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Differentiation of Japanese green tea cultivars as revealed by RFLP analysis of phenylalanine ammonia-lyase DNA

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Abstract Japanese green tea cultivars and 463 local tea plants including mountainous tea, *yama-cha*, were analyzed to determine the process of differentiation of Japanese tea plants using phenylalanine ammonia lyase (PAL) as a DNA marker. The main DNA fragments detected by RFLP analysis, which were named A, B and D, were inherited as multiple allelic genes at one locus. Japanese tea cultivars were divided into five groups according to RFLPs: AA, AB, AD, BD and DD. The AA group included many cultivars selected from local tea plants. The BD group consisted of cv Yabukita or descendants from Yabukita produced by artificial crossing. There was no BB group of cultivars. Allelic frequencies of A, B and D were 0.66, 0.08 and 0.22, respectively, and these values were same in tea plants collected from all regions of Japan. Since the frequencies in *yama-cha* and local tea plants were also the same, it is thought that these teas have the same origin. These results indicate a process of differentiation from the ancestral material presumably introduced from China to the local tea plants and, finally, cultivars which were produced by selecting from local tea plants and crossing.

Keywords Tea · *Camellia sinensis* · RFLP · Phenylalanine ammonia lyase · Allelic frequency

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Introduction

Tea plants (*Camellia sinensis*) are mostly cultivated in Asia, Africa and Russia. The origin of *C. sinensis*, as well as other species of *Camellia*, is thought to be regions around the source of the river Irrawaddy to the south-east of China and Assam in north-east India (Eden 1958). The origin of Japanese tea, however, is not clear. There are currently two hypotheses concerning its origin: i.e. that it was introduced into Japan from China about 1,200–800 years ago by Buddhist priests, or that it is indigenous to Japan. Tea plants, which bear close resemblance to Japanese cultivated teas, can be found on mountainous areas near human settlements in western Japan. These tea plants, called *yama-cha* (meaning "mountainous tea plant"), belong to a group of Japanese local tea plants. According to the above former hypothesis, *yama-cha* originated from cultivated local tea plants that had been introduced from China. However, according to the latter hypothesis, tea plants including *yama-cha* in Japan are indigenous. Although the origin of Japanese tea is not clear, local tea plants were first cultivated, and then several cultivars were later selected from them. Several new cultivars have been developed by artificial crossing. Local tea plants played an important role in the early tea cultivation until the first green tea cultivars were registered in 1953, which subsequently became widespread particularly in western Japan. However, local tea plants are still essential as genetic resources for green tea breeding. Evaluation of such genetic resources and the differentiation of cultivars are important for effective tea breeding.

Morphological characters such as leaf size, leaf shape, length of pistil and flower sizes (Takeda and Toyao 1980) have been used for the classification of tea plants. Chemical compounds contained in the leaves of tea plants are also useful for classification and evaluation (Nagata and Sakai 1984). Since there is no genetic isolation between the two major taxa of tea, *C. sinensis* var. *assamica* and *C.sinensis* var. *sinensis*, most teas exhibit clinal variation in morphological traits. Therefore, new

techniques that facilitate the characterization and determination of the diversity of tea germplasm are required. We have considered molecular biological methods to be suitable for this purpose, and we have cloned several genes that can be used for the characterization and evaluation of tea genetic resources (Matsumoto et al. 1994; Takeuchi et al. 1994). There have been several studies on tea plant identification and characterization using DNA techniques (Tanaka and Yamaguchi 1996; Wachira et al. 1997; Kaundun et al. 2000). We also previously reported that tea phenylalanine ammonia-lyase (PAL) cDNA is a useful DNA marker. The aim of the present study was to determine the genetic diversity of Japanese green tea germplasm using PAL cDNA as a DNA marker and to elucidate the differentiation of cultivars.

Materials and methods

Plant materials

The green tea cultivars used in this study were all derived from Japanese local tea plants and their hybrids. These cultivars are listed in Table 1. Local tea plants were collected from regions in Shizuoka, Kinki, Kochi and Kyushu. The samples collected in Kochi and Kyushu were *yama-cha*. The locations of sampling sites and the number of samples collected at each site are shown in Fig. 1.

DNA extraction and Southern-blot analysis

Genomic DNA was isolated from young tea leaves according to the modified method of Guillermaut and Maréchal-Drouard (1992). Fifteen micrograms of DNA were digested separately with the restriction enzymes *Hin*dIII and *Eco*RV, and fractionated electrophoretically in an 0.8% agarose gel. The DNA was then transferred onto nylon membranes (Hybond N+; Amersham). The PAL cDNA fragment was labeled with ^{32}P dCTP using a DNA labeling kit (Takara shuzou, Japan) for use as a probe. The membranes were hybridized overnight at 65 °C in a hybridization buffer (6 × SSC, 5 × Denhatdt's solution, 0.1% SDS) that contained the labeled probe (Sambrook et al. 1989). After hybridization, the membranes were rinsed twice with the first washing buffer (2 × SSC, 0.1% SDS) for 5 min at room temperature and then rinsed twice with a second washing buffer (0.2 × SSC, 0.1% SDS) for 15 min at 65 °C. After 2 to 7 days, the membranes were developed and several hybridized fragments were detected with the PAL cDNA probe.

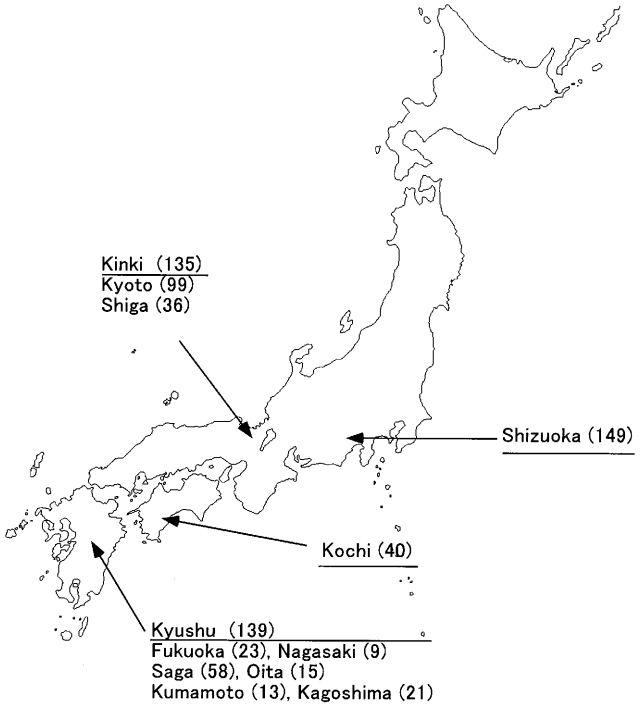

Fig. 1 Locations of sample sites and number of samples from each site used for analysis. Samples collected in Kochi, Fukuoka, Nagasaki, Oita and Kumamoto were *yama-cha* and others were local tea plants

Results

Classification of Japanese green tea cultivars

When the two restriction enzymes *Hin*dIII and *Eco*RV were used separately in RFLP analysis of Japanese green tea cultivars, five patterns were detected in both restriction enzyme experiments, which divided the cultivars into five groups (Fig. 2). Four fragments of the pattern obtained using *Hin*dIII were named A, B, C and D. Fragments A, B and D were inherited as multiple allelic genes of a single locus in a Mendelian fashion. Therefore, we classified the cultivars into the five PAL genotypes: AA, AB, AD, BD and DD (Matsumoto et al. 1994). The AA genotype group included 11 cultivars, while the other genotype groups had only four or five cultivars (Table tablerefT11). Furthermore, most of the cultivars belonging to the AA genotype group had been

Table 1 Classification of green tea cultivars based on the results of RFLP analysis using a PAL cDNA probe

PAL genotype	Cultivar
AA	Miyoshi, Tamamidori, Kyomidori, Samidori, Asahi, Gokou, Komakage, Himemidori, Kuritawase, Ujihikari, Z1
AB	Sayamamidori, Takachiho, Kanayamidoria[a], Fushun[a]
AD	Asatsuyu, Ujimidori, Fukumidoria[a], Meiryoku[a], Syunmei
BD	Yabukita, Surugawase[a], Sayamakaori[a], Toyoka[a], Saemidori[a]
DD	Koyanishi, Rokurou, Natsumidori, Asagiri

[a] Indicate offspring of cv Yabukita

selected from local tea plants about 50 years ago. On the other hand, all of the cultivars in the BD genotype group were either from cv Yabukita or its hybrids. The other genotype groups (AD, AB and DD) included cultivars selected from local tea plants and their hybrids. No cultivars of the BB type were found, as was the case in our previous study. These results indicate that the PAL genotype is related to the origin of the cultivar.

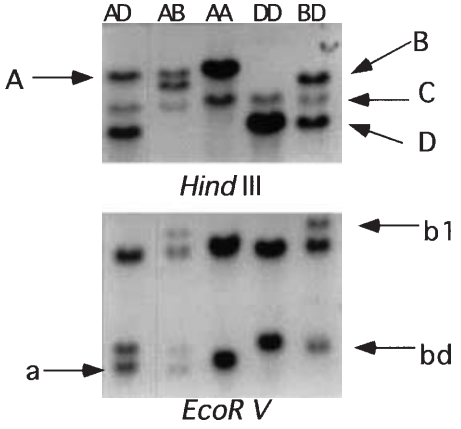

Fig. 2 Five typical hybridized fragment patterns of Japanese green tea cultivars obtained by RFLP analysis using a PAL cDNA probe. Two restriction enzymes, *Hin*dIII (*upper*) and *Eco*RV (*lower*), were used, and both results divided 29 Japanese tea cultivars into the five groups which were composed of the same cultivars. There were close links between A and a fragment and between B and b1 fragment. B and D fragments also connected with bd fragment

Analysis of Japanese local tea plants

The cultivars used in this study were divided into five groups according to the PAL genotype, each group having its own characteristics. For example, the AA group mainly contained old cultivars, while the cultivars in the BD group included Yabukita and its hybrids. To determine the relationship between the PAL genotype and the cultivars, we analyzed the local tea plants, including *yama-cha*. Fragments A, B and D were also detected in the local tea plants as had been revealed in the cultivars (Table tablerefT22). The frequency of A was higher than that of D, and the frequency of B was the lowest. The same result was found in plants from all the regions. The allelic frequencies of A, B and D in plants from Shizuoka, Kinki and Kyushu were 0.63–0.67, 0.06–0.09 and 0.22–0.24, respectively. The allelic frequencies of A, B and D in plants from Kochi (0.73, 0.11 and 0.14, respectively) were distorted owing to the low sample numbers. The mean frequencies for all samples were 0.66(A), 0.08(B) and 0.22(D), which were not significantly different from the frequencies obtained from the individual sampling. In addition to A, B and D fragments, a weak 4.1-kb fragment was also detected (Fig. 3). The frequency of this fragment was higher in the Kinki region which included the Shiga and Kyoto Prefectures when compared to the other regions (Table 2).

Discussion

Tea cultivation in Japan was initially started using local tea plants which were propagated through seed, The first clon-

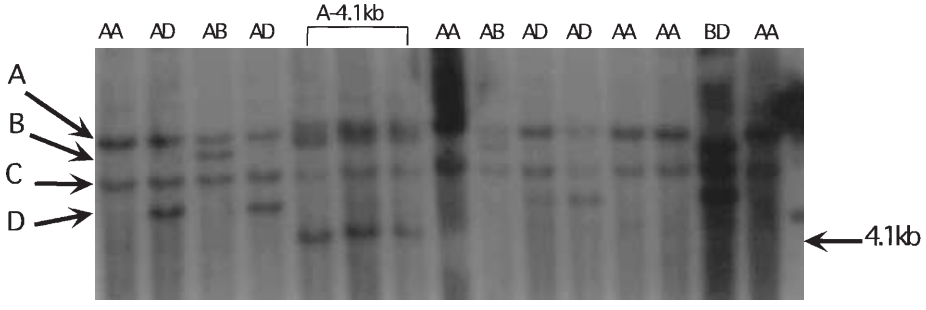

Fig. 3 RFLP patterns including a 4.1-kb fragment detected in a local variety collected in Kyoto Prefecture. The restriction enyzme used was *Hin*dIII and DNA probe used was PAL cDNA

Table 2 Comparision of the numbers of PAL genotypes and allelic frequencies among Japanese local tea plants

Region	No. of PAL genotypes								PAL allelic frequency			
	AA	AB	AD	BB	BD	DD	Other[1]	Total	A	B	D	Other[2]
Shizuoka	70	8	50	1	17	1	2	149	0.67	0.09	0.23	0.01
Kinki	51	18	33	0	4	9	20	135	0.63	0.08	0.22	0.07
Kochi	24	6	4	1	1	2	2	40	0.73	0.11	0.14	0.03
Kyushu	65	8	42	1	6	9	8	139	0.67	0.06	0.24	0.03
Total	210	40	129	3	28	21	32	463	0.66	0.08	0.22	0.03

Other[1] indicates tea plants in which one allele was a 4.1-kb fragment and the other one was A, B or D
Other[2] indicates the frequency of the 4.1-kb fragment

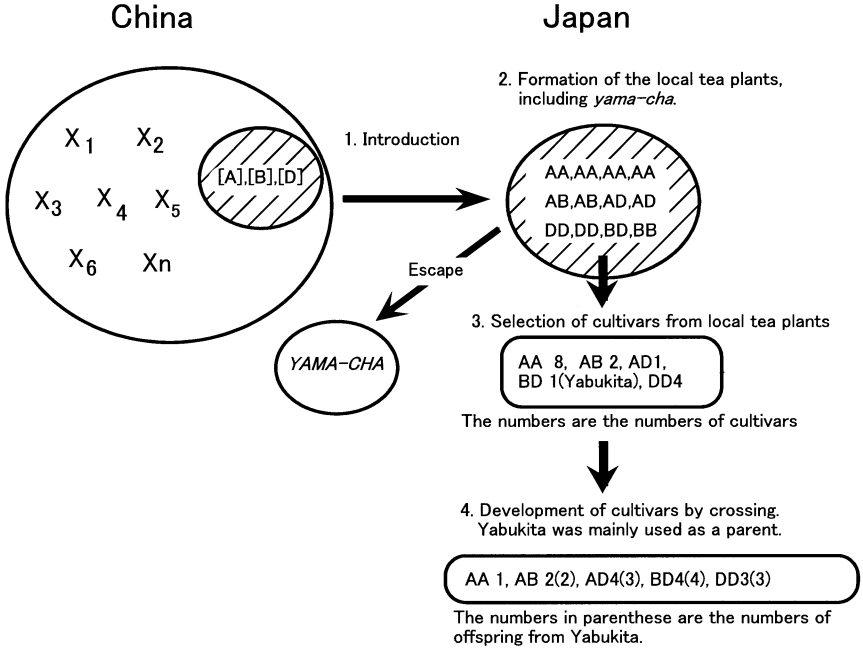

Fig. 4 The process of differentiation of Japanese green tea cultivars based on the results of PAL RFLP analysis. A, B and D are RFLP markers and Xl–Xn indicate other PAL RFLP markers detected in Chinese tea germplasm. Since half of the Japanese local tea plants were AA type, half of the selected cultivars were also AA

al cultivars were selected from these local tea plants resulting in the registration of 21 green tea cultivars during the period 1953 to 1954. Since then, these cultivars have been multiplied and spread by vegetative cuttings. Yabukita, which is one of those selected cultivars, increased dramatically, owing to its good quality and high yield. It now accounts for 84% of all tea crops in Japan and is a good crossing parent. Breeding methods in Japan have been radically changed with their emphasis shifting from selection with the unimproved seedling to crossing. Therefore, most of the new cultivars have now been derived by artificial crossing with the majority having descended from cv Yabukita. Based on these results, we speculated on the process of differentiation of Japanese green tea cultivars (Fig. 4). Half of Japanese local tea plants showed the AA PAL genotype, because the frequency of fragment A was 0.67 and the AA population was estimated to be 0.4489 (= 0.67 × 0.67). When 21 cultivars were selected from local tea plants, half of the population was thought to be AA. We analyzed 19 of these 21 cultivars and found that eight of them were AA. On the other hand, the reason why no cultivars belonging to BB were found was thought to be the low frequency of allele B in the local tea plants (0.08). The possibility of the existence of a BB cultivar is quite low because the theoretical value is 0.0064 (= 0.08 × 0.08). Furthermore, hybrids bred by crossing with Yabukita (BD) have a B or D fragment. The chances of breeding an AA cultivar by artificial crossing became low due to an over reliance on cv Yabukita as a breeding parent, and consequently the ratio of AA cultivars decreased. Inversely, although the possibility of selection of BD cultivars from the original local tea plants was low, the artificially bred cultivars with these genotypes increased due to the use of Yabukita as a breeding parent.

We have studied the genetic diversity of Chinese and Korean local teas, and reported that Chinese teas are more diverse compared with the Japanese germplasm (Matsumoto et al. 1994). While the PAL locus in Japanese germplasm was composed of only three multiple alleles, we have detected more polymorphic fragments in Chinese and Korean germplasm. In this study, a 4.1-kb fragment was also detected at very low frequency. However, the frequency of this fragment was higher in samples from the Kinki region (Kyoto and Shiga) than those from other regions. This fragment was also detected in Chinese and Korean teas at higher frequency. Apart from molecular markers, the length of the pistil is an important and useful morphological descriptor for the characterization and classification of tea plants. The Chinese tea germplasms have a longer pistil often with protruding anthers. On the other hand, the pistil of Japanese germplasm is shorter and hidden by the anthers (Oishi 1959; Toyao et al. 1996). Tea plants which have the long type of pistil similar to the Chinese and Korean germplasm, were more abundant in the Kinki region including the Kyoto and Shiga prefectures. Hybrid cultivars crossed with the Chinese tea also had a long pistil. It is therefore speculated that the origin of the long pistil character and the 4.1-kb DNA fragment is from China. While the reason is unknown, local tea plants in the Kinki region are thought to include more Chinese types than the other regions of Japan.

Matsushita (1978) reported that the distribution of *yama-cha* was limited to regions near human habitation and did not extend deep into forests. In his study, there were no morphological differences and no genetic isolation between *yama-cha* and the local tea plants. Matsushita therefore supported the hypothesis that Japanese tea was introduced as a cultivated plant from China and that *yama-cha* represented escaped plants from the cultivated tea germplasm. In the present study, the PAL allelic frequencies of the local tea plants, including

yama-cha, were the same. These results therefore suggest that *yama-cha* and the local tea plants are of the same character and have the same origin. We also speculate that Japanese tea was introduced from China, because *yama-cha* was found in limited regions near the human settlement, as reported by Matsushita, and because fragments A, B and D were also detected along with some Chinese specific fragments in Chinese tea germplasm (in preparation). In addition to A, B and D, at least seven other different alleles (shown as X1–Xn in Fig. 4) were found in the PAL locus of Chinese tea germplasm which increased the diversity of allelic combinations. Therefore, it was not easy to obtain Chinese tea populations with a composition of only A, B and D alleles, and which would potentially be the ancestor of Japanese tea. A comparision of DNA diversity between Japanese and Chinese germplasm suggests that limited populations of tea from China were introduced into Japan. On the other hand, there are historical records where several Japanese Buddhist priests introduced tea seeds from various regions in China to various regions in Japan about 800–1,200 years ago (Matsuzaki 1992). According to these records, the chances of tea introduction from China to Japan increased, and the process of introduction was complex and should have caused the accumulation of more genetic diversity and imbalance in the genetic frequency of neutral genes against selection. As we showed in this study, the ancestral Japanese teas were from limited populations of Chinese germplasm, and there is no possibility that the ancestral teas were introduced from China many times. Furthermore, not only the main PAL fragments but also fragment B or the 4.1-kb fragment, which was easy to omit due to its low frequency, was maintained equally among the Japanese local tea plants. These findings suggest that, even if the Buddhist priests brought tea seeds, all teas introduced by them did not influence the formation of Japanese local tea plants. Although more scientific evidence is needed to elucidate the origin of Japanese tea plants, the results of this study using PAL as a DNA marker demonstrated that the cultivars differentiated from local tea plants, and that *yama-cha* was an escape plant (Fig. 4).

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