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# Gene Localization on the Chloroplast DNA of the Maiden Hair Fern; *Adiantum capillus-veneris*

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Gene maps were constructed for the inverted repeat region and for the adjacent large single copy region of the chloroplast genome of the maiden hair fern, *Adiantum capillus-veneris* L. Gene order and organization was very different from the typical angiosperm chloroplast genome (*e.g.* tobacco). Elongation of inverted repeat and a minimum of two inversions must be postulated to account for the unusual genome structure.

Key words: Chloroplast DNA — Inverted repeat — *Adiantum* — pteridophytes — Gene order.

Chloroplast DNAs (cpDNAs) from many angiosperms have been extensively analyzed and characterized (reviewed in Palmer 1985a, b; Birky 1988). The high level of conservation of their genome size, organization and gene order has been well documented. All angiosperm cpDNAs are circular and the most noteworthy feature of their organization is the presence of a large inverted repeat (IR) separated by a large and a small single copy region (LSC and SSC) except for some legumes (Kolodner and Tewari, 1979; Palmer and Thompson, 1982) and the conifers (Strauss *et al.*, 1988; Raubeson, 1991). Gene mapping and sequencing studies have shown that gene order is identical in most land plants. However, several species exhibit varying gene orders; these alterations result from inversions (reviewed in Palmer, 1985a, b). The conservative organization of the chloroplast genome makes such rearrangements valuable for phylogenetical studies (Jansen and Palmer, 1987).

Recently, chloroplast DNAs from several ferns have been characterized. Gene order in three species of *Osmunda* (Palmer and Stein, 1982; Stein *et al.*, 1986) is the same as that of the typical angiosperm chloroplast genome (*e.g.* tobacco; Shinozaki *et al.*, 1986). Hasebe and Iwatsuki (1990) showed by cross hybridization experiments that the order of genes in the LSC and SSC of *Adiantum capillus-veneris* was the same as that of the typical angiosperm, but that significant difference of genome structure were found within the IRs. Moreover, similar rearrangements in the IR of chloroplast DNAs were reported in *Pteridium* (Tan and Thomson, 1990), *Cyathea*, and *Polystichum* (Stein *et al.*, 1992). In these genera, the *psb*A gene is duplicated and becomes parts of the IR, whereas in typical angiosperm, *psb*A is part of the LSC close to IR. In the typical angiosperm cpDNA, the 23S rDNA locates closer to the SSC than 16S rDNA does, whereas the two genes are arranged in the reverse order in these fern genera.

These results indicate that inversions may have occurred in the fern genera in comparison with typical angiosperms (Hasebe and Iwatsuki, 1990; Stein *et al.*, 1992).

In this study, we used PCR technique to make specific gene probes, and results of gene mapping show that gene rearrangement of cpDNA of *Adiantum capillus-veneris* has occurred in the IR and adjacent LSC regions.

## Materials and Methods

Chloroplast DNA fragments of *Adiantum capillus-veneris* cloned in pUC12 or 18 (Yanisch-Perron *et al.*, 1985) were used (Hasebe and Iwatsuki, 1990).

The 15 gene probes prepared are listed in Table 1. The probes were essentially made according ot R. Bellamy *et al.* (1990). Ten ng of cloned tobacco cpDNA (Sugiura *et al.*, 1986; Shinozaki *et al.*, 1986; clones supplied by the courtesy of Dr. M. Sugiura, Nagoya University, Nagoya, Japan) were used as template. To label a gene probe, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.12 mM dTTP, 0.06 mM digoxigenin-11-dUTP (Bohringer Mannheim), 1 mM gene specific primers (Table 1), and 0.6 units *Taq* polymerase (Perkin Elmer Cetus) were combined according to the suppliers instructions, and subjected to 25 cycles of PCR amplification with denaturing at 94°C for 1 min, annealing at 65°C for 2 min and extension at 72°C for 3 min. The PCR product was precipitated with ethanol and dissolved in 50 $\mu$l of TE that contained 0.1% SDS by heating at 37°C for 10 min with frequent voltexing.

Hybond-N membrane (Amersham) and Dig-ELISA Kit (Bohringer Mannheim) were used for Southern hybridization and detection. Hybridization was performed at 50°C for 12–18 hr in a hybridization buffer containing 5×SSC and according to suppliers instructions. The membranes were washed in 0.1×SSC and 1% SDS at 50°C twice.

## Results and Discussion

*Physical and Gene mapping of* Adiantum capillus-veneris

Physical maps of *Adiantum capillus-veneris* cpDNA had been constructed for *Pst*I, *Eco*RV, *Hin*dIII, *Stu*I, *Xho*I (Hasebe and Iwatsuki, 1990). Fine maps for additional enzymes were newly made for detailed examination of the gene map in the IR (Fig. 1). For the 6.2 kb *Pst*I fragment, *Cla*I and *Sal*I sites were mapped. For the 11.4 kb *Pst*I fragment, a *Cla*I map was consructed. For the 2.6 kb *Pst*I fragment, a *Dra*I map was constructed and for the 3.2 kb *Eco*RV fragment located within the 9.4 kb *Pst*I fragment, *Sty*I sites were mapped.

Southern hybridization of colned *A. capillus-veneris* cpDNA with tobacco chloroplast gene probes (Table 1) clarified the location of these genes (Fig. 1). For four genes, *psb*A, 16SrDNA, 23SrDNA, and *ndh*B, the direction of transcription was determined by differential hybridization of gene probes at 5′- and 3′-side. When 5′ and 3′ probes were not available, the orientation of genes was estimated based on their cotranscription with the 4 genes (Jansen and Palmer, 1987).

Table 1. Gene probes

| Gene probes | Sequence position* |
|---|---|
| *trn*N | 131731-131750 |
| | 132060-132041 |
| *trn*R | 132301-132320 |
| | 132640-132621 |
| 5SrDNA/4.5SrDNA/23SrDNA(3′) | 132621-132640 |
| | 133631-133650 |
| 23SrDNA(5′) | 135464-135483 |
| | 136190-136171 |
| *trn*A/*trn*I/16SrDNA(3′) | 136171-136190 |
| | 138581-138600 |
| 16SrDNA(5′)/*trn*V | 139401-139420 |
| | 140270-140251 |
| 3′*rps*12 | 141631-141650 |
| | 142190-142171 |
| *rps*7 | 142527-142546 |
| | 142960-142941 |
| *ndh*B(5′) | 143501-143520 |
| | 144165-144146 |
| *ndh*B(3′) | 144689-144708 |
| | 145559-145530 |
| *trn*L | 145881-145900 |
| | 146309-146327 |
| *trn*H | 155771-155790 |
| | 529-510 |
| *psb*A(3′) | 510-529 |
| | 1000-981 |
| *psb*A(5′) | 985-1004 |
| | 1727-1710 |
| *trn*K | 1810-1842 |
| | 2286-2266 |
| *rps*16 | 4861-4880 |
| | 5540-5521 |

* Sequence position exhibits position of gene specific primer on tobacco chloroplast genome (Shinozaki *et al.*, 1986)

*Comparison of gene order between* Adiantum capillus-veneris *chloroplast DNA and tobacco chloroplast DNA*

Comparison of the *A. capillus-veneris* and tobacoo cpDNA in the IR and adjacent LSC, revealed differences in gene copy number, and many changes in gene location and orientation.

The *psb*A and *trn*H genes are single genes and locates in LSC of tobacco cpDNA,

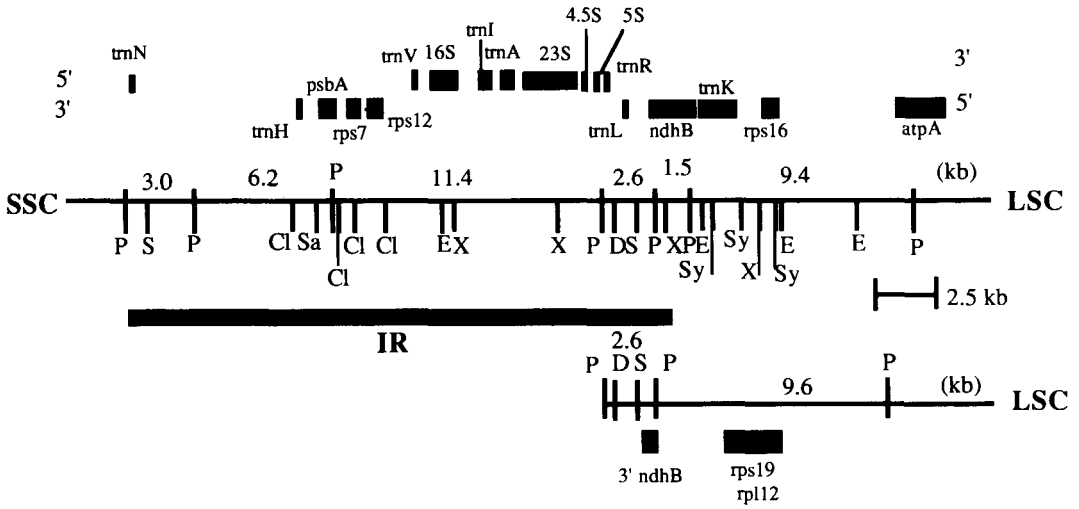


Fig. 1. Physical and gene maps of the *Adiantum capillus-veneris* chloroplast DNA inverted repeat region and the adjacent large single copy regions. Upper slender line is the map of the side containing *rps*16 and *atp*A genes and lower slender line is the other side. The long solid black line between physical maps indicates the minimum extent of the inverted repeat. Restriction sites in this map are: *Pst*I(P), *Stu*I(S), *Cla*I(C), *Sal*I(Sa), *Eco*RV(E), *Xho*I(X), *Dra*I(D), *Sty*I(Sy). Lengths of *Pst*I fragments are indicated above physical map. The short solid lines above maps show the position and orientation of the gene probes listed in Table 1 and adjascent genes as shown are cited from Hasebe and Iwatsuki (1990). The lengths of the lines are taken from the sequence data of tobacco.

but in *A. capillus-veneris*, they are duplicated genes located in the IR. Duplication of *psb*A is observed in some leptosporangiate ferns and appears to be a synapomorphic character in the group (Stein *et al.*, 1992).

The *ndh*B gene is located in the IR of tobacco cpDNA. In our experiments, the *ndh*B(3′) tobacco gene probe hybridized with the *Sal*I-*Pst*I fragment produced from the cloned 2.6 kb *Pst*I fragment and bordering on the 1.5 kb *Pst*I fragment. The *ndh*B(5′) hybridized with the *Xho*I-*Pst*I fragment generated from the 1.5 kb *Pst*I fragment and bordering on the 9.4 kb *Pst*I fragment, and with the *Pst*I-*Eco*RV fragment digested from the cloned 9.4 kg *Pst*I fragment and bordering on the 1.5 kb *Pst*I fragement. Since the border between IR and LSC is located on the 1.5 kb *Pst*I fragment (Hasebe and Iwatsuki, 1990) in *A. capillus-veneris*, the *ndh*B gene extends from the LSC into the IR and the whole 3′ region which we used as probe is located in IR. The other border of the LSC and IR, the 9.6 kb *Pst*I fragment is located next to the 2.6 kb *Pst*I fragment which is part of the IR and contains 3′ region of *ndh*B (Fig. 1). Since the *ndh*B(5′) gene probe does not hybridize to the 9.6 kb *Pst*I fragment, the 5′ region of *ndh*B is not on this side of the LSC, but only on the other side of the LSC near *rps*16 and *atp*A genes.

The *rps*16 gene was found in the tobacco chloroplast genome, but not in the *Marchantia polymorpha* coloroplast genome. In *A. capillus-veneris*, a sequence

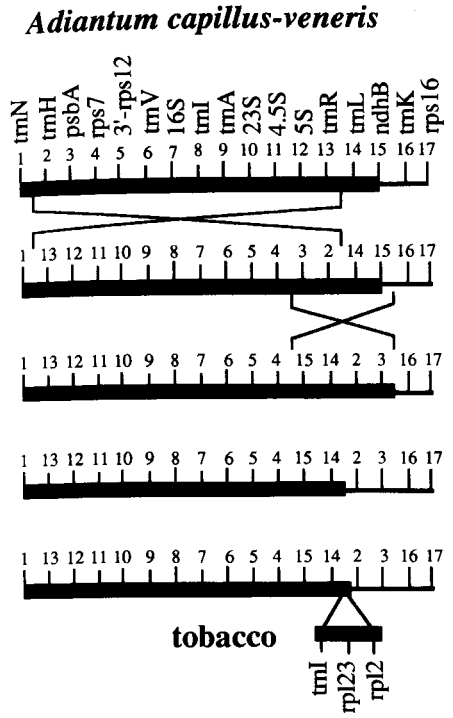


Fig. 2. Model for the relationships between *Adiantum capillus-veneris* and tobacco cpDNA in inverted repeat region.

homologous to the tobacco *rps*16 gene was detected by Southern hybridization. Since the hybridized band was not detected under more stringent conditions (55°C, 0.1×SSC), it is speculated that the sequence of the *A. capillus-veneris rps*16 gene is highly diverged from that of tobacco. To confirm that this gene in *A. capillus-veneris* is functional unlike *Marchantia*, it will be necessary to sequence the *A. capillus-veneris* gene.

Homologous regions to ORFs 581 and 1708 were not detected by our Southern hybridization condition. Even under low stringency conditions (hybridization at 45°C and washing with 2×SSC), no hybridization signal was detected.

Fig. 2 shows a hypothesis which most parsimoniously explains the rearrangements of gene order and orientation between cpDNA of *A. capillus-veneris* and tobacco. These rearrangements are of four types; (1) expansion of the LSC and the resulting singleness of three genes which are located in IR region of tobacco (*trn*I, *rpl*23 and *rpl*2): (2) expansion of the IR and the resulting duplication of *trn*H and *psb*A: (3) an inversion whose ends are located between *psb*A and *rps*7, and between *ndh*B and *trn*K: (4) an inversion whose ends are located between *trn*N and *trn*H, and between *trn*R and *trn*L.

In summary, the unusual gene order of *A. capillus-veneris* cpDNA was characterized in detail by using PCR generated gene probes and fine restriction maps of *Adiantum capillus-veneris* cpDNA. We demonstrated that the *ndh*B gene was only partially duplicated and confirmed the unusual genome organization described earlier (Hasebe and Iwatsuki, 1990). This gene order has already proven to be phylogenetically informative (Stein *et al.*, 1992) in linking four families of leptosporangiate ferns.

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## References

BELLAMY, R., C. INGLEHEARN, D. LESTER, A. HARDCASTLE AND S. BHATTACHARYA. 1990. Better fingerprinting with PCR. Trends in Genet. **6**: 32.

BIRKY, C.W. Jr. 1988. Evolution and variation in plant chloroplast and mitochondrial genomes. *In* L.D. GOTTLIEB and S.K. JANE, ed., Plant Evolutionary Biology, pp. 23-53. Chapman and Hall, London.

HASEBE, M. AND K. IWATSUKI. 1990. Chloroplast DNA from *Adiantum capillus-veneris* L., a fern species (Adiantaceae); clone bank, physical map and unusual gene localization in comparison with angiosperm chloroplast DNA. Curr. Genet. **17**: 359-364.

JANSEN, R.K. AND J.D. PALMER. 1987a. Chloroplast DNA from lettuce and *Barnadesia* (Asteraceae): structure, gene localization, and characterization of a large inversion. Curr. Genet. **11**: 553-564.

——— AND J.D. PALMER. 1987b. A chloroplast DNA inversion marks an ancient evolutionary split in the sunflower family (Asteraceae). Proc. Natl. Acad. Sci. USA **84**: 5818-5822.

KOLODNER, R. AND K.K. TEWARI. 1979. Inverted repeat in chloroplast DNA from higher plants. Proc. Natl. Acad. Sci. USA **76**: 41-45.

LIDHOLM, J., A.E. SZMIDT, J.-E. HALLGREN AND P. GUSTAFSSON. 1988. The chloroplast genomes of conifers lack one of the rRNA-encoding inverted repeats. Mol. Gen. Genet. **212**: 6-10.

PALMER, J.D. 1985a. Comparative organization of chloroplast genomes. Ann. Rev. Genet. **19**: 325-354.

———. 1985b. Evolution of chloroplast and mitochendrial DNA in plants and algae. *In* R.J. MACINTYRE, ed., Molecular Evolutionary Biology, pp. 131-240. Plenum, New York.

——— AND D.B. STEIN. 1982. Chloroplast DNA from the fern *Osmunda cinnamomea*: Physical organization and comparison to angiosperm chloroplast DNA. Curr. Genet. **5**: 165-170.

——— AND ———. 1986. Conservation of chloroplast genome structure among vascular plants. Curr. Genet. **10**: 823-833.

——— AND W.F. THOMPSON. 1982. Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell **29**: 537-550.

RAUBESON, L.A. 1991. Structural variation in the chloroplast genome of vascular land plants. Ph.D. Dissertation. Yale University.

SHINOZAKI, K., M. OHME, M. TANAKA, T. WAKASUGI, N. HAYASHIDA, T. MATSUBAYASHI, N. ZAITA, J. CHUNWONGSE, J. OBOKATA, K. YAMAGUCHI-SHINOZAKI, C. OHTA, K. TORAZAWA, B.Y. MENG, M. SUGITA, H. DENO, T. KAMAGASHIRA, K. YAMADA, J. KUSUDA, F. TAKAIWA, A. KATO, N. TOHDOH, H. SHIMADA AND M. SUGIURA. 1986. The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J. **5**: 2043-2049.

STEIN, D.B., J.D. PALMER AND W.F. THOMPSON. 1986. Structural evolution and flip-flop recombination of chloroplast DNA in the fern genus *Osmunda*. Curr. Genet. **10**: 835-841.

———, D.S. CONANT, M.E. AHEARN, E.T. JORDAN, S.A. KIRCH, M. HASEBE, K. IWATSUKI, M.K. TAN AND J.A. THOMSON. 1992. Structural rearrangement of the chloroplast genome provide an important phylogenetic link in ferns. Proc. Natl. Acad. Sci. USA **89**: 1856-1860.

STRAUSS, S.H., J.D. PALMER, G.T. HOWE AND A.H. DOERKSEN. 1988. Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. Proc. Natl. Acad. Sci. USA **85**: 3898-3902.

SUGIURA, M., N. ZAITA, M. KUSUDA AND M. KUMANO. 1986. Clone bank of the tobacco (*Nicotiana tabacum*) chloroplast genome as a set of overlapping restriction endonuclease fragments: mapping of eleven ribosomal protein genes. Plant Science **44**: 211-216.

TAN, M.K. AND J.A. THOMSON. 1990. *In* J.A. THOMSON and R.T. SMITH, ed., Bracken Biology and Management, pp. 95-103. Australian Institute of Agriculture Science, Sydeny.

WHITE, E.E. 1990. Chloroplast DNA in *Pinus monticola*. 1. Physical map. Theor. Appl. Genet. **79**: 119-124.

YANISCH-PERRON, C., J. VIEIRA AND J. MESSING. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene **33**: 103-119.

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