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Deletions/insertions, short inverted repeats, sequences resembling *att*-lambda, and frame shift mutated open reading frames are involved in chloroplast DNA differences in the genus *Oenothera* subsection *Munzia*

J. vom Stein and W. Hachtel

Botanisches Institut der Universität Bonn, D-5300 Bonn 1, Federal Republic of Germany

Summary. A restriction fragment length mutation has been mapped in the large single copy region of the chloroplast DNA from two *Munzi-Oenothera* species. Fragments containing the deletion/insertion were cloned, further analysed by additional restriction enzymes, and sequenced. A deleted/inserted 136 bp sequence was identified upstream of the 5′ end of a tRNA-Leu (UAA) gene and presumably is located in the spacer between this gene and a tRNA-Thr (UGU) gene. The endpoints of the 136 bp sequence are covered by short inverted repeats. Complementary inverted repeats are present in the middle of the deleted/inserted sequence. The repeats are part of sequences resembling the lambda chromosomal attachment site (*att*-lambda) which is essential for site specific recombination in the lambda/*Escherichia coli* system. Possible interactions of the repeats during the deletion/insertion process are discussed. The spacer also contains a 1 bp deletion/insertion within an open reading frame (ORF). Due to this frame shift mutation the ORF sizes are quite different between the two *Oenothera* species.

Key words: *Oenothera* (subsection *Munzia*) chloroplast DNA – Deletion/insertion – Inverted repeat – *att*-lambda – Open reading frame

Introduction

Much of the variation in sequence complexity of angiosperm chloroplast DNA (cpDNA) appears to be the result of rather small length mutations. By far the most common of these events are small deletions and insertions 1–10 base pairs in size. At our present knowledge these occur predominantly in noncoding regions. Larger length mutations, 50–1200 bp in size, occur at a significantly lower frequency. Such events, however, are likely to provide an important component of cpDNA size and sequence complexity (Palmer 1985). Often, these mutations have been found to cluster in "hotspots" (Salts et al. 1984; Palmer et al. 1985). Small length mutations might occur mainly by slippage and mispairing during DNA-replication and repair. Additional, yet undocumented processes – e.g. recombination processes (Bowman and Dyer 1986) – might account for the large and moderate-sized length mutations.

Offprint requests to: W. Hachtel

To reveal underlying processes responsible for cpDNA variation and to evalute the possible biological significance of this variability, we continued sequence studies to determine the structural basis of length differences between the cpDNAs of two closely related species in the genus *Oenothera* subsection *Munzia* and their reciprocal hybrids (Alt et al. 1982; vom Stein and Hachtel 1986). These differences have been characterized as deletions/insertions and localized within five regions scattered along the plastome (vom Stein and Hachtel 1986). A 24 bp sequence duplication/deletion was found in the large inverted repeats within the intron of the *ndhB* gene (vom Stein and Hachtel 1988). The predicted secondary structure of the *ndhB*-intron is altered by this duplication/deletion. This might affect the splicing process of the *ndhB*-transcript. Furthermore, the short direct repeat shows remarkable similarity to the lambda chromosomal attachment site (*att*-lambda) which is essential for site specific recombination in the phage lambda/*Escherichia coli* system (Nash 1981). In the work described here a deletion/insertion in the large single copy region was analysed in more detail.

Materials and methods

Chloroplast DNA cloning. Cp DNA was prepared from *Oenothera odorata* "Erlangen" Haustein (*Oe. picensis* subsp. *picensis*) and *Oe. berteriana* "Erlangen" Haustein (*Oe. villaricae* Dietrich) as described earlier (vom Stein and Hachtel 1986). Restriction endonuclease digests were performed as specified by the supplier (Boehringer Mannheim). Agarose gel electrophoresis followed standard procedures (Maniatis et al. 1982). CpDNA restriction fragments from *Oe. berteriana* and *Oe. odorata* were cloned in the plasmid pUC19 (Yanisch-Perron et al. 1985). *E. coli* strain JM 103 (Vieira and Messing 1982) was transformed according to the standard $CaCl_2$ protocol (Maniatis et al. 1982). Recombinant clones were analysed by DNA preparation from 1 ml cultures as described by Holmes and Quigley (1981) or by colony hybridization directly on dried-down agar plates (Hiesel and Brennicke 1983). Plasmid DNA was isolated according to Brennicke (unpublished). *E. coli* cells were pelleted from 500 ml cultures and incubated with lysozyme (2 mg/ml) on ice for 30 min. Triton X-100 was added (final concentration 1%) and the mixture immediately centrifuged. DNA was isolated from the supernatant by CsCl density gradient centrifugation in a Beckman VTi65 vertical rotor (150000 × *g*, 18 h).

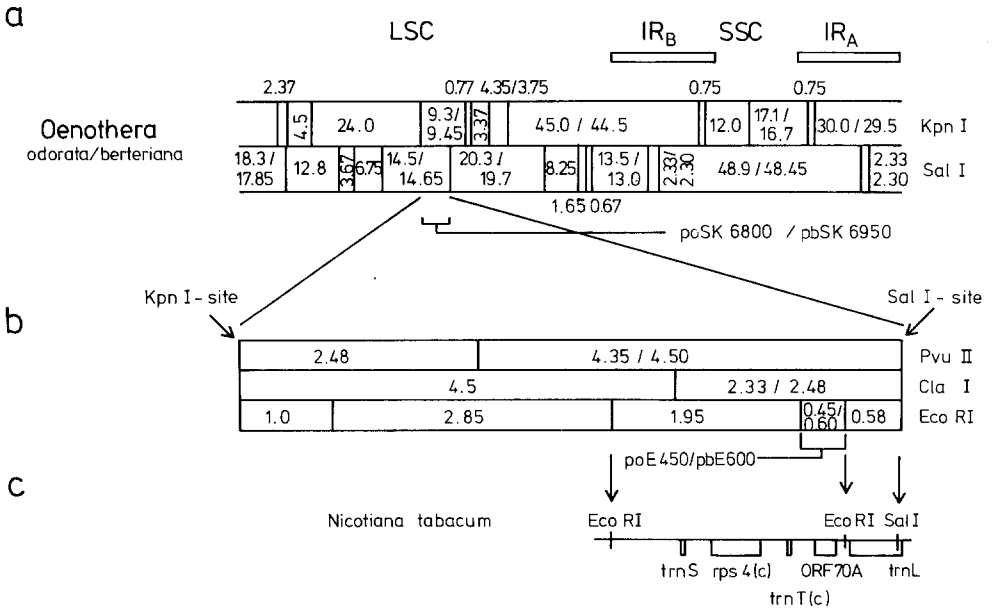

Fig. 1a–c. Detailed mapping of a deletion/insertion in the large single copy of the chloroplast (cp) DNA from *Oenothera odorata* and *Oe. berteriana* and comparison with *Nicotiana tabacum* cpDNA. **a** *Sal*I and *Kpn*I map of the whole plastome from *Oe. odorata*/*Oe. berteriana*. **b** Detailed *Pvu*II, *Cla*I and *Eco*RI map of a single *Sal*I-*Kpn*I fragment containing the deletion/insertion. Fragment sizes are given in kbp. Fragments containing two fragment sizes are involved in deletion/insertion. Cloned fragments containing the analyzed deletion/insertion are marked with plasmid names. **c** Comparison of the region containing the deletion/insertion with *Nicotiana* cpDNA (Shinozaki et al. 1986). Common restriction sites are marked by *vertical arrows* (LSC, SSC large and small single copy region; IR inverted repeat)

DNA sequencing. Nucleotide sequences of the plasmids poE450 and pbE600 (Fig. 1) were determined by the pUC19-dideoxy method (Sanger et al. 1977; Chen and Seeburg 1985) using the pUC sequencing kit from Boehringer Mannheim. The sequences of the plasmids have been analysed independently from different clones and different plasmid DNA preparations. Additional oligonucleotides (5′→3′: AGAAATTGAATATTGC, GGGTCTCCGATAGAA, ATGACAAGTAATAGAA) were synthesized with the Cyclone™ DNA Synthesizer from BioSearch as specified by the supplier and used as primers for sequencing parts of the plasmids poSK6800 and pbSK6950 (Fig. 1). Sequencing polyacrylamide gel electrophoresis was performed in the Biorad Sequence Gene Cell. Gels were subjected to autoradiography with a Kodak X-Omat AR film. Sequence data were handled with the Sequence Analyse Package described by Stephens (1985).

Results

Mapping and sequencing of a deletion/insertion

A restriction fragment dimorphism of a *Sal*I-*Kpn*I fragment has been localized in the large single copy of *Munzia-Oenothera* cpDNA (vom Stein and Hachtel 1986). The size of the fragment has been shown to be 6.8 kb in *Oe. odorata* and 6.95 kb in *Oe. berteriana* (Fig. 1). We supposed that this length difference results from a deletion/insertion. Both fragments have been cloned (poSK6800/pbSK6950) and mapped with the restriction endonucleases *Cla*I, *Pvu*II and *Eco*RI (Fig. 1). The smallest fragments containing the region of variability are a 450 bp *Eco*RI fragment in *Oe. odorata* and a 600 bp *Eco*RI fragment in *Oe. berteriana*. Neither restriction site alterations nor length differences between fragments outside of these *Eco*RI fragments have been detected with agarose gel electrophoresis. The *Eco*RI fragments containing the deletion/insertion were cloned (poE450/pbE600) and sequenced in both 5′ and 3′ directions using the corresponding primers for the pUC19 vector plasmid. Additionally, the adjoining regions in the 5′ and 3′ directions were sequenced using synthesized oligonucleotides as primers. Comparison of sequences (Fig. 2) shows that the length difference is mainly caused by a 136 bp deletion/insertion at position 118 in *Oe. odorata* and between positions 112 and 248 in *Oe. berteriana*.

The borders of the deleted/inserted 136 bp sequence lie within a short inverted repeat 5′-AAATGAT-3′ (Fig. 2, *Oe. berteriana*). In *Oe. odorata* only one shortened copy 5′-AAATGA-3′ of this repeat is present at the deletion/insertion site (Fig. 2). In the middle of the deleted/inserted sequence in *Oe. berteriana* two adjoining sequences, 3′-TTACTA-5′ and 5′-TTCTA-3′, are present which show an almost perfect complementarity to the inverted repeats at the borders. Thus, they form a second set of inverted repeats including only a single mismatch (Fig. 2).

The deletion/insertion presumably is located within the spacer between the genes for tRNA-Thr (UGU) and tRNA-Leu (UAA). We have determined the 5′ exon of tRNA-Leu (UAA) (Fig. 2) but not yet the tRNA-Thr (UGU) gene, which probably is at a greater distance in *Oenothera* than in *Nicotiana*. However, the comparison of restriction sites of several endonucleases (Fig. 1) and hybridization experiments (not shown) demonstrate that the relative location of this sequence in the plastome is the same in *Nicotiana* and *Oenothera*. Additionally, the analysed sequence shows significant homology to the *Nicotiana* sequence (Shinozaki et al. 1986; Fig. 3). The level of homology is different in the various regions of the analysed sequence. Near the 5′-exon of tRNA-Leu (UAA) the homology is 70%–95%. In the middle part of the spacer region between the two

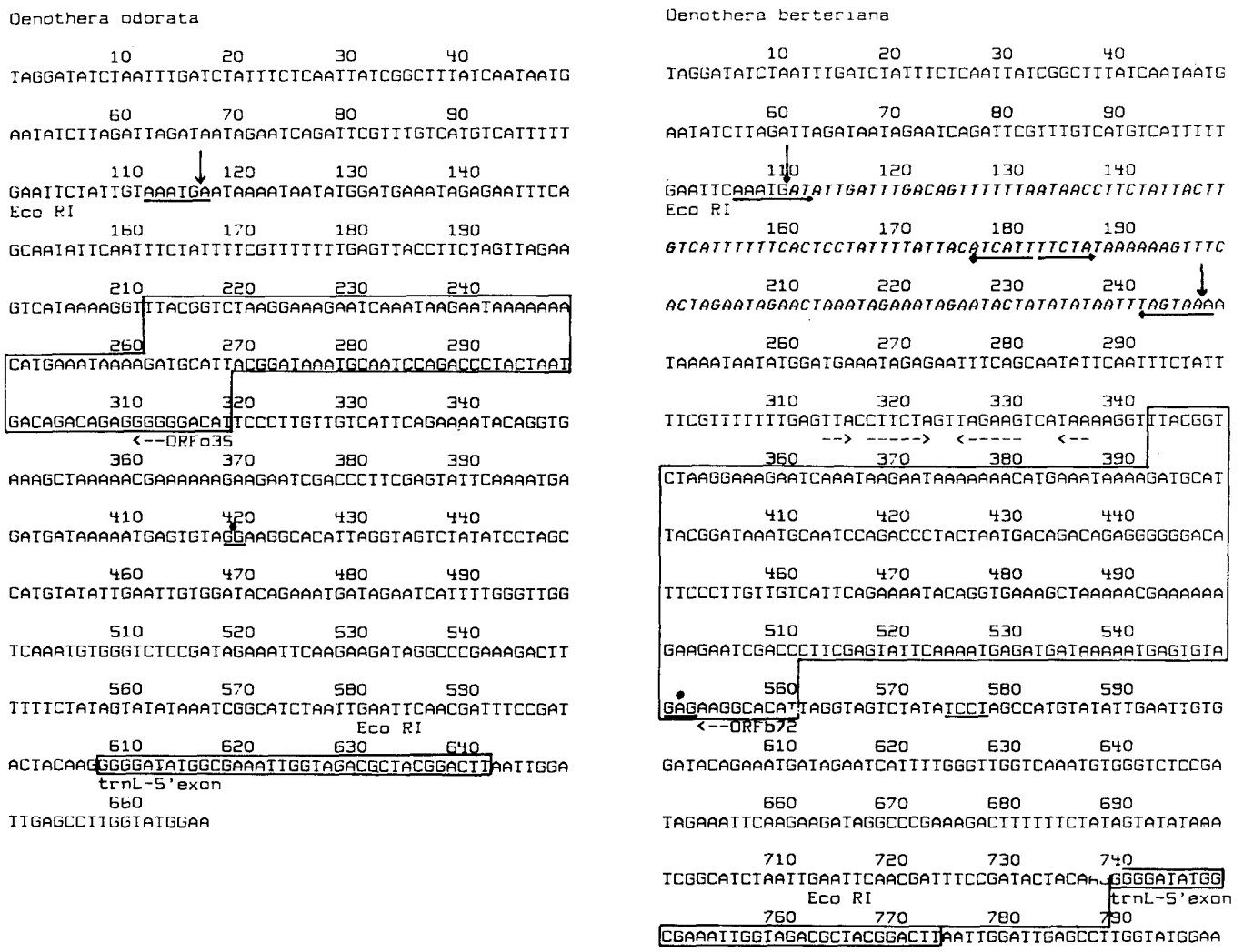

Fig. 2. Chloroplast DNA sequences from *Oenothera odorata* and *Oe. berteriana* containing deletions/insertions. The 136 bp insertion/deletion positions are marked by *vertical arrows*. The inverted repeats in *Oe. berteriana* are indicated by *horizontal arrows*, and the corresponding single copy sequence in *Oe. odorata* is underlined with a *solid line*. The deleted/inserted sequence in *Oe. berteriana* is given in *italics*. The 1 bp deletion/insertion is *underlined* and marked with a *dot*. The sequences of ORFo35, ORFb72 and the 5′exon of tRNA-Leu (UAA) are *boxed*. A sequence that might function as a ribosomal binding site is *underlined*. Elements of a possible hairpin termination structure are underlined with *broken arrows*

tRNA genes the homology is only 35%–50%. Only 4 bp upstream of the 5′-exon of tRNA-Leu (UAA) a 56 bp sequence is present in *Nicotiana* but absent in *Munzia-Oenothera*.

Open reading frames (ORFs)

In the cpDNA of *Nicotiana tabacum* the spacer between tRNA-Thr (UGU) and tRNA-Leu (UAA) contains an open reading frame with 70 codons (ORF70A) between positions 48933 and 49145 (Shinozaki et al. 1986). The ORF70A is not present in the analysed sequence of both *Munzia-Oenothera* species. The homology of ORF70A to the analysed *Oenothera* sequence is only 40%–60% (Fig. 3). Instead, the analysed sequence of *Oe. berteriana* contains an open reading frame with 72 codons (ORFb72) between positions 562 and 344 on the complementary strand in relation to ORF70A (Fig. 2). Upstream of the 5′ end of ORFb72 there is a putative ribosomal binding site: the sequence 5′-AGGA-3′ (-17 to -14 with respect to the start codon ATG) is complementary to the sequence TCCT in the position −6 to −3 relative to the 3′ end of *N. tabacum* 16 S rRNA (positions 104241 to 104244 according to Shinozaki et al. 1986). Downstream of the 3′ end of ORFb72 a putative termination hairpin structure was found (Fig. 2). Comparison of the analysed sequences between *Oe. berteriana* and *Oe. odorata* shows that there is a 1 bp deletion/insertion within ORFb72 at position 552/421 (Figs. 2, 4). The deletion of a single A base in *Oe. odorata* leads to a frame shift and almost halves the ORF size (Fig. 6). Due to this frame shift mutation the start codon of ORFb72 cannot be used in *Oe. odorata* so that the reading frame starts at the next start codon at position 320 (Figs. 2, 5). The reading frame in *Oe. odorata*, therefore, has only 35 codons (ORFo35) which are identical to the last 35 codons of ORFb72 (Fig. 5).

Discussion

The cpDNA differences between the two *Munzia-Oenothera* species described in this report are caused by one 136 bp and one single bp deletion/insertion. These have been local-

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Oen          IAGGAIAICIAATTTGAICIAI?I?ICICAATTAICGGCTTTAICAAIAAIGAA
             * *** **** * ** ** **  * * ***** **     *  ** * *  ****
Nic   48627 TTGGA?AICTTAGII?AI?IAACIAI?ICAAI?AI????I??AI?A?I??IGAA

IAI?CTIAGAI??IA?G?AI?AAIA??G??A?AICAGA?II?CG?TTTGICAIGICATTTTTGAAI
*** ** ***   ** * ** **** *  * ** *** ** ** *** ***   ** ** * ***
IATTCI?AGAACAIAAGGAIIAAIAIAGCGAIAI?AGAATTICGATTTAICA???CAAITCI?AAI

T?CIATTGIAA?A?IGAAIAAAAI?AA?IA?IGGAI?G?AAAIA??G??AGA?AII?I????????
  * *** *** * * ****  ** ** ** ** ** * *****  *  * * *** *
AACAAITCIAAIACI?AAIAIIAIIAAAIAGIG?AIIGIAAAIAIIGIIA?AIAITCITTTTTTTT

CAG???CAAI???A?I??ICAAI?IICIAIII??I??CGITTTTTTG??AGII?A???CCIICIAG
**    * **   * *  * *** **** ***  *  * ********  * ** *   **** * *
CAIIIICCAIIIGAAIGGIAAAIGIICTTTTTCAIIIC?IIIIIIIIGICAIIIGAAAICCIIII?G

?II????AG?A?AGI?CAIAAAAGGIIIACGGICIAAGGAAAGAAICAAAIAAGAAIAAAAAAACA
 **     *  * *** * ** *   ****   ****   *     *** *** * *         *
AIIIIIIAIIACAGIIC?IAIAI??IIIAI??ICIAI??A?????ICAIAIAI?A?I????????C?

IGAAAIAAAAGAIGCAIIACGGAIAAAIGCAAICCAGACCCIACIAAI????????G?AC??????A
*    *        **** *  ***  *  * * ** *   * *****          *      *
IC???I????????CAII?CI?AIAIII??AIIICAAAI???I?CIAAIIGIIIAAIGGAAIGGIIA

G??A???C?AGAGGGGGGACAIICCCIIGIIGICAIICAGAAAAIACAGGIGAAAGCIAAA?A?AC
*  *   * * * * *  ******* *    * * **       * **** ***** * ** * *
GIIAIAACIA?AIGAG??ACAIICC?ICC??G?C?II????????I?CAGGCGAAAG?IGAAGAIA?

GAAAAAAGAAGAAICGACCCIICGAGIAIICAAAAI?GAGAIGAIAAAAAIGAGIGIAGGAAG?GC
 ****** ********** *** ******* **** ** ***  **** ** *   ****** *
?AAAAAA?AAGAAICGACCGIICAAGIAIICCAAAIIGA?AIGGCAAAA?IG?GC??AGGAAGAGA

?ACAIIAGGIAG?IC???IAIAICCIAGCCAIGIAIAIIGAAIIGIGGAIACAGAAAIGAIAGAAI
 **** *  *** *    *****  *  **** ********** **** * ********* ***
GACAI?A??IAGAIGGGGIAIAIA?I??CCAICIAIAIIGAAIIGCGGAIICCGAAAIGAIAAAAI

CAIIIIGGGIIGGICAAAIGIGGGICICCGAIAGAA?AI??ICAAGAAGAI?A??G?GCCCGAAAG
****** * **** ****    ******* ****** **  * ***** ** * * **   ****
CAIIIIIGAIIGGACAAAAAAAGGICICCIAIAGAAGAIAGIIAAGAAAAICAAAGAGGA?GAAAA

?ACIIIIIICIAIAGIAIAIAAAICGGCAICIAAIIGAAIICAACGAIIICC?G?AIACIACAAGG
 ** **** * *  * ***  ****** ******* ******** * ** ** * ***    ****
CACGIIII?CGA??G?AIAGGAAICGGIAICIAAI?GAAIICAAIGGII?CCAGIAIA----AAGG
                                                          56bp
GGGAIAIGGCGAAAIIGGIAGACGCIACGGACIIAAIIGGAIIGAGCCIICGCIACGGACIIAAII
*************** ************************************************
GGGAIAIGGCGAAAICGGIAGACGCIACGGACIIAAIIGGAIIGAGCCIICGCIACGGACIIAAII

GGAIIGAGCCIIGGIAIGGAA  670
**********************
GGAIIGAGCCIIGGIAIGGAA  49347
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Fig. 3. Comparison of *Oenothera odorata* and *Nicotiana* chloroplast DNA sequences covering the 5′ end of tRNA-Leu (UAA) and part of the spacer between tRNA-Leu (UAA) and tRNA-Thr (UGU). The *Nicotiana* sequence shown is between nucleotide positions 48627 and 49347 on the tobacco plastome, according to Shinozaki et al. (1986). The end of tRNA-Leu is labeled by a *triangle* in both sequences. To achieve maximal alignment, gaps indicated by a *question mark* are inserted

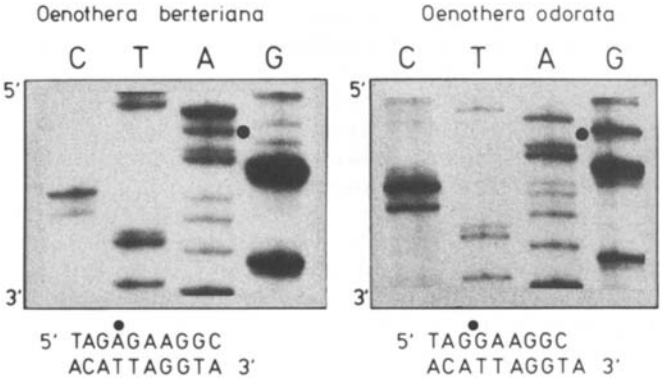

Fig. 4. Electrophoretic separation of sequencing products showing the 1 bp deletion/insertion. The deletion/insertion is marked with a *dot* in the gel and above the sequence

ized upstream of the 5′ end of a tRNA-Leu (UAA) gene and are presumably located in the spacer between this gene and a tRNA-Thr (UGU) gene, as shown by comparison with the tobacco cpDNA sequence. From the work of Brennicke and coworkers, additional evidence is available that this plastome region in the genus *Oenothera* is a relative hot spot. About 800 bp upstream of the 136 bp deletion/insertion a 758 bp sequence is located which was found to be also present in mitochondrial DNA of *Oe. berteriana* (Schuster and Brennicke 1987). Furthermore, the 618 bp *Eco*RI fragment from *Oe. berteriana* containing the analysed deletion/insertion hybridizes with a 10 kbp *Bam*HI and a 7.2 kbp *Hin*dIII fragment of the mitochondrial DNA from *Oe. berteriana* (W. Schuster, personal communication). Comparative sequence analysis must prove whether parts of the 618 bp *Eco*RI fragment in fact have been transferred from chloroplasts to mitochondria or even vice versa. Nevertheless, these findings together can be taken as a hint of recombinogenic activity of this plastome region.

Moreover, our analysis reveals possible elements of a recombination pathway. In *Oe. berteriana* the borders of the deleted/inserted 136 bp sequence lie within a short inverted repeat. There is another short inverted repeat in the middle part of the deleted/inserted sequence. The sequences of both inverted repeats are complementary so that they might interact and be involved in the deletion/insertion process. It is well known that direct and inverted repeats are able to cause loop structures in double-strand DNA which may cause deletion or inversion events (Kleckner 1981). A possible structure of an intermediate in the process leading to a deletion of the 136 bp sequence is shown in Fig. 6b. Both short repeats might form heteroduplices to bring the

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ORFb72          •              20              30              40              50
ATG TGC CTT CTC TAC ACT CAT TTT TAT CAT CTC ATT TTG AAT ACT CGA AGG GTC GAT TCT
Met Cys Leu Leu Tyr Thr His Phe Tyr His Leu Ile Leu Asn Thr Arg Arg Val Asp Ser

            70              80              90              100             110 ORFo35
TCT TTT TTC GTT TTT AGC TTT CAC CTG TAT TTT CTG AAT GAC AAC AAG GGA ATG TCC CCC
Ser Phe Phe Val Phe Ser Phe His Leu Tyr Phe Leu Asn Asp Asn Lys Gly Met Ser Pro

            130             140             150             160             170
CTC TGT CTG TCA TTA GTA GGG TCT GGA TTG CAT TTA TCC GTA ATG CAT CTT TTA TTT CAT
Leu Cys Leu Ser Leu Val Gly Ser Gly Leu His Leu Ser Val Met His Leu Leu Phe His

            190             200             210
GTT TTT TTA TTC TTA TTT GAT TCT TTC CTT AGA CCG TAA
Val Phe Leu Phe Leu Phe Asp Ser Phe Leu Arg Pro ***
```

Fig. 5. Nucleotide and amino acid sequence of ORFb72 on the chloroplast (cp) DNA of *Oenothera berteriana* and of ORFo35 on the cpDNA of *Oe. odorata*. The frame shift mutation is due to the deletion of a T-A base pair marked by a *dot*. ORFo35 is underlined by a horizontal *bracket*

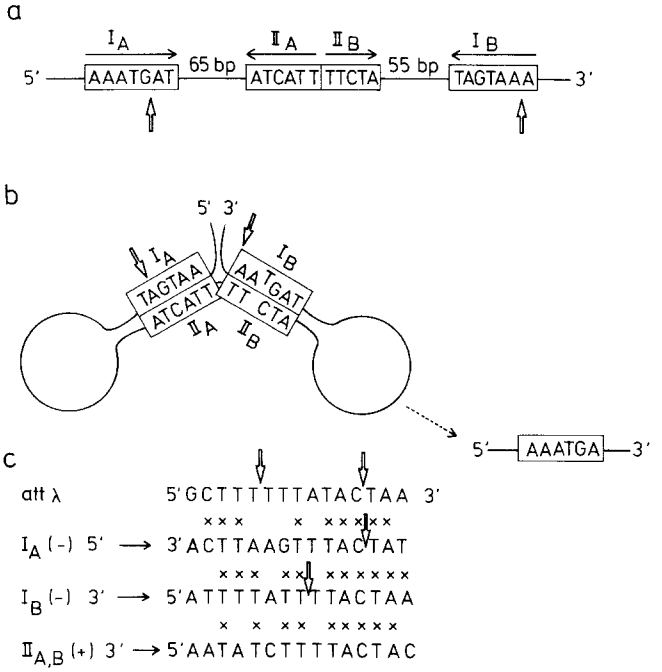

Fig. 6a–c. Possible interaction of the two sets of inverted repeats and similarity of sequences surrounding the inverted repeats to the lambda chromosomal attachment site (*att*-lambda). **a** Schematic location and orientation of the repeated sequences in the deleted/inserted sequence of *Oenothera berteriana*. **b** Proposed heteroduplex and loop structure of a possible intermediate in a deletion process. **c** Similarity of sequences surrounding the inverted repeats to *att*-lambda. Common base pairs are marked with *stars* between the lines. (−), (+) indicates the strand which was used for comparison. *Vertical arrows* indicate borders of the deleted/inserted sequence and the possible crossing-over points in the *att*-lambda sequence, respectively

borders of the deleted/inserted sequence together. Furthermore, the short inverted repeats and their surrounding sequences show remarkable similarity to the lambda chromosomal attachment site (*att*-lambda; Fig. 6c) which is essential for the site specific recombination pathway of phage lambda integration (Landy and Ross 1977). Similarities range from 9 to 11 bp in common with the 15 bp core sequence of *att*-lambda. The motif 5′-TACTA-3′ is common to all sequences compared in Fig. 6c. One of the borders of the deleted/inserted 136 bp sequence in the *Oe. berteriana* cpDNA is identical with one of the crossing-over points in the lambda integration system. In a previous paper (vom Stein and Hachtel 1988) we reported on a short direct repeat resembling *att*-lambda which occurs as a deletion/insertion in the cpDNA of *Oe. berteriana* ad *Oe. odorata*, respectively. Howe (1985) has demonstrated that an *att*-lambda homologous sequence from wheat cpDNA can function as an attachment site in vivo. In the lambda integration system several protein factors are involved, mainly to stabilize the attachment of heterologous sequences (for references see Nash 1981). Presumably, proteins also are involved in DNA recombination processes in chloroplasts, since the inverted repeats present in *Oenothera* cpDNA are rather short and the proposed secondary structure (Fig. 6b) per se might not be stable enough.

In the cpDNA from *Oe. berteriana* we detected an open reading frame of 216 bp (ORFb72) which starts 178 bp upstream of the 5′ end of a tRNA-Leu (UAA) gene (Fig. 2). This ORF is absent in the cpDNA from *Oe. odorata* due to a frame shift mutation. Only a part of ORFb72 is preserved (ORFo35). ORFo35 probably has no function because of the absence of a ribosomal binding site. The presence of a number of unassigned ORFs in the cpDNA of *Nicotiana* (Shinozaki et al. 1986) and *Marchantia* (Ohyama et al. 1986) is indicative of several rarely expressed genes which might play a regulatory rather than a structural role. However, sequence comparison of ORFb72 with the whole plastome of *N. tabacum* did not reveal any significant homology. To answer the question whether the observed frame-shift mutation is of any biological significance, therefore, identification of the transcript and polypeptide possibly encoded in ORFb72 is necessary.

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