Theor Appl Genet (1996) 92:10–14

Y.-O. Lee · A. Kanno · T. Kameya

The physical map of the chloroplast DNA from *Asparagus officinalis* L.

Received: 16 June 1995 / Accepted: 23 June 1995

Abstract The genus *Asparagus* consists of 100–300 species of both dioecious and hermaphrodite plants. Since there are diploid, tetraploid, and hexaploid plants in this genus, RFLP (restriction fragment length polymorphism) analysis of chloroplast DNA (ctDNA) is suitable for examining the phylogenetic relationships. We have constructed a physical map of the ctDNA of garden asparagus (*A. officinalis* L. cv 'Mary Washington 500 W') using five restriction endonucleases, namely, *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Xho*I. Asparagus ctDNA was digested with restriction enzymes and cloned into plasmid and λ phage vectors, and a clone bank was constructed that covered 70% of the genome. A physical map was constructed by Southern hybridization of total DNA from asparagus with homologous and heterologous probes. The asparagus ctDNA was about 155 kb long and it contained two inverted repeats (23 kb each) separated by a large single-copy region (90 kb) and a small single-copy region (19 kb). Fifteen genes, encoding photosynthesis-related proteins, rDNAs, and tRNAs, were localized on the physical map of asparagus ctDNA. Comparing the length and the gene order of asparagus ctDNA with that of other plants, we found that asparagus ctDNA was similar to tobacco ctDNA but different from rice ctDNA. The restriction patterns of the ctDNAs from several varieties of *A. officinalis* and three species of *Asparagus* were analyzed. The restriction patterns of the varieties of *A. officinalis* were very similar, but polymorphisms were detected among the three species of *Asparagus*.

Key words Chloroplast DNA (ctDNA) · Physical map · Garden asparagus · *Asparagus officinalis* L. · Chloroplast genes

Communicated by F. Salamini

Y.-O. Lee (✉) · A. Kanno · T. Kameya
Institute of Genetic Ecology, Tohoku University, Sendai 980-77, Japan

Introduction

The genus *Asparagus* is distributed mainly on dry land on the Old World, and it includes 100–300 species (Bailey 1944; Chittenden 1956; Ohwi 1965). Garden asparagus (*A. officinalis* L.) belongs to the genus *Asparagus* and is regarded as an economically important horticultural crop. *A. officinalis* L. is a dioecious species. The male and female flowers at early developmental stages possess both carpels and stamens; sex differentiation appears to be the result of the selective abortion of carpels in male flowers and of stamens in female flowers (Lazarte and Palser 1979; Caporali et al. 1994). *A. officinalis* is generally propagated by seeds, which are usually mixtures with equal numbers of seeds of either sex (Rick and Hanna 1943). Sexual dimorphism in this species is controlled by the genetic factors X and Y (Reimann-Philip et al. 1959); female plants are homogametic (conventionally XX), while males are heterogametic (XY) for sex chromosomes (Rick and Hanna 1943, reviewed by Bracale et al. 1991). The sex chromosomes are homomorphic (Loptien 1979), indicating that the system for sex determination in *A. officinalis* evolved relatively recently (Dellaporta and Calderon-Urrea 1993).

Asparagus species are classified into four sections: Euasparagus, Asparagopsis, Kodiastigma, and Myrsiphyllum (Bailey 1944). The species in Euasparagus, which includes *A. officinalis*, are dioecious, and the plants in the other three sections are hermaphrodites. Thus, it is of great interest to determine the period at which sex differentiation occurred in the genus *Asparagus*; i.e., when Euasparagus diverged from the other three sections.

In investigations of phylogenetic relationships in the genus *Asparagus*, restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (ctDNA) allows us to compare the varieties and species since *Asparagus* includes diploid, tetraploid, and hexaploid plants (Sen 1978; Kar and Sen 1985). In the present

study, we constructed a physical map of the ctDNA of *A. officinalis* cv 'Mary Washington 500 W' and compared it with similar maps of ctDNA from several other cultivars and other species.

Materials and methods

Plant materials

The cultivars of *Asparagus officinalis* L. used in this work were 'Mary Washington 500 W', 'Hokkai 100', 'Goldschatz', 'Fruit', and 'UC157', the seeds of which were generously provided by Dr. Asano (Hokkaiseikan Co, Hokkaido, Japan). Another cultivar, 'Million', was generously provided by Dr. T. Yamaguchi (Fukukaen Nursery & Bulb Co, Mie, Japan). Two horticultural species, namely, *A. plumosus* (var 'nanus') and *A. scandens*, which were kindly provided by Dr. K. Sudo (National Research Institute of Vegetables, Ornamental Plants and Tea, Mie, Japan), were also used. All of the plants were grown in a greenhouse.

Preparation of chloroplast and total DNAs

Chloroplast DNA was extracted from young green cladophylls ("leaves") of *A. officinalis* L. cv 'Mary Washington 500 W' that were harvested 2–3 months after germination, as described by Hirai et al. (1985). Total DNA was extracted from 1 g of mature green cladophylls by the method of Honda and Hirai (1990).

Restriction endonuclease analysis

Chloroplast DNA was digested overnight with *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Xho*I (Takara Shuzo Co, Japan) at 37 °C. Restriction fragments were seperated by electrophoresis on 0.7% agarose gels, and ethidium bromide-stained bands were visualized with a UV transilluminator. Fragments of *Hin*dIII-digested λ phage DNA were used as molecular-weight markers.

Cloning of ctDNA and construction of a physical map

Asparagus ctDNA was digested with *Hin*dIII and *Xho*I and cloned into the appropriate sites of the pUC119 and pBluescript SK+ vectors, respectively. The ctDNA was also digested with *Xho*I and cloned into the λ DASH II vector in accordance with the manufacturer's instructions (Stratagene, USA). Recombinant clones were selected by colony or plaque hybridization with clones of rice ctDNA as probes. These clones were kindly provided by Dr. A. Hirai (The University of Tokyo, Japan). Using these homologous and heterologous probes, we constructed a physical map of asparagus ctDNA and determined the locations of the genes on the ctDNA.

Results

Restriction endonuclease analysis and estimation of genome size

Restriction fragment patterns of asparagus ctDNA, after digestion with *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Pst*I + *Sal*I, respectively, are shown in Fig. 1. The length of the chloroplast genome was estimated to range from 154.4 kb (from the *Bam*HI digest) to 155.7 kb (*Sal*I digest), as indicated in Table 1. From these values, the length of asparagus ctDNA was estimated to be approximately 155 kb.

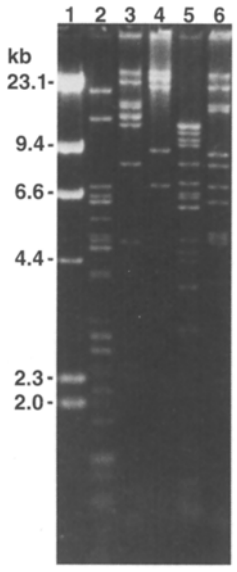

Fig. 1 Restriction fragment patterns of asparagus ctDNA generated by digestion with *Bam*HI (*lane 2*), *Pst*I (*lane 3*), *Sal*I (*lane 4*), *Hin*dIII (*lane 5*), and *Pst*I + *Sal*I (*lane 6*). *Lane 1* shows molecular weight marker of λDNA digested with *Hin*dIII

Cloning and the physical map of ctDNA from *Asparagus officinalis* L.

Asparagus ctDNA was digested with restriction enzymes, and the resulting fragments were cloned into plasmid (pUC119 and pBluescript SK+) and λ phage (λ DASH II) vectors. A clone bank was constructed that covered 70% of the genome. Some of the plasmid clones of the ctDNA are shown in Fig. 2. The restriction sites of the inserted fragments and entire ctDNA were confirmed by digestion with *Hin*dIII alone or *Hin*dIII in combination with *Bam*HI, *Pst*I, *Sal*I, and *Xho*I, and by Southern hybridization with total DNA from asparagus. From the results obtained from homologous and heterologous hybridization, recognition sites of the five restriction enzymes were confirmed in the asparagus ctDNA. A physical map of asparagus ctDNA for the five restriction endonucleases was then constructed (Fig. 3A).

On the basis of the results from homologous and heterologous hybridization, the genome of asparagus ctDNA could be divided into three parts: a large single-copy region of 90 kb; a small single-copy region of 19 kb; and a pair of inverted-repeat regions of approximately 23 kb each between the two single-copy regions. Fifteen genes were mapped on the physical map of asparagus ctDNA by Southern hybridization (Fig. 3A). From the hybridization patterns, the gene order of asparagus ctDNA was found to be the same as that of tobacco ctDNA (Shinozaki et al. 1986) but different from that of rice ctDNA (Hiratsuka et al. 1989): unlike the latter, asparagus ctDNA did not have a large inversion around the *rpoB/C* genes (Fig. 3B).

Differences among individuals, varieties, and species

Since *A. officinalis* L. is a dioecious plant and some of the cultivars are F_1 hybrids, differences among individuals

Table 1 Molecular size of restriction fragments of asparagus ctDNA generated by digestion with *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Xho*I

*Bam*HI		*Pst*I		*Sal*I		*Hin*dIII		*Xho*I	
Frag.	kb	Frag.	kb	Frag.	kb	Frag.	kb	Frag.	kb
B1	21.0	P1	34.0	S1	80.0	H1	13.0(2)	X1	27.5
B2	13.5	P2	24.0	S2	36.0	H2	11.5	X2	22.0
B3	7.1	P3	17.0	S3	22.0	H3	10.0(2)	X3	16.0
B4	6.6	P4	16.0	S4	9.1	H4	9.4	X4	13.0
B5	6.2(2)	P5	14.0(2)	S5	7.1	H5	8.2	X5	9.8
B6	5.7	P6	13.0	S6	0.8	H6	7.4	X6	9.1(3)
B7	5.1	P7	8.2	S7	0.7	H7	6.6	X7	6.5
B8	5.0	P8	4.9			H8	6.4	X8	4.9
B9	4.7(2)	P9	2.7			H9	5.8(2)	X9	4.4(2)
B10	4.2	P10	2.5			H10	5.1	X10	3.2
B11	4.1	P11	2.3			H11	4.6	X11	3.0(2)
B12	3.5	P12	1.3			H12	4.4	X12	1.8
B13	3.0(2)	P13	1.0			H13	3.9(2)	X13	1.5(2)
B14	2.9(4)					H14	3.1(2)	X14	1.3(2)
B15	2.4					H15	2.9	X15	1.0(3)
B16	2.3					H16	2.4		
B17	2.2(2)					H17	2.1		
B18	1.9					H18	1.8		
B19	1.6(6)					H19	1.7		
B20	1.4(2)					H20	1.3(3)		
B21	1.2(2)					H21	1.2		
B22	1.1(3)					H22	1.1(3)		
B23	1.0(3)					H23	0.7		
B24	0.9(2)								
B25	0.8(4)								
B26	0.7(3)								
Total	154.4	154.9		155.7		155.2		155.4	

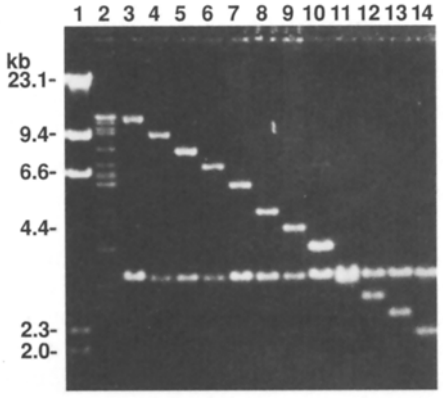

Fig. 2 Clone bank of ctDNA from *Asparagus officinalis* L. The clones and ctDNA were digested with *Hin*dIII and subjected to electrophoresis on a 0.7% agarose gel. *Lane 1* molecular weight marker of λDNA digested with *Hin*dIII, *lane 2* ctDNA of *A. officinalis* digested with *Hin*dIII, *lanes 3–14* plasmid clones of the ctDNA from *A. officinalis* L.

are possible even in a single cultivar. In order to investigate the differences among individuals, cultivars, and species, restriction fragment patterns were analyzed with three endonucleases, namely, *Bam*HI, *Hin*dIII, and *Xho*I. For a study of differences among individuals, we examined four female and four male plants of *A. officinalis* L. cv 'Mary Washington 500 W' (Fig. 4A). The restriction patterns were identical, and no differences were detected among these eight individuals. To study differences among cultivars and species, we examined six cultivars of *Asparagus officinalis* L. and another two species of *Asparagus*, namely, *A. plumosus* (var 'nanus') and *A. scandens* (Fig. 4B). The six cultivars gave the same patterns of bands, indicating that the genetic distance between them was very close. In contrast, the patterns of bands for the three species of *Asparagus* were slightly different. *A. officinalis* and *A. plumosus* belong to Euasparagus and Asparagopsis, respectively (Bailey 1944). *A. scandens* did belong to section Asparagopsis (Bailey 1944), but Clifford and Conran (1987) have recently classified it into Myrsiphyllum. Our results indicate that the genetic distance between these sections is relatively close.

Discussion

The length of the chloroplast genome from *Asparagus officinalis* L. and the gene order

The length and the gene order of asparagus ctDNA were the same as those of tobacco but different from those of rice (Fig. 3B). Asparagus is a monocot, as is rice. However, asparagus ctDNA does not include three major inversions around the *rpoB/C* genes, a characteristic of ctDNAs in the Gramineae (Quigley and Weil 1985;

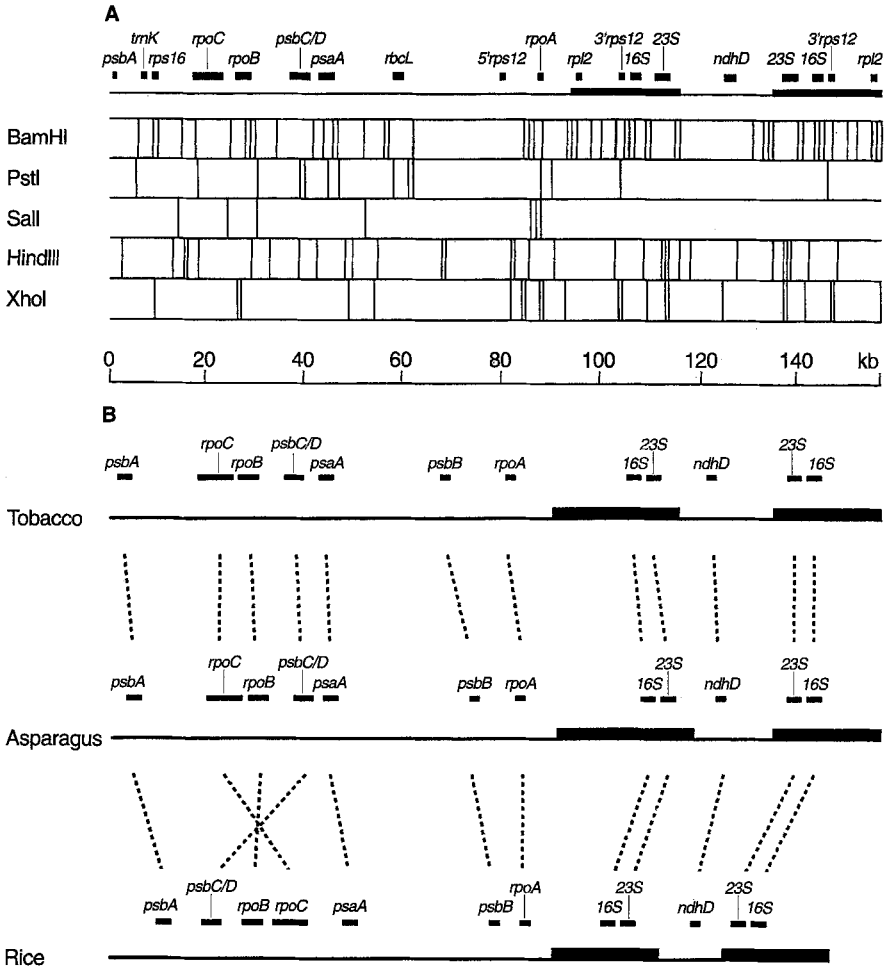

Fig. 3 **A** Linear physical map of asparagus ctDNA showing the restriction sites of five endonucleases, namely, *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Xho*I. *Long heavy lines* over the map indicate locations of inverted repeats (IR), and the genes are indicated by *short heavy lines*. **B** Comparison of the gene order of ctDNA from tobacco, asparagus, and rice

Hiratsuka et al. 1989; Doyle et al. 1992). Doyle et al. (1992) also found this inversion in genera that represent all subfamilies of grasses and in the non-grass families Restionaceae, Ecdeiocoleaceae, and Joinvilleaceae, but not in any other monocot, such as Liliales. The gene order of ctDNA from asparagus, which is classified as a member of Liliaceae, is not consistent with this earlier observation. The gene order of onion ctDNA is also inconsistent with this observation (Katayama et al. 1991).

Differences among individuals, varieties, and species of *Asparagus*

Interspecific variations in ctDNA are common although evolutionary changes in ctDNA have occurred much slower than those in nuclear and mitochondrial DNA (Banks and Birky 1985; Palmer et al. 1985). In particular, *A. officinalis* is well-known to harbor large variations, and it is difficult to breed for the following reasons. (1) *A. officinalis* is a dioecious species that can not breed by self-pollination. Therefore, it is very difficult to establish a breeding line. (2) It takes 3 years to grow one generation, so a long time is required for estimation of a specific character. Therefore, the restriction fragment patterns of the ctDNA of eight individual plants and six cultivars of *A. officinalis* were examined after digestion by three restriction enzymes. Each individual and cultivar gave the same banding pattern (Fig. 4A,B). This result indicates that there are few or no differences in ctDNAs among individuals and among the cultivars of *A. officinalis* that we used. Additionally, three species of *Asparagus* were compared in terms of the restriction patterns of their ctDNAs (Fig. 4B). The patterns generated by the three species, namely, *A. officinalis*, *A. plumosus*, and *A. scandens*, were slightly different. As mentioned above, these three species belong to different sections in *Asparagus* and so the genetic distances among these sections seem to be relatively close.

Taxonomic classification of the genus *Asparagus* has led to the identification of four sections: Euasparagus, Asparagopsis, Kodiastigma, and Myrsiphyllum (Bailey 1994). However, phylogenetic relationships among species of *Asparagus* are not well-understood. The present study demonstrates that these four sections are closely related. We are now trying to elucidate phylogenetic relationships in the genus *Asparagus* by RFLP analysis of ctDNA and to determine the period of timing of sex differentiation in *Asparagus*.

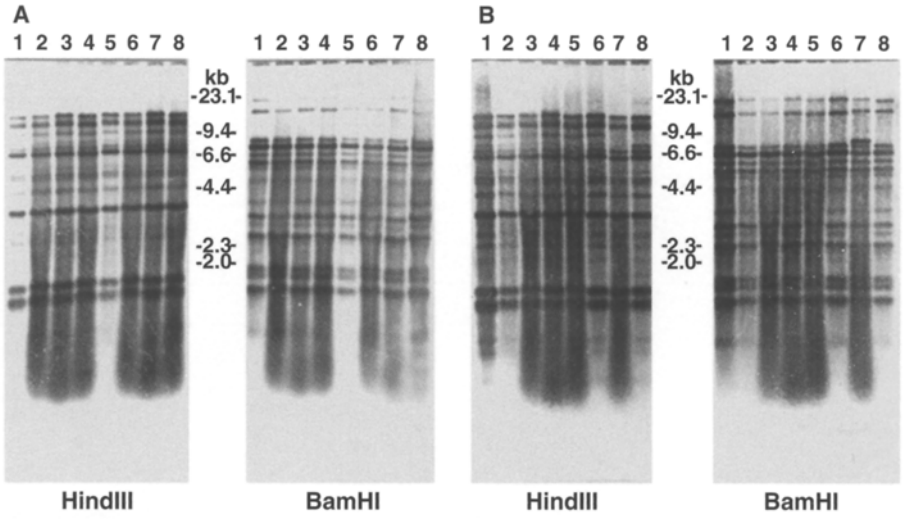

Fig. 4 **A** Southern blot analysis of total DNA from four female and four male specimens of *Asparagus officinalis* L. cv 'Mary Washington 500 W' with the entire rice ctDNA as probe. The restriction enzymes used were *Hin*dIII and *Bam*HI. *Lanes 1–4* and *5–8* show females and males, respectively. **B** Southern blot analysis of total DNA from cultivars of *A. officinalis* (*lanes 1–6*) and *Asparagus* species (*lanes 7 and 8*) with all of the cloned fragments of rice ctDNA as probe. The restriction enzymes used were *Hin*dIII and *Bam*HI. *Lane 1 A. officinalis* cv 'Mary Washington 500 W', *lane 2* cv 'Million', *lane 3* cv 'UC157', *lane 4* cv 'Hokkai 100', *lane 5* cv 'Goldschatz', *lane 6* cv 'Fruit', *lane 7 A. plumosus* var 'pyramidalis', and *lane 8 A. scandens*

Acknowledgments We thank Dr. Asano (Hokkaiseikan Co, Hokkaido, Japan), Dr. T. Yamaguchi (Fukukaen Nursery & Bulb Co, Mie, Japan), and Dr. K. Sudo (National Research Institute of Vegetables, Ornamental Plants and Tea, Mie, Japan) for providing the seeds and or plants of *Asparagus* species. We are also grateful to Dr. A. Hirai (The University of Tokyo, Japan) for providing the plasmid clones of rice ctDNA used in this study and for critical reading of the manuscript, as well as to Mr. H. Tohkairin for his collaboration in culturing the plants. This work was supported by the grant-in-aid for Center of Excellence (COE) from the Ministry of Education, Science, Sports and Culture, Japan.

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