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The structure of the chloroplast genome in members of the genus *Asparagus*

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Abstract In a previous study we constructed a physical map of the chloroplast DNA (ctDNA) of garden asparagus (*Asparagus officinalis* L. cv 'Mary Washington 500W'; Lee et al. 1996). In the present study we have constructed and compared *Hin*dIII and *Xho*I restriction maps of the ctDNAs of eight species of *Asparagus*: namely, *A. officinalis*, *A. schoberioides*, *A. cochinchinensis*, *A. plumosus*, *A. falcatus*, *A. sprengeri*, *A. virgatus* and *A. asparagoides*. The ctDNA of *A. officinalis* has 32 and 23 sites that are recognized by *Hin*dIII and *Xho*I, respectively. Taking the physical map of the ctDNA of *A. officinalis* as a standard, we found that the ctDNAs of *A. falcatus*, *A. sprengeri*, and *A. asparagoides* each had one additional *Hin*dIII site and lacked one *Xho*I site. We also detected two relatively large deletions of nucleotides in the ctDNA from *A. cochinchinensis* by sequencing analysis. Both of these deletions were located in a non-coding region between the *ndhC* and *trnV* genes and were 95 bp and 347 bp in length, respectively. The regions around the deletions exhibited strong homology, and short direct-repeat sequences were detected at the borders of the deletions, an indication that these deletions were the result of intramolecular recombination mediated by the direct repeats.

Key words *Asparagus* · Chloroplast DNA (ctDNA) · Deletion · Direct repeat

Introduction

The structure and organization of chloroplast DNA (ctDNA) have been studied in many land plants and algae (Whitfeld and Bottomley 1983; Palmer 1985). Phylogenetic relationships between closely related species or genera have been deduced from the results of analyses of restriction fragment length polymorphisms (RFLPs) in many groups of plants (e.g. Palmer 1985; Ogihara and Tsunewaki 1988; Chase and Palmer 1989; Shimizu 1993; Bogler and Simpson 1995; Kato et al. 1995; Pillay and Hilu 1995). Such analyses have revealed several types of variation in chloroplast genomes; namely, inversions (Howe 1985; Hiratsuka et al. 1989; Doyle et al. 1992), translocations (Bowman et al. 1988; Ogihara et al. 1992), rearrangements of gene order (Palmer and Thompson 1982; Palmer et al. 1987; Strauss et al. 1988), and insertions and/or deletions (e.g. Zurawski et al. 1984; Ogihara et al. 1988; vom Stein and Hachtel 1988), as well as base substitutions (Palmer and Zamir 1982; for a review see Zurawski and Clegg 1987).

Garden asparagus (*Asparagus officinalis* L.) is an economically important horticultural crop. The genus *Asparagus*, which includes garden asparagus, consists of 100–300 species. The plants in this genus include herbaceous perennials, as well as tender woody shrubs and vines, and they are distributed mainly on dry land in the Old World (Bailey 1944; Chittenden 1956; Ohwi 1965). The species of *Asparagus* have been classified into four Sections: Euasparagus, Asparagopsis, Kodiastigma and Myrsiphyllum (Bailey 1944). The species in Euasparagus are dioecious while those in the other three Sections are hermaphrodite.

We previously cloned and constructed a physical map of the ctDNA from garden asparagus (*A. officinalis* L.) and found that the length of the ct genome and the order of genes in asparagus ctDNA were the same as in tobacco (Lee et al. 1996). In the present study we have examined and compared the ctDNAs from various species of *Asparagus*. In addition to the gain or loss of restriction sites, we detected two deletions of relatively long regions of nucleotides that appeared to have been mediated by short direct-repeats in the ctDNA from *A. cochinchinensis*.

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Materials and methods

Plant materials

The species of *Asparagus* that we used in this study are shown in Table 1. The Sections of *Asparagus* were classified by Bailey (1944). The cultivar of *A. officinalis* employed was 'Mary Washington 500W'. *A. schoberioides* and *A. cochinchinensis* are distributed in East Asia (Ohwi 1965). *A. schoberioides* was provided by the Medicinal Plant Garden of Kumamoto University, Japan. *A. cochinchinensis* was provided by Toyama Medical and Pharmaceutical University, Japan, and by Kyoritsu College of Pharmacy, Japan. The other species that we examined are all ornamental and/or medicinally important. *A. plumosus* was supplied by Fukukaen Nursery and Bulb Co., Mie, Japan, and the Faculty of Science of Tohoku University, Japan. *A. asparagoides* and *A. virgatus* were obtained from the National Research Institute of Vegetables, Ornamental Plants and Tea, Mie, Japan. All of the plants were grown in a greenhouse.

Preparation of total DNA and Southern hybridization

Total DNA was extracted from 1 g of the mature green cladophylls of eight species in the genus *Asparagus* (Honda and Hirai 1990). Each sample of total DNA was digested for 2–3 h with an appropriate restriction enzyme (Takara Shuzo Co., Japan) at 37°C. Restriction fragments were separated by electrophoresis on 0.7% agarose gels and bands of ethidium bromide-stained fragments were visualized with a UV transilluminator. Fragments in a *Hin*dIII digest of λ DNA and a *Hae*III digest of X174 were used as markers.

Southern hybridization was performed with a non-radioactive DNA labelling and detection kit (Boehringer Mannheim, Germany). Cloned fragments of ctDNA from asparagus (*A. officinalis*; Lee et al. 1996) and rice (*Oryza sativa*; Hirai et al. 1985) were used as probes.

The polymerase chain reaction (PCR), cloning and sequencing

PCR was performed with Takara Ex *Taq* DNA polymerase (Takara Shuzo Co.). Enzymes were added after heating of samples for 5 min at 95°C and 5 min at 50°C. PCR was conducted for 30 cycles, with each cycle consisting of 1 min at 72°C, 1 min at 94°C and 2 min at 50°C. The reaction mixture was finally incubated for 10 min at 72°C. The sequences of the primers were as follows: P1, 5′- CAACTGGTACCATATAGAAG-3′; P2, 5′-CGAGAAGGTCTACGGTTCG-3′; P3, 5′-CGAACCGTAGACCTTCTCG-3′; P4, 5′-GCACTTGAAATAGCCGAAGC-3′; P5, 5′- GCTTCGGCTATTTCAAGTGC-3′; and P6, 5′-GGGAGCTCCTCTCAGTGTTC-3′. The products of PCR were cloned into the pCR2.1 vector with an original TA cloning kit (Invitrogen, U.S.A.), and sequencing was performed with an automated DNA sequencer (Applied Biosystems).

Table 1 The species of *Asparagus* examined in the present study

Species	Section[a]	Dioecious(D)/ hermaphrodite(H)
A. officinalis	Euasparagus	D
A. schoberioides	Euasparagus	D
A. cochinchinensis	Euasparagus	D
A. plumosus	Asparagopsis	H
A. falcatus	Asparagopsis	H
A. sprengeri	Asparagopsis	H
A. virgatus	Kodiastigma	H
A. asparagoides	Myrsiphyllum	H

[a] Bailey (1944)

Results

Variation among ctDNAs in the genus *Asparagus*

Total DNA was prepared from the green cladophylls of eight species of *Asparagus*. After digestion with *Hin*dIII and *Xho*I, Southern hybridization was performed with cloned fragments of asparagus and rice ctDNAs as probes. Figure 1 shows the Southern hybridization patterns obtained with the *Pst*I-1 fragment of rice ctDNA used as a probe. Employing the physical map of the ctDNA from *A. officinalis* (Lee et al. 1996) as a standard, we constructed the *Hin*dIII and *Xho*I restriction maps of the ctDNAs from the other seven species of *Asparagus* and compared them (Fig. 2A, B). We detected two types of variation, which were presumed to be mutations: the gain and/or loss of restriction sites and the deletion of nucleotides. In the ctDNA from *A. officinalis*, 32 and 23 sites were recognized by *Hin*dIII and *Xho*I, respectively. The ctDNAs from *A. falcatus*, *A. sprengeri*, and *A. asparagoides* each had one additional *Hin*dIII site and lacked one *Xho*I site. The second type of variation, namely deletion, was found in the ctDNA from *A. cochinchinensis*. Compared with the length of the *Hin*dIII-1 and *Xho*I-1 fragments of the ctDNA from *A. officinalis* (Lee et al. 1996), the

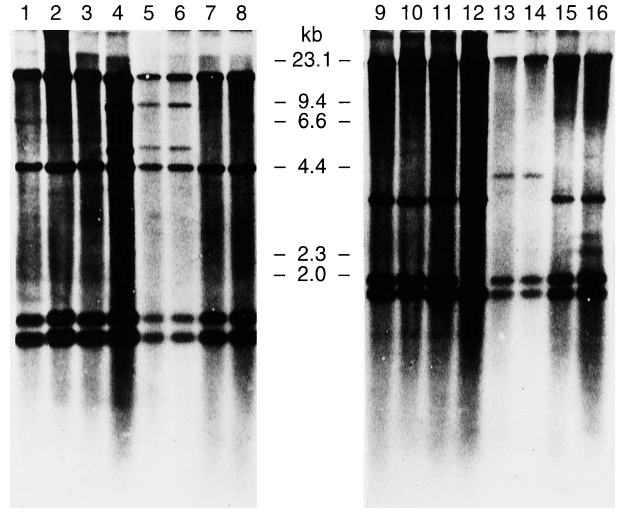

Fig. 1 Southern-blotting analysis of the total DNA from eight species of *Asparagus* with *Hin*dIII (*lanes 1–8*) and *Xho*I (*lanes 9–16*), and the *Pst*I-1 fragment of rice ctDNA, as probes. *Lanes 1 and 9*, *A. officinalis*; *lanes 2 and 10*, *A. cochinchinensis*; *lanes 3 and 11*, *A. schoberioides*; *lanes 4 and 12*, *A. asparagoides*; *lanes 5 and 13*, *A. falcatus*; *lanes* 6 *and* 14, *A. sprengeri*; *lanes* 7 *and* 15, *A. plumosus*; *lanes 8 and 16*, *A. virgatus*

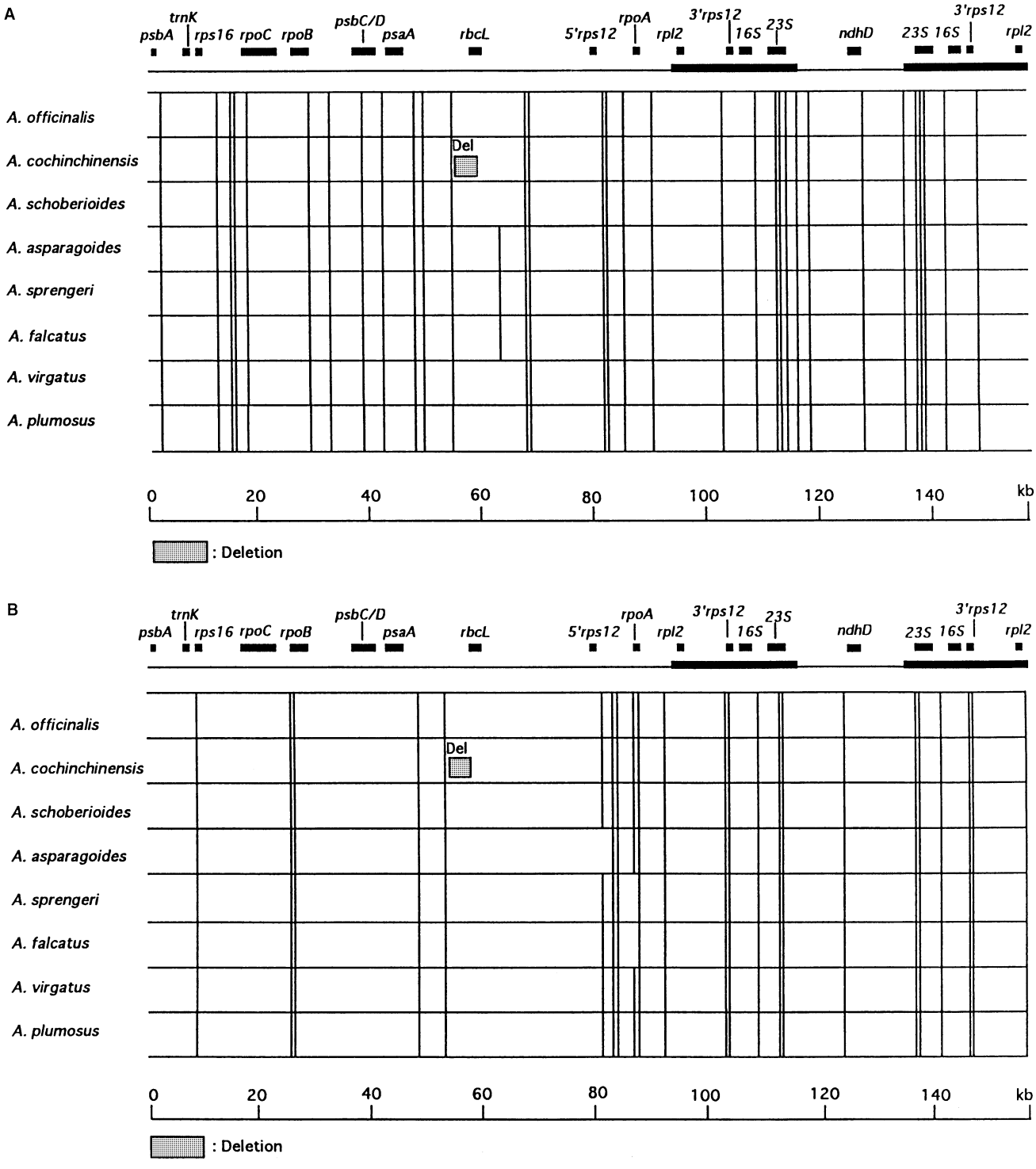

Fig. 2 Restriction maps of ctDNAs from eight species of *Asparagus* obtained with *Hin*dIII (A) and *Xho*I (**B**). The *long bold lines* above the map indicate the locations of the inverted repeats (IR) and genes are indicated by *short bold lines*. A deletion is shown by a *shaded box*

corresponding fragments from *A. cochinchinensis* were about 0.5 kb shorter. Since we detected these size variations by Southern hybridization after electrophoresis on 0.7% agarose gels, it seemed likely that all deletions (or insertions) of more than 200 bp would have been detected.

Analysis of the deletions in the ctDNA from *A. cochinchinensis*

As described above, we identified deletions in the *Hin*dIII-1 and *Xho*I-1 fragments of the ctDNA from

A. cochinchinensis. The *Hin*dIII-1 fragments of the ctDNA from *A. officinalis* and from *A. cochinchinensis*, which are about 13 kb in length, are included in the corresponding *Xho*I-1 fragments. Since the *rbcL* gene (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase) is located in the center of the *Hin*dIII-1 fragment (Lee et al. 1996), we performed Southern hybridization using rice ctDNA probes of about 20 kb in length from the region around the *rbcL* gene to clarify the precise location of the deletion(s). Total DNA was prepared from *A. officinalis* and *A. cochinchinensis* and digested with *Pst*I and *Hin*dIII. We then performed Southern hybridization using seven probes derived from rice ctDNA. These probes included the region from IRF170 (intron-containing reading frame) to the *petA* gene (for cytochrome f) and the sum of their lengths was about 20 kb.

Some of the results of Southern hybridization are shown in Fig. 3. In addition to probe A, no variations were found with four probes (data not shown). Two bands can be seen in lane 6 but there is only one band in lane 5. However, this variation seemed to be caused by the gain or loss of a restriction site that resulted from a base substitution since the sum of the sizes of the fragments in lane 6 is equal to that of the fragment in lane 5. Comparing the sizes of the smaller fragments in lanes 3 and 4, we found that the fragment in lane 4 was about 0.5 kb shorter than that in lane 3, an indication that this variation was caused by a deletion. These results indicated that a deletion had occurred within a *Pst*I-*Hin*dIII fragment of about 3.9 kb.

In order to detect and clone the precise site(s) of deletion(s), we constructed six primers (Materials and methods; Fig. 4A) and amplified fragments by PCR using total DNA from *A. officinalis* and from *A. cochinchinensis* as the template. As shown in Fig. 4B, only one PCR product band was detected in each lane. The sizes of the products of PCR were the same when P3 and P4 or P5 and P6 were used as primers. However, the sizes were different when P1 and P2 were used as primers. From the analysis with restriction endonucleases, we located a *Hin*dIII site on the product of PCR with P1 and P2 and a *Pst*I site on the product of PCR with P5 and P6. These results indicated that the deletion(s) in the *Pst*I-*Hin*dIII fragment of about 3.9 kb

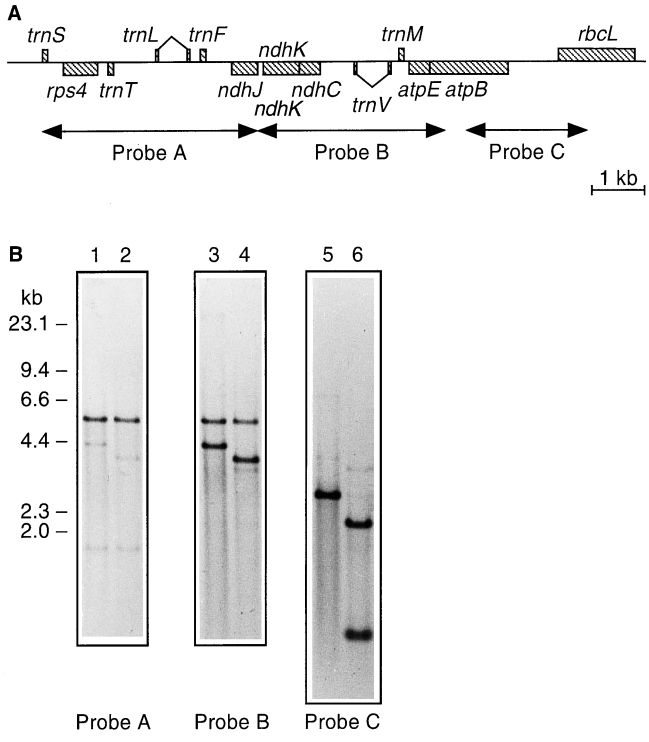

Fig. 3A A map showing genes and the locations of probes that correspond to fragments of rice ctDNA. **B** The results of Southern hybridization. Total DNA from *A. officinalis* (*lanes 1, 3 and 5*) and from *A. cochinchinensis* (*lanes 2, 4 and 6*) was digested with *Pst*I and *Hin*dIII. The probe used is indicated under each Southern blot. Fragments of *Hin*dIII-digested λ DNA were used as markers

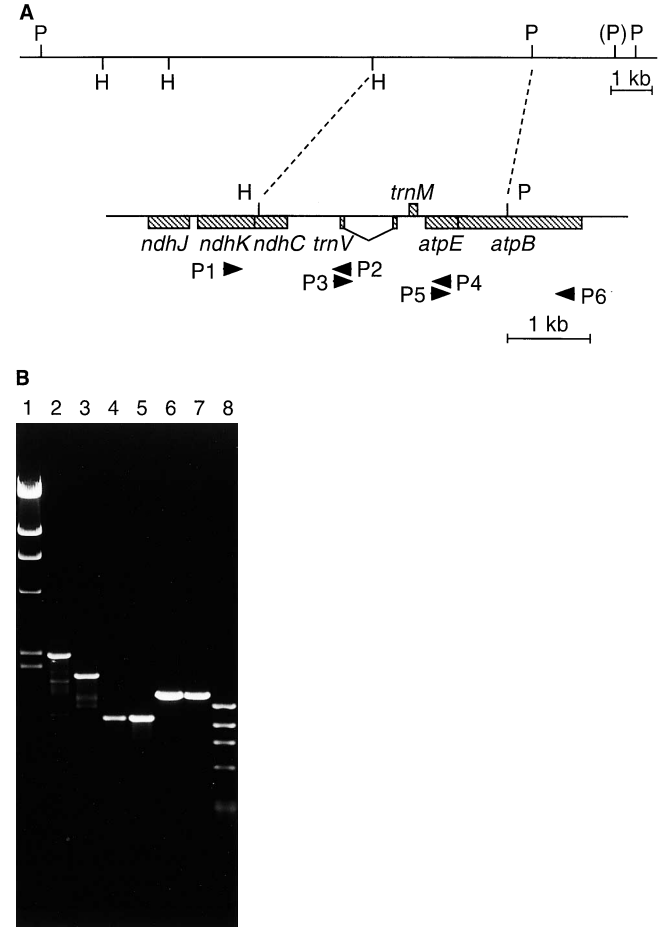

Fig. 4 Results of PCR. **A** A map showing genes and the locations of primers. **B** The products of PCR. Total DNA from *A. officinalis* (*lanes 2, 4 and 6*) and from *A. cochinchinensis* (*lanes 3, 5 and 7*) was used as a template. Primers P1 and P2 (*lanes 2 and 3*), P3 and P4 (*lanes 4 and 5*), and P5 and P6 (*lanes 6 and 7*) were used as primers. *Lanes 1 and 8* show fragments in a *Hin*dIII digest of λ DNA and a *Hae*III digest of ϕX174, respectively

Fig. 5 Sequencing analysis. **A** The locations of the deletions (*Del A* and *Del B*) found in the ctDNA of *A. cochinchinensis*. *P1* and *P2* refer to primers shown in Fig. 4. **B** Alignment of ctDNA sequences around the deletions. Deletions mediated by short direct-repeats (*Del A* and *Del B*) are boxed. *Arrows* show tandemly repeated sequences. Short direct-repeats found at the borders of the deletions are *double-underlined*

was located between the *ndhK* gene (for a component of NADH dehydrogenase) and the *trnV* gene (for $tRNA^{Val}$). This conclusion was supported by the fact that the product of PCR with DNA from *A. cochinchinensis* as template in lane 2 was about 0.5 kb shorter than the product with DNA of *A. officinalis* as template in lane 1. The products of PCR in lanes 1 and 2 were cloned into the pCR2.1 vector and DNA sequences were determined as described in Materials and methods.

We aligned the DNA sequences around the deletion(s) in the ctDNAs of *A. officinalis* and *A. cochinchinensis*. Two deletions of more than 50 bp were found in the non-coding region between the *ndhC* and *trnV* genes and they were 95 bp and 347 bp in length, respectively. We designated them Del A and Del B (Fig. 5A, B). The regions around the deletions exhibited strong homology. At the borders of both Del A and Del B, we detected direct repeats of A(T/C)CCG and AA(T)GAGG, respectively; an indication that these deletions were probably the result of intramolecular recombination mediated by these direct-repeat sequences. In addition we also detected small deletions or insertions upstream of Del A. These deletions or insertions were 13 bp and 4 bp long in the ctDNAs of *A. officinalis* and *A. cochinchinensis*, respectively, and both of them represented one copy of the tandemly repeated sequences.

Discussion

Restriction mapping of the ctDNAs of the various species of *Asparagus* revealed one additional *Hin*dIII site and one fewer *Xho*I site in the ctDNAs from some species when the physical map of the ctDNA from *A. officinalis* was taken as a standard. These variations can be assumed to be the result of base substitutions. In addition, two deletions were found in the ctDNA from *A. cochinchinensis* by sequencing analysis. The restriction maps of the ctDNAs suggested that the various species of *Asparagus* examined in this study are very closely related. However, a previous analysis of phylogenetic relationships within this genus, derived from the restriction sites in ctDNAs, yielded values for interspecific divergence ($100 \times p$) that ranged from 0.4 to 2.4 (Lee et al. 1997). In other genera of plants, reported values of interspecific divergence ($100 \times p$) range from 0.0 to 0.3 in *Zea* and 0.24 to 1.0 in *Aegilops*/*Triticum* (Doebley et al. 1987b; Ogihara and Tsunewaki 1988). Nevertheless, four and 14 deletions or insertions were reported in *Zea* and *Aegilops*/*Triticum*, respectively (Doebley et al. 1987a; Ogihara and Tsunewaki 1988). The deletions in *Zea* were 80–250 bp long and those in *Aegilops*/*Triticum* were 100–900 bp long. Therefore, it is possible that there are many more small deletions and insertions in the ctDNA of *Asparagus*. We detected size variations of more than 200 bp in our analysis with 0.7% agarose gels. Deletions of less than 200 bp might be detectable in the ctDNAs from *Asparagus* species on polyacrylamide gels or on gels with a higher percentage of agarose.

Sequencing analysis can reveal three types of variation: (1) base substitutions; (2) deletions or duplications of tandem repeats; and (3) deletions mediated by short direct-repeats. Deletions or duplications of tandem repeats were reported previously in barley and maize (Zurawski et al. 1984) as well as in wheat (Ogihara et al. 1991, 1992). This type of variation seems to be caused by slippage during DNA replication (Farabaugh et al. 1978). We found two deletions of 95 bp and 347 bp in length, respectively, that were located in the non-coding region between the *ndhC* and *trnV* genes in the ctDNA from *A. cochinchinensis*. Since the order of genes around this region in the ctDNA of asparagus was found to be almost the same as that in rice by Southern hybridization (Fig. 3), and since this region is transcribed polycistronically from the *atpB* genes in rice (Kanno and Hirai 1993), it seems likely that transcription in this region is not affected by these deletions. At the borders of the deletions we found short direct-repeats, which indicated that the deletions had been mediated by these sequences. Two deletions mediated by short direct-repeat sequences have been reported in wheat (*Aegilops*/*Triticum*; Ogihara and Tsunewaki 1988) and four others in rice (*Oryza*; Kanno and Hirai 1992; Kanno et al. 1993). The lengths of the direct-repeat sequences and the sizes of the deletions found in the ctDNA of *A. cochinchinensis* were almost same as those found in rice and wheat, suggesting that the deletions in *A. cochinchinensis* might have occurred by a similar mechanism to that proposed by Ogihara et al. (1988). The direct repeats found in rice and wheat were AT-rich, while those found in *A. cochinchinensis* were not. Another type of deletion, which was not flanked by direct repeats, has been reported in maize (Doebley et al. 1987a) and potato (Kawagoe and Kikuta 1991). The lengths of these deletions were 80 bp and 83 bp in maize and 241 bp in potato. Therefore, it remains a possibility that the deletions found in the ctDNA from *A. cochinchinensis* were not caused by recombination that was mediated by short direct-repeats. It is now important to analyze many examples of deletions in ctDNA and to determine the characteristics of the direct-repeat sequences so that the mechanisms for such deletions can be clarified.

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