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The plastome of *Citrus*. Physical map, variation among *Citrus* cultivars and species and comparison with related genera

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Summary. A physical plastome map was constructed for *Citrus aurantium*, and the plastomes of species and cultivars of *Citrus* and of two *Citrus* relatives were analysed by Southern blot-hybridisation of labelled total tobacco cpDNA to digests of total *Citrus* DNA. A resemblance was found between the plastomes of cultivars of *C. limon* (lemon), *C. sinensis* (orange), *C. aurantium* (sour orange), *C. paradisii* (grapefruit) and *C. grandis* (pomello). The plastomes of other *Citrus* types such as mandarin (*C. reticulata*) and citron (*C. medica*) differed from each other as well as from the plastomes of the aforementioned group. The plastomes of *Poncirus trifoliata* and *Microcitrus* sp. are distinct from each other as well as from the *Citrus* types.

Key words: Chloroplast DNA – *Citrus* – Restriction mapping – Plastomes

Introduction

The species and cultivars of the genus *Citrus* (with the possible exception of the grapefruit, *C. paradisii*) originated in a large area ranging from the Himalayas in the North-West to New Caledonia and Australia in the South-East (Vardi and Spiegel-Roy 1978).

Apomixis and many centuries of cultivation and hybridisation have complicated *Citrus* taxonomy with the result that very different systems have been proposed and there has been a large diversity in the number of species recognised: from 159 (Tanaka 1969) to 16 (Swingle 1967) and 3 (Barret and Rhodes 1976). A comparison of endonuclease restriction patterns of cpDNA has been used to investigate taxonomic problems in some genera: *Nicotiana* (Rhodes et al. 1981), *Coffea* (Berthou et al. 1983) and *Lycopersicon* (Palmer and Zamir 1982). Physical maps have been constructed for the plastomes of many plant species (Dyer 1984) but none, so far, for any "woody species". In this study we have constructed a physical restriction map of the *C. aurantium* plastome and investigated differences in chloroplast DNA restriction patterns, within the genus *Citrus* as well as between *Citrus, Poncirus* and *Microcitrus,* as a source of phylogenetic information. In our previous work on *Citrus* we developed protoplast-to-plant systems in this genus (Vardi et al. 1982) and more recently have used the "donor-recipient" protoplast-fusion method (Galun and Aviv 1986) to produce inter-cultivar *Citrus* cybrids (Vardi et al., in preparation).

The results of the present study constitute a useful tool for the identification of plastomes in cybrid plants of *Citrus*.

Materials and methods

Chloroplast DNA extractions

Chloroplast DNA was isolated using a modification of the procedure of Frankel et al. (1979). Leaves from 2–4-month old plants or the soft, young leaves from mature trees were used. In both cases the leaves were previously darkened for 2 to 6 days. All procedures until lysis were carried out at 4 °C. For each sample 25 g of leaves were washed and finely chopped with 75 ml Buffer A (Sorbitol, 350 mM; MES, 50 mM; EDTA, 2 mM; $MgCl_2$, 1 mM; K_2HPO_4, 0.8 mM; $MnCl_2$, 1 mM; NaCl, 50 mM; ascorbic acid, 5 mM; B-mercaptoethanol, 7 mM; spermine 1 mM; spermidine 1 mM; PVP, 2 mg/ml; pH 6.1) in a Waring Blender, fitted with razor blades, for 5×5 s bursts at 4/5 of line voltage. The material was then strained through 4 layers of gauze and 2 of Miracloth and the filtrate centrifuged for 2 min at 250×g (Sorval HG4L). The supernatent was centrifuged for 5 min at 1,600×g (Sorval HG4L). The resultant pellet was resuspended gently with a soft paintbrush in 40 ml Buffer B (Sorbitol, 350 mM; hepes, 50 mM; EDTA, 2 mM; $MgCl_2$, 1 mM; $MnCl_2$, 1 mM; K_2HPO_4, 0.5 mM; NaCl, 50 mM; spermine, 1 mM; spermidine, 1 mM; PVP, 2 mg/ml; BSA 1 mg/ml; pH 7.6) and recentrifuged for 5 min at 1,600×g

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(Sorval HG4L). The pellet was then resuspended in 2 ml Buffer B and loaded onto a discontinuous gradient, (25%, 40%, 60% sucrose in Buffer B) in an SW 28 polyallomer tube and centrifuged for 1 h, 72,000×g (Beckman).

Two chloroplast bands appeared and were removed separately with wide mouthed Pasteur pipettes into siliconized 30 ml Corex tubes. They were incubated with 50 µg/ml proteinase K for 4 min at room temperature, then 0.2 M NaOH and 2% sarkosyl (final concentration) were added. After 30 min of lysis at 37 °C the lysate was centrifuged for 30 min at 16,300×g (Sorval HB4). The supernatant was extracted twice with freshly distilled phenol, and once with chloroform and isoamyl alcohol (24 : 1 v/v). The DNA was ethanol precipitated from the aqueous phase. Yields of about 2 µg DNA/g starting tissue were obtained.

Total DNA isolation

Total plant DNA was isolated using the method of Fluhr (1983) with minor modifications. Two hundred mg of young leaf tissue was homogenised on ice, using a mortar and pestle, with 100 mg PVP (P-6755, Sigma) in 5 ml buffer A (as for chloroplast DNA extraction but with 400 µg/ml ethidium bromide in place of spermine and spermidine).

Sodium dodecyl sulphate and proteinase K were added to a final concentration of 0.5% and 50 µg/ml, respectively, and lysis was carried out in the dark for 1 h at 37 °C. The lysate was centrifuged 12,000×g (Sorval SS34), and the supernatant removed to a Corex tube. It was then extracted once with distilled phenol (freshly neutralised with an equal volume of 0.1 M Tris base) twice with chloroform and isoamyl alcohol (24 : 1, v/v) and once with distilled ether. The ether was totally removed by evaporation with nitrogen and the extract was centrifuged at 4 °C for 5 h at 192,000×g in SW 50.1 polycarbonate tubes (Beckman). The pellet was resuspended for 24 h in 100 µl TE buffer.

The average yield of total DNA was 1.5 µg/mg starting tissue.

Enzymes and gel electrophoresis

Restriction endonucleases (from Biolabs or Anglian) were used singly or in combination (double digestions), according to the suppliers instructions, to digest 2.0 µg DNA in 60 µl assay volumes. After digestion, a 0.6 volume of liquified sample buffer (0.4% sea plaque agarose, 10% glycerol and 0.02% bromophenol blue) was added to each reaction and the samples were run on agarose gels (0.5 to 1.2% seakem ME type 2) in TAE running buffer (40 mM Tris acetate, pH 7.8, 20 mM sodium acetate, 2 mM EDTA).

DNA transfer and hybridisations

DNA transfer to nitrocellulose was done according to the method of Wahl et al. (1979). Nick translation was carried out with modifications to the procedure of Maniatis et al. (1982). 0.5–1 µg of DNA was digested with 25 µg DNAase for 1 min at 37 °C in 20 µl of reaction mixture containing 50 mM tris pH 7.8, 5 mM $MgCl_2$ 10 mM B–mercaptoethanol, 3.75 mM unlabelled nucleotides and 20 µCi labelled nucleotide (Amersham). Then 5 units of DNA polymerase I (Biolabs) were added and incubated for 6 min at 15 °C. This gave an incorporation of 50–90% of the labelled nucleotide into the probe. The reaction was terminated with 80 µl of 0.1% SDS; 40 µl sonicated salmon sperm DNA were then added; the probe was boiled for 10 min and then frozen.

Hybridisation reactions were in 6×SSC, 0.08% PVP-360, 0.08% Ficol 400, 0.08% BSA (Fraction V, Sigma) 50 mM phosphate buffer (pH 6.8) and 200 µg/ml denatured and sonicated salmon sperm DNA. The nitrocellulose blots were soaked in 2×SSC for 2 min and prehybridized in 5–15 ml of hybridisation buffer for at least 2 h at 65 °C in heat-sealable bags. The probe was added together with fresh hybridisation buffer and hybridisation carried out overnight at 65 °C.

After hybridisation the blot was washed, at room temperature, four times for 15 min in 0.1% SDS, 2×SSC then at 50 °C twice for 15 min in 0.1% SDS, 0.1×SSC and finally rinsed in 0.1×SSC. Several exposures of varying duration were made onto X-ray films.

Results

The sizes of the fragments generated by PvuII, XhoI and PstI digestion of *C. aurantium* cpDNA were calculated from molecular-size markers (e.g. HindIII digested lambda phage). The average size of the *C. aurantium* plastome, calculated by summation of the fragments is 166.5 kb (Fig. 1, Table 1).

A physical map of the *C. aurantium* plastome was constructed using heterologous probes from *N. tabacum* (Fig. 2i). Table 2 shows how the *C. aurantium* fragments hybridised with these probes. Using these data many of the *C. aurantium* fragments could be sequentially aligned. Thus, for example, the *C. aurantium* fragment X2A hybridised with *N. tabacum* probes Ps2A

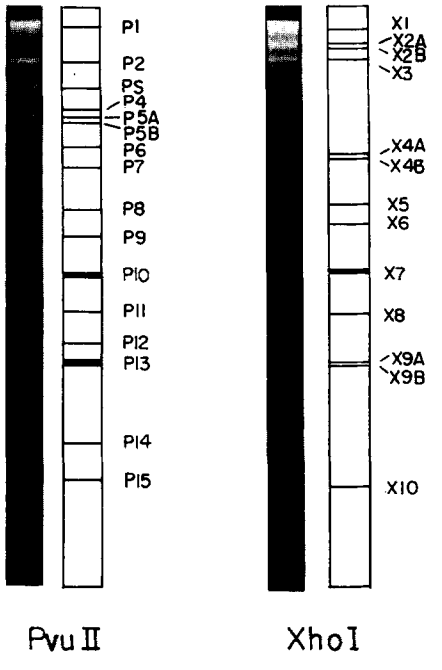

Fig. 1. *C. aurantium* cpDNA restriction patterns. *C. aurantium* cpDNA was cut with two restriction endonucleases and the fragments separated on an 0.8% agarose gel. For fragment sizes see Table 1. Some of the smaller bands are difficult to see on these fluorographs but were revealed in parallel fluorographs; only fragments subsequently verified by southern blotting (see Fig. 4) were presented in the schemes

and Ps2B. In addition X10 hybridised with probe Ps2B and X1 with Ps2A, Ps12, Ps6, Ps4 and Ps3A. Ps3A also hybridised with X6 and X2B. X2B hybridised with *N. tabacum* probe Ps10. The order of the PstI cut *N. tabacum* fragments in Ps2B, Ps2A, Ps12, Ps6, Ps4, Ps3A, Ps10. Therefore we assumed the order of the XhoI cut *C. aurantium* fragments to be X10, X2A, X1, X2B, X6, X2.

Table 1. Molecular sizes of cpDNA fragments produced by three restriction endonucleases

PvuII		XhoI		PstI	
Fragment no. (dose)	Size (kbp)	Fragment no. (dose)	Size (kbp)	Fragment no. (dose)	Size (kbp)
P1	46.0	X1	45.6	Ps1A	40.5
P2	19.0	X2A	28.2	Ps1B	35.0
P3	14.4	X2B	26.0	Ps2A	22.5
P4	11.6	X3	21.0	Ps2B	19.0
P5A	10.5	X4A	7.8	Ps3	16.0
P5B	10.3	X4B	7.7	Ps4	12.5
P6	8.6	X5	6.3	Ps5	7.8
P7	7.5	X6	5.7	Ps6	6.3
P8	5.9	X7 (x2)	4.2	Ps7	2.8
P9	5.2	X8	3.5	Ps8	1.9
P10 (X2)	4.2	X9 (x2)	2.7	Ps9	1.2
P11	3.8	X10	1.2	Ps10	1.0
P12	2.9				
P13 (x3)	2.7				
P14	2.0				
P15	1.8				
Total	166.1		166.8		166.5

Hybridisation of the *N. tabacum* probes to double digestions (with pairs of enzymes) of *C. aurantium* cpDNA fragments helped to align the three restriction maps with each other (Fig. 2ii). It also allowed the clarification of those regions which could not be interpreted using the results of hybridisations to the single digests, mainly the inverted repeat regions and the area of the long single copy (LSC) hybridising with *N. tabacum* probe Ps3A. A physical map was consequently constructed (Fig. 3).

Heterologous gene-probes were used to map four genes on the *C. aurantium* plastome. The probes used were *rbc* L, the gene for the large subunit of RuBP carboxylase (LS) from maize (McIntosh et al. 1980), the genes for the alpha and beta subunits of ATP-synthetase (*atp* A and *atp* B from tobacco, prepared by H. Fromm in our department) and an internal sequence of the 32 Kd protein gene *psb* A from *Spirodela* (Fluhr et al. 1983). Another probe was used which contained *atp* B + *rbc* L and the respective intervening region. Figure 3 shows how these probes hybridised.

The results of the mapping work showed that the plastomes of *N. tabacum* and *C. aurantium* are essentially co-linear and that all of the major fragments generated by endonuclease digestion of *C. aurantium* cpDNA hybridise with at least one of the *N. tabacum* probes. Therefore, total *N. tabacum* cpDNA was used as a probe to hybridise with the cpDNA in digestions of total cellular DNA extracted from leaves of the different *Citrus* types.

One of the hybridisation patterns given by this method is shown in Fig. 4. A schematic diagram com-

Table 2. Hybridisation of *N. tabacum* plastome probes to *C. aurantium* fragments cut with PstI, PvuII and XhoI

N. tabacum Probes[a]	Endonucleases		
	PvuII	XhoI	PstI
Ps 9	P1	X9	PS7
Ps 8	P1	X9	PS6
Ps 2B	P1	X2, X10	PS2B, PS6
Ps 2A	P7, P9, P12, P13, P14, P15	X2, X1	PS2A
Ps 12	P4	X1	PS10
Ps 6	P4	X1	PS3
Ps 4	(P4), P5	X1	PS3, PS8
Ps 3A	P3, P5, P11	X1, X2, X6	PS4, PS5
Ps 10	(P1), P2, P3	X2, X3	PS9, PS1
Ps 5	P1, P2	X2, X3	PS1
Ps 7	P1, P2	X2, X3	PS1
Ps 1	(P1), (P2), P5, (P6), (P8[b]), P10, P13	(X2), (X3), X4, (X5), X7, (X8)	PS1
Ps 3B	(P1), (P2), (P5), (P6), (P8), P10, P13	(X2), (X3), X4, (X5), X7	PS1
Ps 11	P1, P2	X2, X3	PS1

[a] Order of fragments on the *N. tabacum* plastome
[b] Very weak hybridisation
Fragments in parentheses hybridised weakly to the probe (although hybridisation strength could only be assessed relatively within each set of hybridisations)

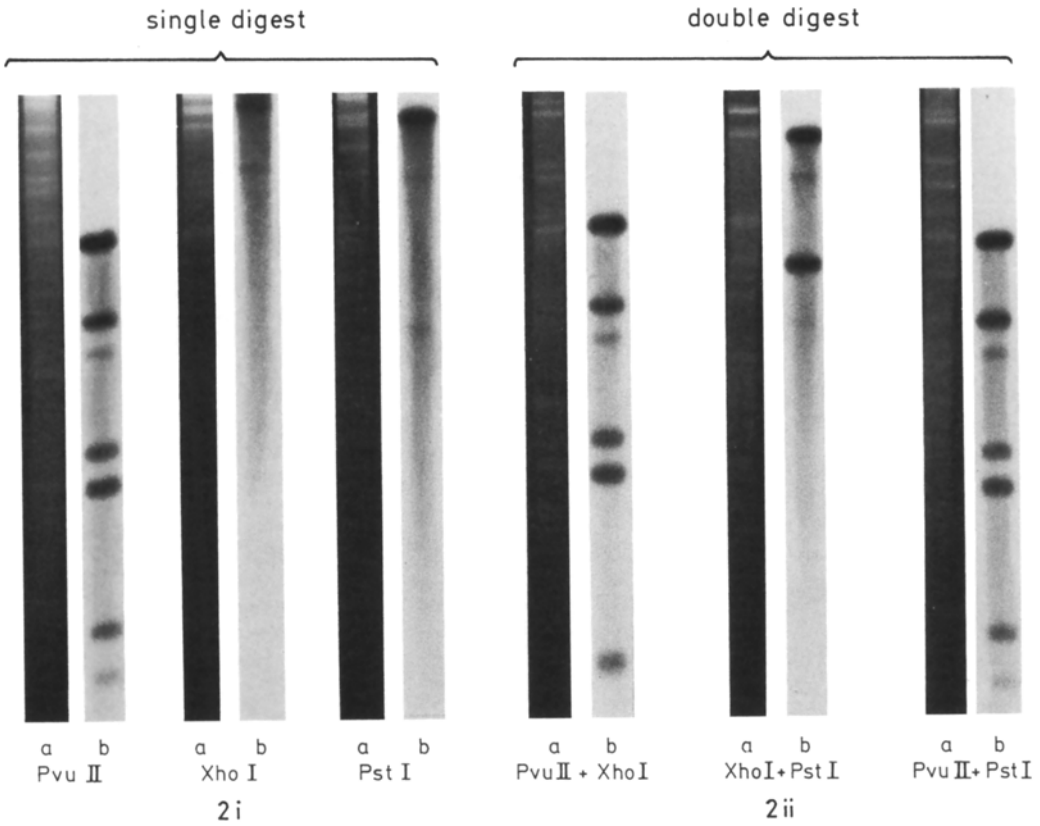

Fig. 2. **i** Hybridisation of *N. tabacum* Ps2A probe to digests of *C. aurantium* cpDNA. The identity of the fragments hybridising to the probe can be found in Table 2. Note the fainter band hybridising below the second band of PvuII, which is probably not a cpDNA fragment (see "Discussion"): *a* ethidium bromide stained agarose gel of electrophoresed fragments; *b* autoradiographs from hybridisation of the Ps2A probe to the same gel. **ii** Hybridisation of *N. tabacum* Ps2A probe to double digests of *C. aurantium* cpDNA: *a* ethidium bromide stained agarose gel of electrophoresed fragments; *b* autoradiographs from hybridisation to the same gel

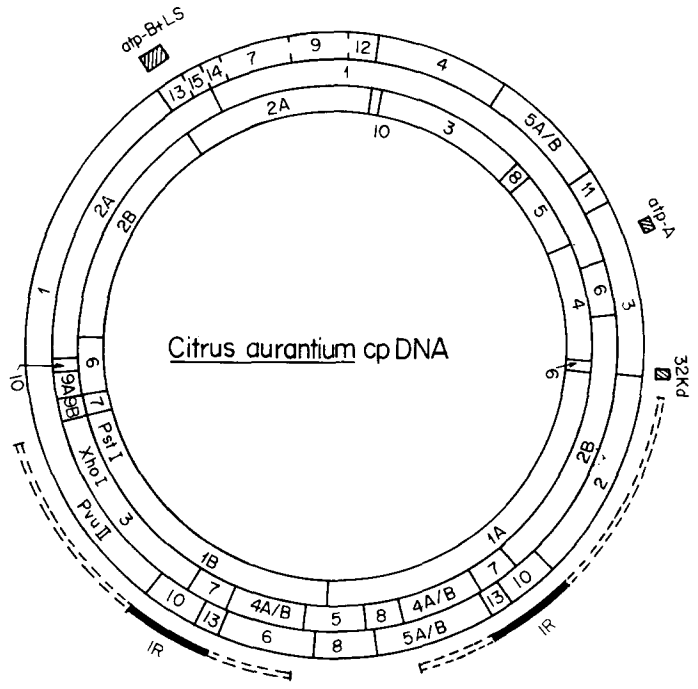

Fig. 3. Circular restriction map of *C. aurantium.* Genes are represented by shaded boxes. Black lines show the inverted repeats and broken lines indicate their limits. The order of PstI fragments: Ps7, Ps9, Ps12, Ps13, Ps14 and Ps15 could not be determined conclusively

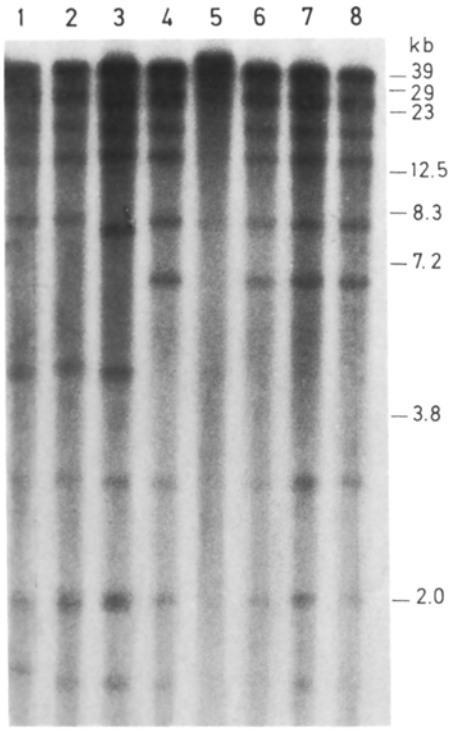

Fig. 4. *N. tabacum* cpDNA was used as a probe to hybridise with total cellular DNA of *Citrus* and its relatives (PstI digest): *1 Poncirus trifoliata; 2 Microcitrus* sp.; *3 C. medica; 4 C. paradisii; 5 C. reticulata; 6 C. aurantium; 7 C. grandis; 8 C. limon*

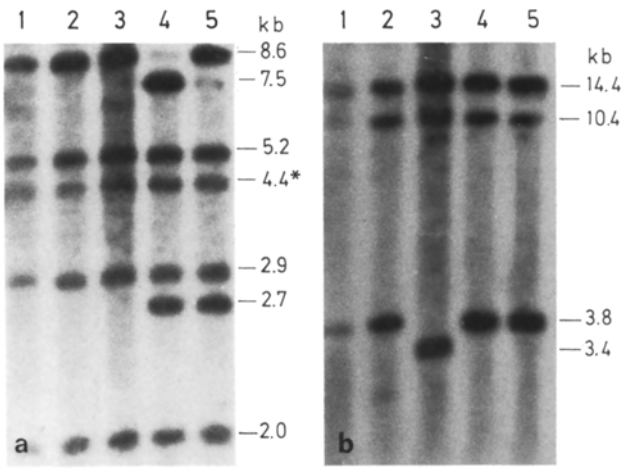

Fig. 6 a, b. Probes used to differentiate between some of the *Citrus* species and *Citrus* relatives cut with PvuII. **a** Ps2A probe; **b** Ps3A probe: *1 Poncirus trifoliata; 2 Microcitrus* sp.; *3 C. medica; 4 C. paradisii;* (same plastome as the *C. aurantium* group of *Citrus* types); *5 C. reticulata.* The band at 4.4 Kb is probably not a cpDNA fragment

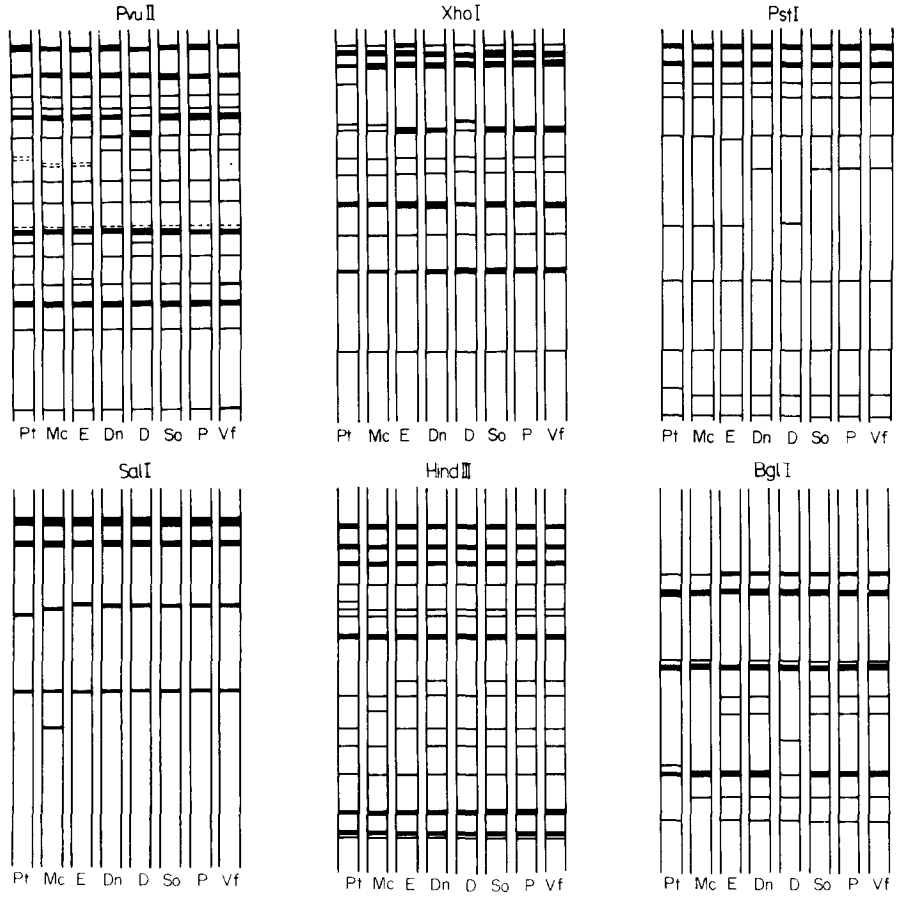

Fig. 5. Schemes of hybridisations of labelled *N. tabacum* cpDNA to total cellular DNA of *Citrus* and its relatives. The positions and intensities of the resultant bands are shown. *Broken lines* indicate bands which are probably not cpDNA: Vf *C. limon;* P *C. grandis;* So *C. aurantium;* D *C. reticulata;* Dn *C. paradisii;* E *C. medica;* Mc *Microcitrus* sp.; Pt *Poncirus trifoliata*

piled from several such patterns, is given in Fig. 5 together with the patterns given by 5 other restriction endonucleases. These patterns as well as hybridisation data from *C. sinensis* (not shown) indicate that the plastomes of the four types, *C. aurantium, C. grandis, C. sinensis, C. limon* and *C. paradisii* are at least similar if not identical. No difference could be seen in either the number of bands or their stoichiometries.

The plastomes from this group of *Citrus* types are not only similar to each other but differ from those of the other types tested. Each of the others, *C. medica, C. reticulata, Poncirus trifoliata* and *Microcitrus* sp., gave slightly different cpDNA patterns after restriction and electrophoresis, indicating dissimilar plastomes.

Hybridisation of *N. tabacum* probes Ps2A and Ps1 to PvuII digestions of total cellular DNA, isolated from leaves, gave different patterns for *C. reticulata, C. medica* and the *Citrus* cultivars having *C. aurantium* types plastomes (Fig. 6). These differences may therefore be exploited to identify the plastomes in cybrid plants.

Discussion

There is considerable sequence homology between the cpDNA of even unrelated plant species. Bisaro and Siegel (1980), using data obtained from heteroduplex formation, calculated some 50% homology between cpDNA of most families of dicotyledons. In addition, Palmer and Thompson (1981) compared the plastomes of seven quite disparate angiosperms and found them, with two exceptions, to be essentially colinear. The exceptions being wheat (a monocotyledon) and the legumes, which each had an inversion in its cpDNA relative to the other angiosperms. It is therefore possible to use cloned fragments from one species as hybridisation probes to map the plastome of another species even from a different family. Thus Palmer et al. (1983) used cloned mung bean (*Vigna racliata*) cpDNA probes to map the common bean (*Phaseolus vulgaris*) plastome and Perl-Treves and Galun (1985) used probes of *Nicotiana tabacum* to map the *Cucumis melo* plastome.

In this work cloned probes from *N. tabacum* cpDNA were used to map the plastome of *C. aurantium*. A restriction map was constructed, based on data from hybridisation of the probes to electrophoresed plastome fragments derived from single digestions or double digestions with pairs of the enzymes XhoI, PvuII and PstI (see Fig. 3). The map for PvuII could not be unequivocally determined as a considerable number of small fragments were produced after digestion of *C. aurantium* with PvuII, several of which hybridised to the same *N. tabacum* probes. Furthermore, both XhoI and PstI digestions gave large fragments in this area so double digestions were of little help in determining the precise sequence of these fragments.

On the basis of the XhoI and PstI maps (as well as parts of the PvuII map) the plastome of *Citrus* (*Rutaceae*) was deduced to be essentially colinear with that of *Nicotiana tabacum* (*Solanaceae*) but larger than the latter species, 166 kb compared to an average of 160 kb for *N. tabacum*. The absence of small bands hybridising near the edges of the two inverse-repeat (IR) regions made it difficult to determine the boundaries of the IRs. This could probably be amended by the use of additional restriction endonucleases which would generate smaller fragments of *C. aurantium* cpDNA in this area.

Palmer (1983) observed that the short single copy (SSC) region exists in both orientations with equal frequency. When *Phaseolus vulgaris* was cut with an enzyme which cleaved once in the SSC region and had no restriction sites within the IR, four fragments were generated after electrophoresis where two might have been expected. These four bands had half the intensity of the other bands and represented the two orientations of the SSC. A similar phenomenon was also observed with the cyanelle DNA of *Cyanophora paradoxa* (Bohnert and Leffelharht 1982). The map constructed in this work shows that PstI has one cleavage site in the SSC of *C. aurantium* and none within the IR regions. However, electrophoresis gives only two bands, Ps1A and Ps1B, even when they were run on an 0.5% agarose gel (which gave a better separation of large bands). This is probably because, although the PstI restriction site in the SSC is asymmetric, the sites in the long single copy (LSC) are in identical (or almost identical) places and both orientations of the SSC region would generate the same fragment sizes.

CpDNA extractions from several of the *Citrus* types as well as from *Microcitrus* and *Poncirus trifoliata* were not of sufficient quality to enable comparison of their plastomes directly from cpDNA restriction patterns; as has been done with other species: *Coffee* (Berthou et al. 1983), *Beta* (Mikami et al. 1984), *Lycopersicon* (Palmer and Zamir 1982). Therefore, the patterns for comparison were produced by hybridisation of *N. tabacum* probes to total cellular DNA digestions separated by agarose gel electrophoresis. Using this method the cpDNA restriction patterns could be compared.

The disadvantage of this method is the possibility that certain sequences in the nuclear and mitochondrial component of the total DNA digestion might also hybridise with the *N. tabacum* cpDNA probes. Sequence homologies have been reported between nuclear and chloroplast genomes in spinach (Timmis and Scott 1983) and between the mitochondrial and chloroplast genomes of maize (Stern and Lonsdale 1982). Stern and Palmer (1984) reported numerous sequence homologies between cpDNA of mung bean and mtDNA from corn, mung bean, pea and spinach. They claimed that every cpDNA sequence they used (covering most of the mung bean plastome) hybridised with at least one mtDNA restriction fragment, even from distantly related species. An example of such a homology can be seen when the *N. tabacum* Ps2A probe is used to hybridise with the PvuII digests of *C. aurantium* cpDNA (Fig. 2). Amongst the other fragments lighting-up was a faint band of 4.4 kb. This band was also seen, with a

greater intensity, after hybridisation of Ps2A to DNA digests of *Microcitrus, P. trifoliata* and several of the *Citrus* types (Fig. 6). A faint band appeared in this position when total DNA was hybridised to the *N. tabacum* cpDNA probe (Fig. 5; represented by a dotted line). It was decided that this was not a cpDNA restriction fragment as it appeared only very faintly, if at all, on ethidium bromide stained agarose gels of PvuII restricted cpDNA and did not fit in with the mapping data. This fragment is therefore probably either a mitochondrial or a nuclear sequence homologous to part of the *N. tabacum* plastome.

It is therefore only possible to decide conclusively that the plastomes of certain *Citrus* species (i.e. *C. aurantium, C. paradisii, C. grandis* and *C. limon*) are alike, and to identify plastome differences from the absence of known cpDNA fragments. Fragments which are definitely cpDNA can be ascertained, for *C. aurantium* at least, from the mapping data for PvuII, XhoI and PstI restrictions. Additional bands might be a result of the *N. tabacum* probe hybridising to different mitochondrial or nuclear sequences. However, only a few bands were observed for *C. aurantium* (and others of the same group) after digestion with XhoI, PvuII and PstI, which were definitely not cpDNA. It might, therefore, be assumed that the majority of the additional bands seen after the hybridisations to the other species are a result of plastome variation.

It is of considerable taxonomic interest to note that the results obtained in this work agree with the findings of Barrett and Rhodes (1976) who, using 146 characters, proposed that *C. grandis, C. medica* and *C. reticulata* are true biological species while *C. aurantium, C. sinensis* and *C. paradisii* are basically hybrids between *C. grandis* and *C. reticulata,* and *C. limon* is probably a three hybrid involving *C. grandis, C. medica* and *Microcitrus* sp. All of this latter group gives the same plastome restriction patterns as *C. grandis* indicating that it was probably involved in each of the original crosses, providing the cytoplasmic content of each of these *Citrus* types. This is in agreement with the interrelationships proposed by Barrett and Rhodes (1976) and the findings of Soost (1964) that several varieties of *C. grandis* are self-incompatible and thus this species was the maternal-parent in crosses which resulted in the present-day cultivated *Citrus* types (e.g. orange, grapefruit, lemon). The other two true species (according to this classification), *C. reticulata* and *C. medica,* as well as the *Citrus* wild-relatives *P. trifoliata* and *Microcitrus* sp., give distinct plastome restriction patterns, as would be expected.

Somatic hybridisation between *Citrus* species and their relatives is a potentially important technique for plant improvement. We have developed methodologies to produce *Citrus* plants from isolated protoplasts (Vardi et al. 1982) and recently succeeded in obtaining cybrids by protoplast fusion (Vardi et al. in prep.). A method has been developed for the identification of the nuclear component of the resulting cybrids (Ben-Hayyim et al. 1982). One of the aims of the present work was to find cpDNA probes which show differences in patterns of endonuclease digestions of total cellular DNA, thus allowing plastome identification. Such a system has been developed for plastome analysis in *Nicotiana* (Fluhr et al. 1983).

CpDNA restriction patterns obtained using five endonucleases showed no differences between the plastomes of *C. aurantium, C. grandis, C. paradisii* and *C. limon.* There are, however, dissimilarities in the restriction patterns of the other species examined. Of the three enzymes used for the mapping, PvuII gave the greatest number of differences in restriction patterns. Comparison of these differences with the order of fragments in the *C. aurantium* map made it seem likely that *N. tabacum* probes Ps2A and Ps1 could be used to distinguish between plastomes of the *C. aurantium* type and each of the other species. Subsequent hybridisation of these two probes to restriction endonuclease digestions of total cellular DNA gave sufficient differences to allow identification of the plastomes of *C. medica, C. reticulata* and the *C. aurantium* group.

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