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Organelle DNA polymorphism in apple cultivars and rootstocks

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Summary. Restriction fragment length polymorphisms (RFLPs) have been used to detect chloroplast (cp) and mitochondrial (mt) DNA variation among 18 apple cultivars and three rootstocks. The distribution of RFLP patterns allowed the assignment of these genotypes into three groups of cytoplasmic relatedness. Our results also demonstrate maternal inheritance of cp- and mtDNAs in apple. Thus, the organelle DNA assay provides a convenient and reliable method to assess cytoplasmic diversity within the apple germ-plasm collection and to trace the maternal lineages involved in the evolution of apple.

Key words: Apple – Chloroplast DNA – Mitochondrial DNA – RFLP – Cytoplasmic diversity

Introduction

Since the early demonstration of cytoplasmically inherited susceptibility to the southern corn-leaf blight in hybrid maize production, attention has been paid to the genetic vulnerability of cytoplasmic uniformity (Gracen and Grogan 1974; Fragoso et al. 1989). Most plant breeders agree that the identification and preservation of cytoplasmic diversity offer the best guarantee against the possibility of future epidemics. Restriction fragment length polymorphism (RFLP) analysis has been used to compare organelle DNAs from a wide range of plant species (see the reviews by Leaver and Gray 1982; Hanson and Conde 1985; Pring and Lonsdale 1985; Palmer 1987; Tsunewaki 1989). These comparisons have proven particularly useful in the survey of cytoplasmic variation.

Although the apple (*Malus domestica* Borkh) is one of the most widely grown fruit in the world, little is known about the degree of heterogeneity of organelle DNAs among the apple cultivars. The primary goal of this study was to evaluate cytoplasmic diversity among the apple germ-plasm collection on the basis of chloroplast (cp) and mitochondrial (mt) DNA analysis. This type of research would also provide new insights into the maternal lineages involved in the evolution of apple and its close relatives.

Materials and methods

Preparation of cpDNA

The cultivars and rootstocks used in this study (Table 1) are part of the apple germ-plasm collection at the Experiment Farms, Faculty of Agriculture, Hokkaido University, Japan. CpDNA was isolated from actively growing leaves essentially as described by Mikami et al. (1984).

Preparation of total DNA

Total DNA was isolated using modified procedures of Saghai-Maroof et al. (1984). Approximately 3 g of leaf tissue was ground to a fine powder in liquid nitrogen, and this powder was then transferred to a 50-ml glass centrifuge tube containing 20 ml of buffer (100 m*M* TRIS-HCl pH 8.0, 2% hexadecyltrimethyl ammonium bromide, 1.4 *M* NaCl, 0.2% 2-mercaptoethanol, 20 m*M* EDTA). The tube was incubated at 60 °C for 30 min with occasional swirling, and the contents were then extracted once with chloroform/isoamylalcohol (24:1 by volume). After centrifugation at 1,600 *g* for 15 min, the aqueous phase was removed, and two-third volumes of cold isopropanol were added to precipitate the nucleic acids. Nucleic acids were dissolved in 2 ml of TE (10 m*M* TRIS-HCl pH 8.0, 1 m*M* EDTA), and the DNA was further purified by phenol extraction, ethanol precipitation and CsCl-ethidium bromide equilibrium centrifugation as described by Sugiura and Kusuda (1979).

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Table 1. Summary of results obtained from cpDNA and mtDNA analysis in apple cultivars and rootstocks. Fragment sizes are indicated in kbp

Cultivar or rootstock	Parentage	CpDNA		MtDNA			
		Restriction fragment pattern [b]		Filter-bound DNA hybridized with			
				coxI		*atp9*	
		*Bam*HI	*Eco*RI	*Eco*RI	*Hin*dIII	*Bam*HI	*Eco*RI
Cultivar:							
Golden Delicious	US cultivar	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Jonathan	do	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Fuji	Rolls Janet × Delicious	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Hokuto	Fuji × Mutsu	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Sensyu	Toh-koh × Fuji	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Alps Otome	Fuji × Jonathan	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Mutsu	Golden Delicious × Indo	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Koutoku	Toh-koh × ? [a]	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
Red Gold	Golden Delicious × Richared Delicious	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
McIntosh	Canadian cultivar	2	1	5.9	9.1, 5.0	3.5, 2.8	4.0, 1.7
American Summer Pearmain	US cultivar	2	1	5.9	9.1, 5.0	3.5, 2.8	4.0, 1.7
Delicious	do	3	2	5.9	9.1, 5.0	3.5	1.4
Starking Delicious	Sport of Delicious	3	2	5.9	9.1, 5.0	3.5	1.4
Red Prince	do	3	2	5.9	9.1, 5.0	3.5	1.4
Richared Delicious	do	3	2	5.9	9.1, 5.0	3.5	1.4
Oregon Spur Delicious	do	3	2	5.9	9.1, 5.0	3.5	1.4
Starkrimson Delicious	Sport of Starking Delicious	3	2	5.9	9.1, 5.0	3.5	1.4
Sekai-ichi	Delicious × Golden Delicious	3	2	5.9	9.1, 5.0	3.5	1.4
Rootstock:							
M.9	Jaune de Metz Paradise	3	2	5.9	9.1, 5.0	3.5	1.4
M.26	M.16 (U3.Spath) × M.9	1	1	11.0	10.0, 4.2	3.5, 2.8	4.0, 1.7
6109011	*M. prunifolia* Borkh	3	2	5.9	9.1, 5.0	3.5	1.4

[a] Pollen parent is unknown
[b] Restriction fragment pattern groupings are as shown in Fig. 1

Gel electrophoresis

Restriction endonuclease digestions were carried out under the conditions specified by the enzyme supplier. Five to eight micrograms of total DNA was digested in 100 µl of reaction solution with sufficient enzyme to give complete digestion. The restriction fragments were separated electrophoretically on 0.8% agarose gels in TBE buffer (89 m*M* TRIS, 89 m*M* boric acid, 2 m*M* EDTA pH 8.0) at 30 mA for 16–18 h. The gels were Southern blotted onto nylon membranes (Hybond N, Amersham) according to the manufacturer's recommendations.

Hybridization

Probe DNA was labelled with [^{32}P]-dCTP by random hexamer primer extension (Feinberg and Vogelstein 1983). Hybridizations were done according to the protocol of Maniatis et al. (1982). After hybridization, the filter membranes were washed twice at 65 °C for 20 min and then exposed to Fuji RX X-ray film at −80 °C using intensifying screens.

Mitochondrial genes used in the hybridization studies were: sugar beet *coxI* (1,500-bp *Eco*RI fragment; Senda et al. 1991), sugar beet *coxII* (800-bp *Xho*I-*Bam*HI fragment; Senda et al. 1991), pea *atpA* (1,400-bp *Eco*RI-*Hin*dIII fragment; Morikami and Nakamura 1987a), pea *atp9* (1,300-bp *Hin*dIII-*Bam*HI fragment; Morikami and Nakamura 1987b) and pea *rrn18* (2,600-bp *Bam*HI fragment; Huh and Gray 1982).

Results

Chloroplast genome

Chloroplast DNAs isolated from 18 apple cultivars and three rootstocks were examined using three restriction enzymes, *Bam*HI, *Eco*RI and *Hin*dIII. As seen in Fig. 1 A, *Bam*HI resolved three types of cpDNAs. With the pattern characteristic of 'Golden Delicious' (Fig. 1 A, lane 1) as the standard, the pattern of 'McIntosh' (Fig. 1 A, lane 2) is seen to be missing a 2.5-kbp band and to have an extra band of 2.45 kbp. 'Delicious' cpDNA (Fig. 1 A, lane 3) is characterized by the presence of two additional *Bam*HI fragments of 4.8 kbp and 2.45 kbp and by the absence of the 2.5-kbp and 2.2-kbp *Bam*HI fragments. In addition, *Eco*RI analysis revealed two restriction fragment profiles (Fig. 1 B), whereas the frag-

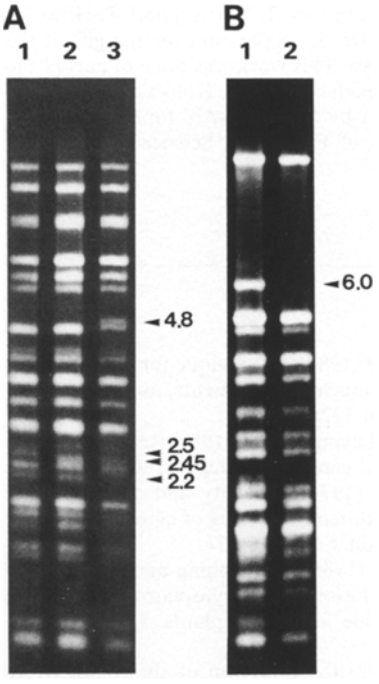

Fig. 1 A, B. *Bam*HI (**Panel A**) and *Eco*RI (**Panel B**) restriction patterns of cpDNAs. The DNAs shown are: *A1* 'Golden Delicious', *A2* 'McIntosh', *A3* 'Delicious', *B1* 'Golden Delicious', *B2* 'Delicious'. The DNA fragments were separated by electrophoresis on a 0.8% agarose gel. Sizes of polymorphic fragments are indicated in kbp

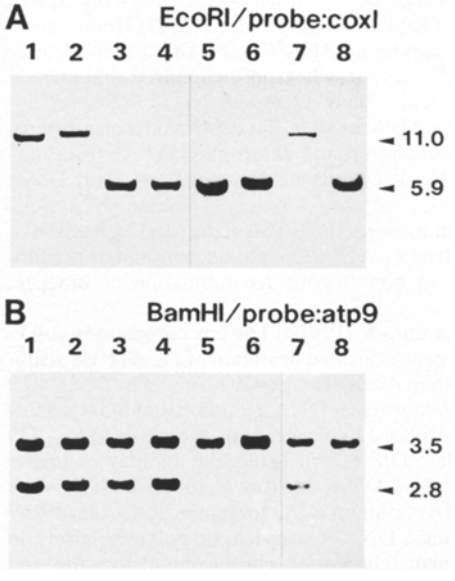

Fig. 2. Autoradiograph identifying mtDNA polymorphism in apple cultivars and rootstocks. The DNAs shown are *1* 'Golden Delicious', *2* 'Red Gold', *3* 'McIntosh', *4* 'American Summer Pearmain', *5* 'Delicious', *6* 'Sekai-ichi', *7* M.26, *8* M.9. Total DNA from each genotype was digested with *Eco*RI (**Panel A**) or *Bam*HI (**Panel B**) and electrophoresed on a 0.8% agarose gel. The gel was blotted onto a nylon membrane filter and hybridized wiht the ^{32}P-labelled *coxI* (**A**) or *atp9* (**B**) probe. Sizes of the hybridization fragments are indicated in kbp

ment patterns generated by *Hin*dIII showed no useful cpDNA variation (data not shown). The distribution of the cultivars and rootstocks among the three unique cpDNA types is shown in Table 1.

The molecular size of the apple chloroplast genome was estimated by summing the sizes of fragments produced by *Bam*HI digestion. When band multiplicity was taken into consideration, the size of 'Golden Delicious', 'McIntosh' and 'Delicious' cpDNAs were 143.4, 143.35 and 143.7 kbp, respectively.

Mitochondrial genome

The mtDNA organization of 21 apple genotypes was studied by Southern hybridization of heterologous mitochondrial gene probes (see Materials and methods) with restriction enzyme digests of total DNA. Figure 2 illustrates the representative profiles of hybridization, which reveal enough variation to characterize three distinct types of the apple mitochondrial genomes. The RFLP data are also compiled in Table 1.

For example, the hybridization of *Eco*RI-digested DNAs to the *coxI* probe detected a polymorphic fragment of 11.0 kbp in 'Golden Delicious', but not in 'Delicious' and 'McIntosh', where a unique fragment of 5.9 kbp was identified instead (Fig. 2A). The same grouping was generated by hybridizing *Hin*dIII-restricted DNA with the *coxI* probe. As presented in Table 1, 8 cultivars ('Jonathan', 'Fuji', 'Hokuto', 'Sensyu', 'Alps Otome', 'Mutsu', 'Koutoku' and 'Red Gold') and a rootstock (M.26) shared the 'Golden Delicious' pattern, whereas the remaining genotypes examined ('American Summer Pearmain', 'Starking Delicious', 'Red Prince', 'Richared Delicious', 'Oregon Spur Delicious', 'Starkrimson Delicious', 'Sekai-ichi', M.9 and *M. prunifolia* Borkh acc. 6109011) showed the pattern identical to that seen with 'Delicious' or 'McIntosh' DNA. Hybridization of the *Bam*HI, *Eco*RI or *Hin*dIII Southern blots with the *atp9* probe allowed us to distinguish the mitochondrial genome of 'McIntosh' and 'American Summer Pearmain' from that of 'Delicious' (Fig. 2B, Table 1; data from *Hin*dIII analysis not shown). On the other hand, no polymorphism was detected when the *coxII, atpA* and *rrn18* sequences were used as probes (data not shown).

One could question whether these RFLPs represent mtDNA because of the widespread sequence homology between mt- and cpDNAs in higher plants (Stern and Lonsdale 1982; Marechal et al. 1987; Schuster and Brennicke 1988). The Southern filter containing *Bam*HI or *Eco*RI fragments of cpDNA (Fig. 1) was hybridized with the *coxI* and/or *atp9* probe, and it failed to hybridize under our hybridization wash conditions (data not shown). This indicates that the RFLPs are not chloroplastic in origin, but represent mtDNA.

Discussion

Our results reveal differences in organelle DNA organization among apple cultivars and rootstocks that can be used to discriminate at least three cytoplasm groups. The groupings of the apple genotypes by cpDNA-mtDNA RFLP analyses agree with each other. This is thus the first report on cytoplasmic diversity within the apple germ-plasm collection, though the specific mutational basis for the cp- and mtDNA RFLPs observed here remains to be characterized.

Most angiosperms exhibit maternal inheritance of the cytoplasmic organelles, with approximately one-third having some degree of biparental inheritance in the chloroplasts (Sears 1980; Whatley 1982). RFLP analyses made it possible to ascertain the inheritance of organelle DNAs in apple. 'Red Gold', the progeny of the cross 'Golden Delicious' × 'Richared Delicious' (a sport of 'Delicious') yielded the restriction patterns coincident with the pattern of cp- and mtDNAs from 'Golden Delicious' alone. Conversely, endonuclease digestion of organelle DNAs from 'Sekai-ichi' ('Delicious' × 'Golden Delicious') was found to produce only the fragment patterns of the maternal parent, 'Delicious'. Intermediate fragment patterns, indicative of a paternal contribution, are not evident; therefore, these data indicate that there is essentially maternal inheritance of cp- and mtDNAs in apple.

One of our aims was to examine the origin and maternal ancestry of economically important apple cultivars. The list of genotypes tested for RFLPs includes several instances in which both the cultivar and its sports were characterized (see Table 1). We found that 'Delicious' and its sports ('Starking Delicious', 'Red Prince', 'Richared Delicious' and 'Oregon Spur Delicious') were not separable from one another in terms of their organelle DNA organizations; neither were 'Starking Delicious' and its sport, 'Starkrimson Delicious.' In our study, three rootstocks were also included: an accession of *M. prunifolia* and the widely used rootstocks, M.9 and M.26 (from the East Malling Research Station, UK), the latter of which are believed to be related to the indigenous European genotypes of 'Paradise' and 'Doucin' apples (Vinterhalter and James 1986). Interestingly, M.9 and *M. prunifolia* shared the same pattern of RFLPs with 'Delicious', while M.26 and 'Golden Delicious' were placed into another cytoplasm group. The evidence favours the hypothesis of separate and presumably independent domestication events in the wild ancestral forms of the taxon. Further investigations are in progress that use a larger number of apple landraces and wild relatives in order to elucidate organelle genome diversity and its evolutionary implications.

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