The Plant Journal (2002) **32**, 175–184

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 *trnI* 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 *trnI* intron, the second *trnI* 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_A, *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 homoplastomic *ycf1* deletion mutants of *Chlamydomonas* and tobacco could not be achieved (Boudreau *et al.*, 1997; Drescher *et al.*, 2000). In IR_B, *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 *et al.*, 1990). 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 homoplastomic 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

ribosome binding site, and was not fused to a promoter or a terminator sequence; in transformed plastome, transcription of *aadA* can be achieved by the promoter of the operon, whereas expression of the other genes of the operon should not be influenced by additional promoter or terminator elements.

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 *rrn16* 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 *trnI* 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_A and IR_B copy of the targeted sequences. In all cases, transformant or wild-type signals were comparable for both sites.

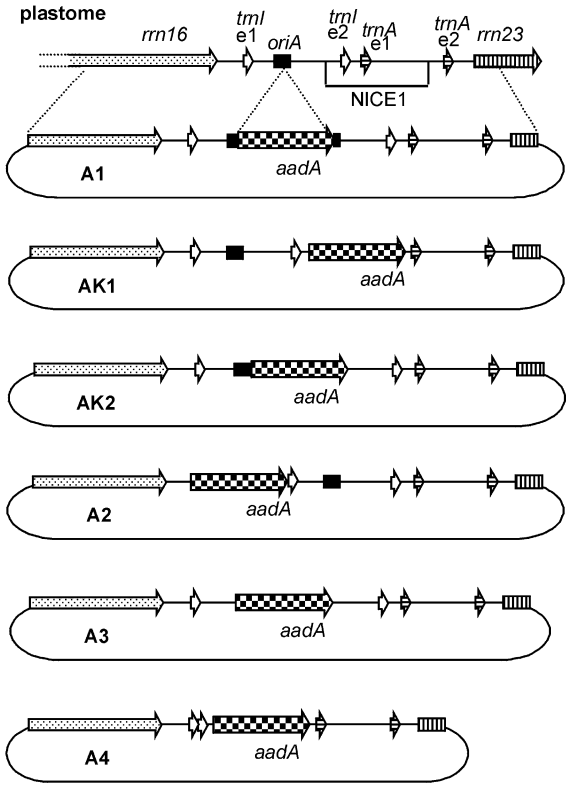


Figure 1. Plastid transformation vectors for analysis of the *oriA* region.
The uppermost panel shows the region of tobacco plastid DNA containing the described *oriA* sequence. Arrows mark the direction of transcription; all of the genes shown are transcribed from the promoter upstream of the *rrn16* gene as an operon. e1 and e2 denote the exons of the *trnI* 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 *trnI* and *trnA* (AK1), upstream of *trnI*, deleting the sequence corresponding to the variable elements of *Oenothera* plastomes (A2), within the *trnI* intron, deleting the complete *oriA*-sequence (A3), and between *trnI* and *trnA*, whereby the two exons of *trnI* are immediately joined, deleting the *trnI* intron including *oriA* (A4).
Black box: *oriA*; white arrows: *trnI* exons; horizontally striped arrows: *trnA* exons; checkered arrow: *aadA*; dotted arrow: 3′-part of *rrn16*; vertically striped arrow: 5′-part of *rrn23*. Drawing is not to scale.

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 *trnI* 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 *trnI* transcript due to insertion of the *aadA* sequence within the intron, a further construct, A4 (Figure 1), was made, where the complete intron sequence

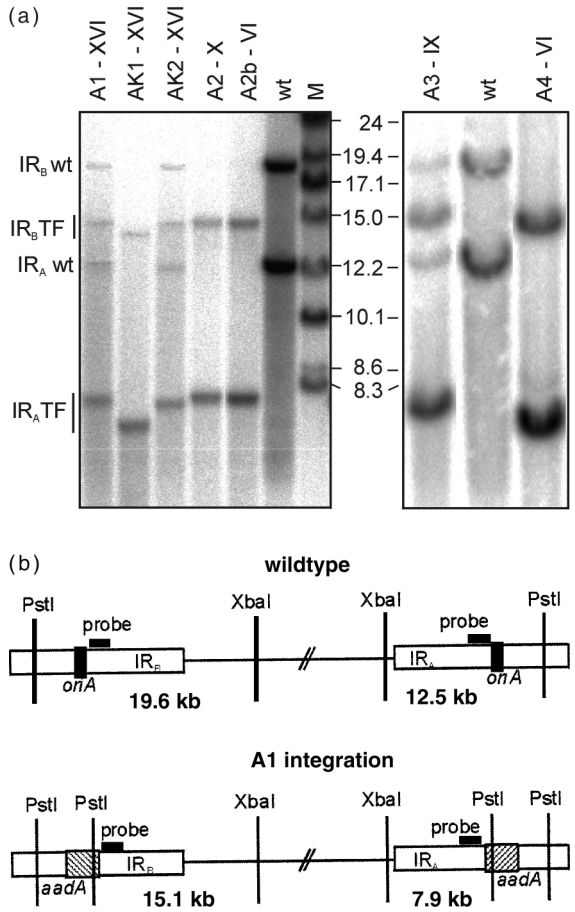


Figure 2. Southern analysis of plastid transformants in the *oriA* region.
(a) Total plant DNA was restricted with PstI and XbaI and hybridised with a fragment corresponding to tobacco plastid DNA 105828–106503 and 136123–136798. Roman numerals give the number of regeneration cycles before analysis. 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_B wt indicates the 19.6 kb fragment of wild-type plastome containing the IR_B copy of *oriA*; IR_A wt indicates the 12.5 kb wild-type fragment from IR_A. IR_B TF and IR_A 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_B fragment and 7.9 kb for the IR_A fragment; AK1 and A4: 14.5 kb and 7.4 kb; AK2 and A3: 15 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.

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.

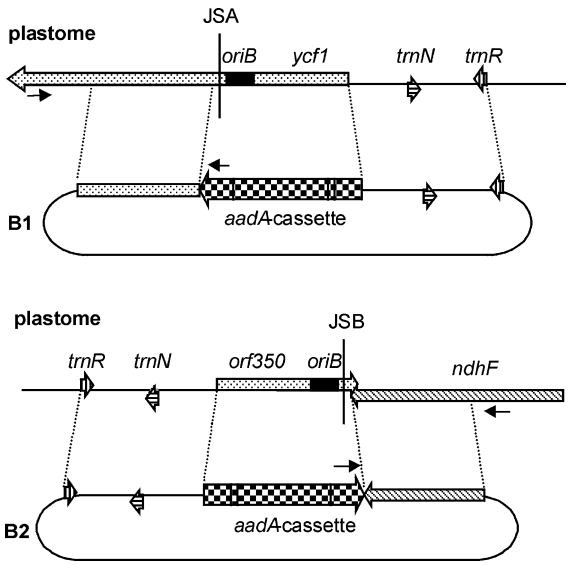
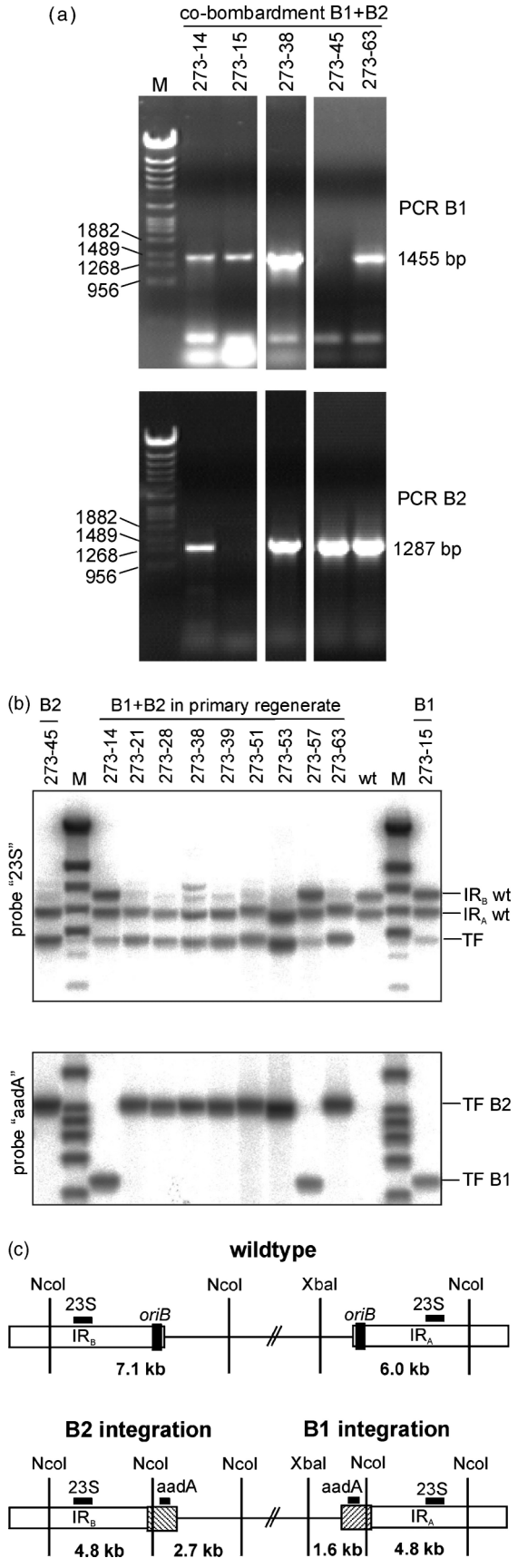


Figure 3. Plastid transformation vectors for analysis of *oriB*.
In the circles, a schematic depiction of the integration cassette of transformation vectors B1 and B2 is given. The targeted region of the plastid genome, containing the *oriB* sequence at the end of Inverted Repeat A or B, respectively, 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: *trnR*; 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.

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_A and contains plastid sequences 131514–132641 (corresponds also to 109985–111112 as within the IR) and 129113–130479 (small single copy region adjacent to IR_A), while the intermediate sequence, representing the N-terminal part of the ycf1 gene including *oriB*, is replaced by the *aadA* coding sequence. This is provided with the tobacco *rrn16* promoter, an artificial ribosome binding site, and a 3′-UTR element from the *Chlamydomonas reinhardtii rbcL* gene. Plasmid B2 (Figure 3), which was designed for deletion of the IR_B copy of *oriB*, differs from B1 in the plastid DNA sequence downstream of the chimeric *aadA* cassette, which corresponds to plastome position 112061–113077 (small single copy region adjacent to IR_B). Hence, plastome transformation with B1 deletes not only one copy of *oriB*, but also the N-terminal part of the essential *ycf1* gene (Drescher *et al.*, 2000), while with B2 only the *orf350* reading frame is concerned, although an identical sequence is deleted.



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 *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 *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 *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 *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 *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 *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 *aadA* at the site targeted by B2; the expected product of 1287 basepairs is indicated. One primer binds within the *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^{TM} DNA Ladder Mix, MBI Fermentas, Vilnius, Lithuania), the length of selected fragments in basepairs is indicated on the left.
(b) Southern analysis of *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 NcoI and XbaI and hybridised with two different probes. The upper panel shows hybridisation with probe ''23S'', corresponding to tobacco plastome sequence 108325 to 109255 and 133371 to 134301. IR_B wt indicates the 7.1 kb fragment of wild-type plastome containing the IR_B copy of *oriB*; IR_A wt indicates the 6.0 kb wild-type fragment from IR_A. The IR_A copy of *oriB* is targeted by B1, the IR_B 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 *aadA* sequence in order to differentiate between the two transgene integration sites. TF B1 indicates the 1.6 kb fragment resulting from *aadA* integration at the site targeted by B1; TF B2 indicates the 2.7 kb fragment resulting from *aadA* integration at the site targeted by B2. M: Lambda DNA Eco130I/MluI, 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 hybrisdised with a probe derived from Lambda DNA in order to detext 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, the 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 (Kunnimalaiyaan and Nielsen, 1997a). Moreover, we conclude that duplication of the ori sequences in tobacco is not important for the rate of 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 heteroplastomic. A homoplastomic 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 heteroplastomic 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 these 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 homoplastomic 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 *trnI*. 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 *trnI* gene product would prevent plastid translation, and therefore also expression of the essential plastid genes *ycf1* and *ycf2*, the transformed plants could not become homoplastomic (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 *trnI* splicing, which still requires some wild-type copies in order to sustain a sufficient level of *trnI* gene product. In A4 transformants, however, where the complete *trnI* 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.

Apart from oriA analysis, our A4 mutants also allow conclusions on maturation of the *trnI* gene product: considering the normal phenotype of the mutants, we propose that removal of the *trnI* 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 *trnI* 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 IR_B could be completely removed, transformation vectors targeting the IR_A copy produced only heteroplastomic lines. This was expected, as deletion of this copy of *oriB* also affects the essential *ycf1* gene, deletion of which had also resulted in heteroplasmy (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 decribed by Drescher *et al.* (2000) part of the *oriB* sequence was also deleted; however, as heteroplasmy was also observed with a further mutant not affecting *oriB*, we do not think that heteroplasmy 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 (gaattcccgggaattc) 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′-ttcggccggaaagaacaccaacggcg-3′) and oA2 (5′-gccggtaccaagccactgcctatgag-3′), whereby a KpnI and an EagI restriction site were added to the ends. The resulting fragment was ligated into the corresponding sites of vector pBluescript II SK- (Stratagene, La Jolla, CA, USA), giving plasmid A0. For construction of plastid transformation vector A1, plasmid A0 was restricted with BamHI, and the overhanging ends were filled in with T4 DNA polymerase; this plasmid was used for insertion of an EcoRV-DraI fragment from pUC16SaadA-Sma, containing the *aadA* coding sequence with the RBS. For construction of vector AK1, the *aadA* fragment was inserted into the NsiI-site of A0 after blunting with T4 DNA polymerase. For construction of AK2, a 40-bp fragment was removed from A0 by restriction with XhoI, and the *aadA* fragment was inserted between the blunted ends.

To construct vector A2, the *aadA* coding sequence with RBS was excised from pUC16SaadA-Sma with SpeI and PstI and ligated into the corresponding sites of vector pBluescript II SK- (Stratagene, La Jolla, CA, USA). The EagI and XbaI restriction sites upstream of *aadA* were then used for insertion of a fragment of plastid DNA corresponding to nucleotides 103415–104427, which was PCR-amplified from isolated tobacco DNA (*Nicotiana tabacum* cv. Petite Havana) with primers oA1 and oA3 (5′-gctctagacctatcaacttgttccgacc-3′). A second fragment of plastid DNA corresponding to nucleotides 104544–106944 was amplified with primers oA4 (5′-aactgcagcacgacgggctattagctc-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′-aactgcagagagggatggggtttctc-3′) or oA6 (5′-aactgcagacatgggggcgaaaaaagg-3′), respectively, and the resulting fragment corresponds to nucleotides 104488–106944 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′-tcctgaggaggagtttggtttc-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′-agcactacatttcgctcatcgc-3′) and oA9 (5′-atcactagttgtaggagggatc-3′). Each of the resulting fragments was ligated independently into the SmaI 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 *trnI* gene, was constructed by modification of plasmid AK1: a PCR fragment containing the 3′-exon of *trnI* and part of the *aadA* sequence was amplified with primers oA8 and oA10 (5′-gggcgaggtctctggttcaagtccaggat-3′) and ligated into the SmaI site of cloning vector pUC18, restoring the SmaI site at the oA10-end. A second PCR fragment containing part of the left flank of AK1 up to the 5′-exon of *trnI* was amplified with primers oA11 (5′-cgtgtttagttgccatcgttgag-3′) and oA12 (5′-ttatcaggggcgcgctctaccactgagctaata-3′), restricted with EcoRI, and inserted into the EcoRI and SmaI restricted vector described above, joining the two *trnI* exons at the SmaI site. A Bpu1102I-BsrGI fragment from the resulting plasmid was ecxised 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′-ggggtaccgaatttgattcacaaagttg-3′) and oB2 (5′-gctctagatgtggtattccacctcttgc-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′-tccccccgggctcagaggattagagcacg-3′) and oB4 (5′-tccccccgggagtccgaccacaacgacc-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 SalI and replacing it with a new plastid DNA flank (129113–130479), which was PCR amplified from tobacco plastid DNA with primers oB5 (5′-cttctgctatctctatccgtgc-3′) and oB6 (5′-agacctcttctggtttcaacagc-3′), ligated into the SmaI site of cloning vector pUC18 so that the oB5-end was next to the SalI site, and excised with Acc65I and SalI.

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 H_2O and put on SCN-medium (Dovzhenko *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 H_2O. The gold pellet was resuspended in 230 μl sterile H_2O and 250 μl 2.5 M $CaCl_2$, 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 macrocarrier. 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; macrocarrier at the top level; leaf piece at the third level.

Two days after bombardment, leaves were cut into small pieces (approximately 3 × 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 (DNeasy 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 × 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 sequence used in the transformation vectors in order to detect plastome insertion. For analysis of B1 transformants, the primers were oIntAAD5 (5′-cggatgtaactcaatcggtag-3′) and oIntB1 (5′-ttgcgcaaaaggaatggtatc-3′); for analysis of B2 transformants, primers oIntAAD5 and oIntB2 (5′-tggggtcttatcgaagcg-3′) were used. For analysis of all transformants in the *oriA* region, primers oIntAAD3 (5′-cactacatttcgctcatcgcc-3′) and oIntA (5′-gctggcggcatgcttaacac-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 α^{32}P 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 × SSC, 0.1% SDS at the same temperature. Hybridisation signals were detected using a phosphoimager (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′-acgagatcaccccaaggac-3′) and oPA2 (5′-tcgccaggttgtctcttgc-3′). *oriB* transformants were analysed with probe '23S', specific to tobacco plastome sequence 108325–109255 and 133371–134301, which was amplified from isolated plastid DNA with primers oPB1 (5′-tctcgccgtgaccttctcttg-3′) and oPB2 (5′-tctgggcactgtctcggagagag-3′), and with probe 'aadA', specific to the *aadA* coding sequence and amplified from plasmid A2 with primers oAAD1 (5′-tgctggccgtacatttgtacg-3′) and oAAD2 (5′-cactacatttcgctcatcgcc-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.

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