriA mutants: In a partially purified replicative system of pea chloroplasts,
plasmids containing both ori sequences from pea showed theta structures, which are usually observed in replicating plastomes (Reddy et al., 1994), whereas plasmids containing the oriA or oriB sequence alone showed intermediates typical for rolling circle replication, which was also observed in plastid DNA by Kolodner and Tewari (1975a). Therefore, the possibility exists that in our tobacco mutants plastome replication occurs from oriB alone via a rolling circle mechanism. We did, however, not find any indication for an altered replication rate in our mutants. A hypothetical explanation for plastome replication involving oriA in mutants containing less than 50% of oriA might be replication of plastome multimers from a single oriA sequence. This would, however, require that only a minority of plastome molecules is present as monomers in these mutants, whereas a monomer content of at least 70% was found in several plant species (Deng et al., 1989; Kolodner and Tewari, 1975b), including tobacco (Rainer M. Maier, Munich University, Germany, pers. comm.). Considering the very low content of wild-type DNA in A4 mutants below detection level, if any at all, this possibility appears negligible.

From oriA analysis, our A4 mutants also allow conclusions on maturation of the trnl gene product: considering the normal phenotype of the mutants, we propose that removal of the trnl intron does not have negative effects on tRNA folding or processing of the ends. This is to our knowledge the first example of removal of a chloroplast intron in a higher plant.

Transformation with our vectors AK1 and A4 also affected the plastome region designated as NICE1. This sequence was found as an extrachromosomal element in plastid transformants and hereafter included in transformation vectors carrying marker genes (Staub and Maliga, 1994; Staub and Maliga, 1995). As these vectors could also be detected as extrachromosomal elements in plastids, it was speculated that they are replicated autonomously, implying that the NICE1 sequence exhibits replication origin activity. Our results demonstrate that plants can have a large insertion (AK1 transformants) or a deletion (A4 transformants) in the NICE1 element without preventing plastome replication; normal regeneration time compared with other transformants indicates that the replication rate is not affected. Interestingly, we could not detect any extrachromosomal transformation vector in plants transformed with A1 or AK2, which contain the intact NICE1 sequence, or in AK1- or A4-transformants (data not shown). Thus, as we did not find any indication for ori activity of the NICE1 element, we consider the alternatively suggested mechanism of integration and excision (Staub and Maliga, 1994) a more probable explanation for the extrachromosomal maintenance of NICE1-containing plasmids. However, we can not definitely exclude the possibility that replicative functionality lies within that sequence element of the NICE1 region (105377–106040) that was maintained in our mutants.

Since all of our vectors designed for deletion of the variable elements of Oenothera (A2, A2a, A2b) produced phenotypically normal, homoplastomic transformants, we conclude that this region does not contain an origin of replication in the tobacco plastome. The question of whether this region contains the replication origin in Oenothera can only be answered when plastid transformation of this species has been achieved. As electron microscopic mapping of a displacement loop within this region did not allow precise localisation (Chiu and Sears, 1992), the replication origin in Oenothera might also be within the trnl intron, analogous to tobacco oriA. As published sequences of the different Oenothera plastid types contain a sequence with high homology to tobacco oriA, one might speculate that this sequence acts as an origin of replication also in Oenothera, although our experiments question the function of the described oriA sequence. The region containing the sequence variations may be involved in the regulation of replication in Oenothera, or the sequence variations may have been generated due to replication slippage (Sears et al., 1996) and influence replication incidentally, thus causing the differences in replication strength between the different plastome types. A regulatory function of this sequence in tobacco appears unlikely from our experiments, since we did not observe any effects on phenotype or regeneration kinetics in our mutants.

OriB transformants

For the case of oriB, deletion of each of the two copies produced different results: while the copy in IRg could be completely removed, transformation vectors targeting the IRg copy produced only heteroplasmic lines. This was expected, as deletion of this copy of oriB also affects the essential ycf1 gene, deletion of which had also resulted in heteroplasmia (Boudreau et al., 1997; Drescher et al., 2000). Therefore, we can not distinguish whether this effect is due to deletion of oriB or inactivation of ycf1. Interestingly, in one of the tobacco ycf1 mutants described by Drescher et al. (2000) part of the oriB sequence was also deleted; however, as heteroplasmia was also observed with a further mutant not affecting oriB, we do not think that heteroplasmia of ycf1 mutants was caused by effects on oriB. Furthermore, these mutants still contained the second copy of oriB, and, as shown in our results with transformation vector B2, replication should not be affected as long as there is one copy of oriB. In addition, our results with B2 show that the truncated version of ycf1, orf350, can be deleted without adverse effects.

Most interestingly, in our attempt to target both copies of oriB by co-transformation, we only obtained lines with transgene integration exclusively at one site, although integration at both sites could be shown in primary regenerates. Regarding the high number of primary co-transformants, we
assume that single chloroplasts of a cell were hit by a gold particle with both plasmids rather than different plastids of one cell. The high rate of co-integration shows that homologous recombination in chloroplasts is very efficient and should also occur between molecules having one integration each, leading to molecules with integration at both targeted sites. The fact that these plastomes were not found in any of the lines after segregation strongly suggests that there is selection against them. The most probable explanation for this is that these plastomes are not replicated, which means that one copy of the deleted sequence, which contains the described oriB, is essential for replication. A method to confirm this result could be co-transformation using two selection markers, which is however, not easily achieved: plastid transformation using the nptII gene conferring kanamycin resistance has proven rather inefficient (Carrer et al., 1993), and the recently described badh selection (Daniell et al., 2001) has not been reproduced and causes substantial costs for the selection agent.

**Experimental procedures**

**Construction of transformation vectors**

Plasmid pUC16SaadA, which contains a selection marker cassette comprising the aminoglycoside 3'-adenyltransferase (aadA) from *E. coli* under the control of the tobacco *rrn16* promoter, a ribosomal binding site, and a 440-bp fragment of the *Chlamydomonas reinhardtii* rbcL downstream region, was described in Koop et al. (1996). An additional SmaI-site was added upstream of the *rrn16* promoter by insertion of a linker oligonucleotide (gaatccggg-gaacct) into the EcoRI-site to give pUC16SaadA-Sma.

For construction of plastid transformation vectors for the *oriA* region, tobacco (*Nicotiana tabacum* cv. Petite Havana) plastid DNA corresponding to positions 103415–106944 of the published sequence (GenBank accession number Z00044) was amplified with PCR from isolated plant DNA with primers oA1 (5'-aatctgacagcagccggtctctg-3') and oA2 and inserted into the PstI and KpnI restriction sites downstream of the aadA coding sequence. For plasmids A2a and A2b, amplification primer oA4 was replaced by oA5 (5'-aatctgacagcagccggtctctg-3') or oA6 (5'-aatctgacagcagccggtctctg-3'), respectively, and the resulting fragment corresponds to nucleotides 104485–108844 or 104466–106944, respectively.

For construction of plasmid A3, part of the left flank of plasmid AK2 excluding the *oriA* sequence was PCR-amplified with primers oA1 (see above) and oA7 (5'-tctctgagggaggaggttttctg-3'), using AK2 as template. In parallel, a second fragment containing the N-terminal part of the aadA sequence was amplified with primers oA8 (5'-agacctagcttgctctagctg-3') and oA9 (5'-ttctctgagggaggaggttttctg-3'). Each of the resulting fragments was ligated independently into the Smal site of cloning vector pUC18 (Stratagene, La Jolla, CA, USA), so that the oA7-end or oA9-end, respectively, was located next to the XbaI site. Both inserts could then be excised with XbaI and Bpu1102I, or XbaI and BsrGI, respectively, and joined together in the Bpu1102I and BsrGI restricted plasmid AK2, resulting in plasmid A3.

Plasmid A4, containing an intron-less *tnl* gene, was constructed by modification of plasmid AK1: a PCR fragment containing the 5'-enon of *tnl* and part of the aadA sequence was amplified with primers oA8 and oA10 (5'-ggtggaggctcttgatcgtcatg-3') and ligated into the Smal site of cloning vector pUC18, restoring the Smal site at the oA10-end. A second PCR fragment containing part of the left flank of AK1 up to the 5'-enon of *tnl* was amplified with primers oA11 (5'-ccctgatgccctgtctctg-3') and oA12 (5'-ttctctgagggaggaggttttctg-3'), restricted with EcoRI, and inserted into the EcoRI and Smal restricted vector described above, joining the two *tnl* exons at the Smal site. A Bpu1102I-BsrGI fragment from the resulting plasmid was excised and used for replacement of the corresponding fragment of plasmid AK1, resulting in A4.

For construction of plastid transformation vector B2, tobacco plastid DNA sequence 112061–113077 was amplified with PCR from isolated tobacco DNA using primers oB1 (5'-ggtggaggctcttgatcgtcatg-3') and oB2 (5'-ggtggaggctcttgatcgtcatg-3'). The resulting fragment was restricted with KpnI and XbaI and inserted into the corresponding sites of plasmid pUC16SaadA-Sma, downstream of the *aadA* gene. A second tobacco plastid DNA fragment (109985–111112) was amplified with primers oB3 (5'-tctctgagggaggaggttttctg-3') and oB4 (5'-tctctgagggaggaggttttctg-3'), restricted with XmaI, and inserted into the corresponding site of the plasmid described above, upstream of the chimeric *aadA* gene, with the oB4-end of the fragment next to the *rrn16* promoter.

Plasmid B1 was constructed from B2 by removing the plastid DNA sequence downstream of *aadA* with Acc65I and Sall and replacing it with a new plastid DNA flank (129113–130479), which was PCR amplified from tobacco plastid DNA with primers oB5 (5'-tctctgatgccctgtctctg-3') and oB6 (5'-ggtggaggctcttgatcgtcatg-3'), ligated into the Smal site of cloning vector pUC18 so that the oB5-end was next to the Sall site, and excised with Acc65I and Sall.

**Transformation of tobacco plastids by biolistic delivery**

Tobacco seeds (*Nicotiana tabacum* cv. Petite Havana) were surface sterilised (1 min in 70% ethanol, 10 min in 5% Dimanin C, Bayer, Leverkusen, Germany), washed three times for 10 min in sterile H2O and put on SCN-medium (Dovzenko et al., 1998). Plants were
grown at 25°C in a 16-h light/8-h dark cycle (0.5–1 W m⁻², Osram L85W/25 Universal-White fluorescent lamps).

Leaves from 4-week-old, sterile grown plants were cut and placed with abaxial side up to solid RMOP-medium (Svab et al., 1990). Gold particles (0.6 micron, Biorad, Hercules, CA, USA) were collected by centrifugation of 35 μl suspension (60 mg ml⁻¹ in ethanol) and washed with 1 ml sterile H₂O. The gold pellet was resuspended in 230 μl sterile H₂O and 250 μl 2.5 M CaCl₂, and 25 μg of plasmid DNA were added. After thoroughly resuspending the mixture, 50 μl 0.1 M spermidin were added, mixed and incubated for 10 min on ice. The coated gold particles were collected by centrifugation, washed twice with 600 μl ethanol, and finally resuspended in 72 μl ethanol. 5.4 μl of gold suspension per bombarded leaf was applied to a macrorosette. Bombardment was carried out with a Bio-Rad PDS-1000/He Biolistic particle delivery system (Bio-Rad, Hercules, CA, USA) using the following parameters: rupture disc 900 psi; vacuum 26–27 inches Hg; macrorosette at the top level; leaf piece at the third level.

Two days after bombardment, leaves were cut into small pieces (approximately 3 x 3 mm) and transferred to RMOP-medium containing 500 μg/ml spectinomycin hydrochloride. Leaf pieces were cut again and transferred to fresh medium after 2 weeks, then every 3 weeks until no further regenerates appeared. Green regenerates were retrieved and transferred to individual plates. The lines were subjected to repeated cycles of shoot generation by cutting small leaf pieces, from which new shoots were regenerated on RMOP-medium with 500 μg ml⁻¹ spectinomycin.

**Molecular analysis of plastid transformants**

For isolation of total plant DNA, 100 mg of fresh leaf tissue were disrupted in 200 μl AP1 buffer (DNasey Plant Mini Kit, QIAGEN, Hilden, Germany) + 1 μl reagent DX (foaming inhibition, QIAGEN) using mixer mill MM 300 (Retsch, Haan, Germany) in a 1.5 ml microcentrifuge tube with one 3 mm tungsten carbide bead (2 x 1 min at 25 Hz). DNA was then purified using the DNeasy Plant Mini Kit. Alternatively, a protocol using cetyltrimethylammonium bromide (CTAB) was used as described in Koop et al. (1996).

PCR analysis was performed according to standard protocols, using a primer binding within the aadA coding sequence in combination with a primer binding in the plastome near the insertion site, but outside of the transformation vectors in order to detect plastome insertion. For analysis of B1 transformants, the primers were oIntAAD5 (5'-cctgcgtagctcttgctg-3') and oIntB1 (5'-tggggtcttatccactacatttcgctcatcgcc-3'); for analysis of B2 transformants, primers oIntAAD5 and oIntB2 (5'-tggggtcttatc-184) were used. For analysis of all transformants in the oriA region, primers oIntAAD3 (5'-cacattctgctgcatcgc-3') and oIntA (5'-gtcgtggcagctcactaac-3') were used.

For Southern analysis, 3 μg of total plant DNA per analysed plant were digested with restriction enzyme and separated on a 0.6%-agarose gel. DNA was denatured and transferred to a positively charged nylon membrane (Hybond-N+, Amersham, Little Chalfont, UK) according to a standard protocol. Probes were PCR-amplified, labeled with 32P using Klenow fragment, and hybridised to the membrane in 250 mM sodium phosphate, 7% SDS at 65°C. Washing was carried out with 0.5 x SSC, 0.1% SDS at the same temperature. Hybridisation signals were detected using a phosphiomager (FujiFilm BAS 1500, Fuji Photo Film, Tokyo, Japan). The probe used for analysis of the oriA region was specific to tobacco plastome sequence 105828–106503 and 136123–136798 and amplified from plasmid A2 with primers oPA1 (5'-cagcagattcaccacaagc-3') and oPA2 (5'-tgcaggtgtctcttgctg-3'). oriB transformants were analysed with probe ‘23S’, specific to tobacco plastome sequence 108325–109255 and 133731–134301, which was amplified from isolated plastid DNA with primers oPB1 (5'-tctgcgtagctcttgctg-3') and oPB2 (5'-tgcaggtgtctcttgctg-3'), and with probe ‘aadA’, specific to the aadA coding sequence and amplified from plasmid A2 with primers oAAD1 (5'-tgcaggtgtctcttgctg-3') and oAAD2 (5'-cactacattctgcatcgc-3'). Filters were also hybridised with a probe derived from Lambda DNA in order to detect size marker bands.

**Acknowledgements**

This work was supported through Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 184. The expert technical assistance by Katharina Yeiser is gratefully acknowledged.

**References**


