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Cytoplasmic DNA Variation and Relationships in Cereal Genomes

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Summary. Chloroplast (cp) and mitochondrial (mt) DNAs were isolated from four cereal genomes (cultivated wheat, rye, barley and oats) and compared by restriction nuclease analysis. Cleavage of cp and mt DNAs by Sal I, Kpn I, Xho I and EcoR I enzymes indicated that each cereal group contains specific cytoplasmic DNAs. A phylogenetic tree of cereal evolution has been obtained on the basis of cp DNA homologies. It is suggested that wheat and rye diverged after their common ancestor had diverged from the ancestor of barley. This was preceded by the divergence of the common ancestor of wheat, rye and barley and the ancestor of oats.

The molecular weight of the different cp DNAs was determined from the Sal I and Kpn I patterns. cp DNAs from wheat, rye, barley and oats appeared to be characterized by a very similar molecular weight of about $80\text{-}82.10^6$ d.

In the case of the mt DNAs, the great number of restriction fragments obtained with the restriction enzymes used prevented precise comparisons and determination of molecular weights.

Key words: Cereal evolution – Chloroplast DNAs – Mitochondrial DNAs – Restriction enzymes – DNA molecular weight

Introduction

The large subunit of ribulose-biphosphate carboxylase encoded by the chloroplast DNA (cp DNA) has been the first cytoplasmic marker widely used in taxonomic and phylogenetic studies of higher plants (Kung 1976). Recent developments in plant molecular biology, namely the isolation of organelle DNA by CsCl-ethidium bromide gradient centrifugation and the restriction endonucleases analysis of the purified DNAs, have shown that cp DNA and mitochondrial (mt) DNAs are specific of the plant species from which these DNAs have been extracted (Atchison et al. 1976; Vedel et al. 1976; Quétier and Vedel 1977). The restriction fragments patterns obtained by gel electrophoresis of the cleaved fragments can serve as fingerprints of the native cp and mt DNA molecules in a manner analogous to the fingerprints resulting from trypsin digestion of the RubPcase large subunit. This provides a method for comparing cp and mt DNAs of higher plants. Specific hydrolysis of cp and mt DNAs by restriction nucleases has been used recently to establish taxonomic relationships in the *Triticum* (Vedel et al. 1978) and *Zea* (Timothy et al. 1979) genera.

Nuclear DNA homology between the cereal genomes has been studied previously by renaturation kinetics, providing a phylogenetic scheme of the evolution of cereal nuclear DNAs based on repeated sequences (Flavell et al. 1977). We present here a restriction enzyme analysis of cytoplasmic DNAs from cultivated wheat, rye, barley and oats to explore phylogenetic relationships between cereals and to compare them with those deduced from repeated sequence analysis of nuclear DNA homology.

Material and Methods

Species Examined

Triticum aestivum (wheat) variety 'Capitole', *Secale cereale* (rye) variety 'Petkus', *Hordeum vulgare* (barley) variety 'Betzes' and *Avena sativa* (oats) variety 'Crin noir' were grown on vermiculite in the greenhouse.

Isolation of cp and mt DNAs

Chloroplast and mitochondrial DNAs were isolated as previously described by using CsCl-ethidium bromide gradients (Herrmann et al. 1975; Kolodner and Tewari 1975). However, an important

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modification has been introduced in the present work: cp and mt DNAs have been prepared from the same extract of a given species. Chloroplast and mitochondria were prepared from 10 to 12 days-old green seedlings. After homogenization of the fresh material and filtration as previously described (Vedel et al. 1976; Quétier and Vedel 1977), the organelle suspension was centrifuged at 50 g, 10 min (IEC, rotor 269). The supernatant was spun at 1000 g, 12 min (IEC, rotor 269); the pellet represented the crude chloroplast fraction. The supernatant was then centrifuged at 1500 g, 10 min and the pellet discarded. The last supernatant was spun at 20,000 g, 15 min (Sorvall, rotor SS34) and the pellet represented the crude mitochondrial fraction. Chloroplast and mitochondrial pellets were then purified, the corresponding DNAs isolated and specifically cleaved by restriction nucleases as described (Vedel et al. 1976; Quétier and Vedel 1977). The restriction enzymes used were Sal I, Xho I, EcoR I, prepared by us according to Gingeras et al. (1978) and Greene et al. (1978), and Kpn I (New England Biolabs, USA). The restriction fragments were separated by electrophoresis in 0.7% agarose, 40 cm long vertical slab gels. Gel staining and ultraviolet fluorescence photography have been described (Vedel et al. 1976; Quétier and Vedel 1977).

Molecular Weight Determinations

The molecular weights of the DNA restriction fragments were measured by using Hind III fragments of phage λ DNA as references. The molecular weight of the seven Hind III λ DNA fragments are respectively: 15.1, 6.2, 4.2, 2.7, 1.4, 1.2 and 0.34 × 10^6 d (Fiandt et al. 1977). The molecular weight of a given cp DNA was obtained by summing the molecular weights of the fragments located on a given pattern, and in the case of Sal I and Kpn I, band multiplicity was determined by microdensitometry of the u.v. fluorescence photographs (Joyce loebl microdensitometer Mark III) and was taken into account.

Results

Specific Cleavage of Cereal cp DNA by Restriction Enzymes

Restriction endonuclease fragments analysis of cp DNAs isolated from the four cultivated cereals revealed that each cereal contains a specific cp DNA. In the case of the Sal I and Xho I patterns obtained with rye, barley and oat cp DNAs, the heaviest band represents undigested DNA. Such a band was observed neither with Sal I and Xho I patterns from wheat cp DNA, nor with all the Kpn I and EcoR I patterns. Varying the conditions of the enzymatic hydrolysis did not permit the complete removal of this 'residual native DNA', the reason for its undigestibility remaining unclear.

However, cp DNA comparisons are not hampered by this residual native DNA, the differences that characterize each pattern being highly reproducible. The undigested DNA band is not taken into account in the following comparisons (Table 1). Sal I and Kpn I enzymes generated 9 to 11 cp DNA fragments (Figs. 1, 2) against 15 to 17 with Xho I and 23 to 26 with EcoR I enzymes. Amongst the 16 cp DNA patterns presented here, only 2 appeared identical, namely the Sal I patterns of the wheat and rye cp DNAs.

Taking the wheat cp DNA as a reference, barley cp DNA contains one specific and nine common fragments whereas oat cp DNA exhibits six specific and five common fragments (Sal I patterns, Fig. 1). In the case of the Kpn I analysis, cp DNAs from rye, barley and oats are characterized respectively by one, two and two specific

Table 1. Distribution of restriction fragments obtained by hydrolysis of wheat, rye, barley and oats cp DNA by Sal I, Kpn I, Xho I and EcoR I enzymes

Enzymes		Wheat	Rye	Barley	Oats
	T	11	11	10	11
Sal I	C		11	9	5
	S		0	1	6
	T	11	10	10	9
Kpn I	C		9	8	7
	S		1	2	2
	T	16	17	16	15
Xho I	C		16	15	10
	S		1	1	5
	T	26	23	23	24
EcoR I	C		22	20	17
	S		1	3	7

T = total number of fragments
C = number of common fragments with wheat
S = number of specific fragments (by comparison to wheat)

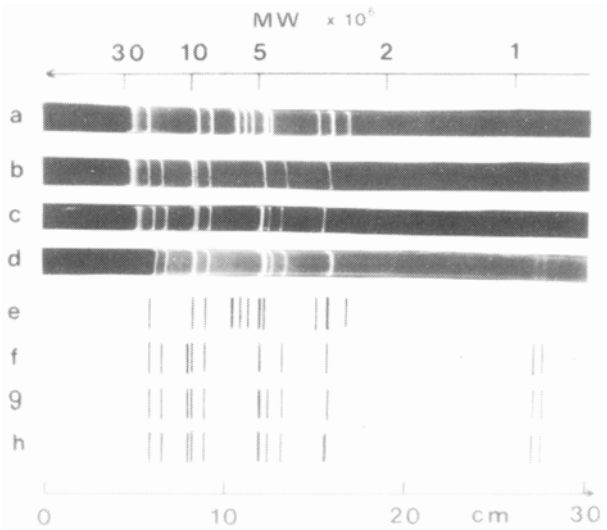

Fig. 1. Agarose slab gel electrophoresis of Sal I digests of cp DNAs from: a oats; b barley; c rye; d wheat. Schematic representations of these Sal I patterns are given in e-h

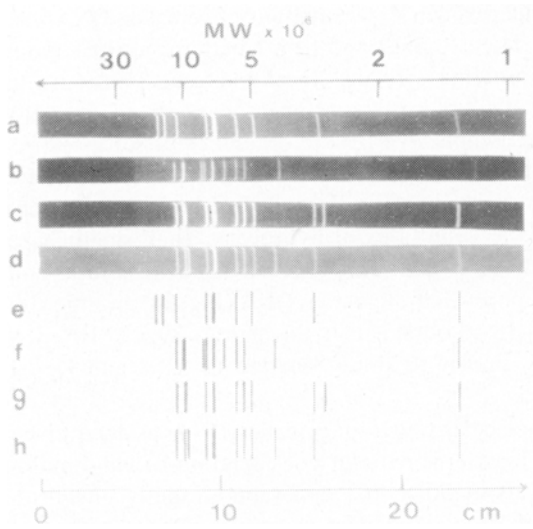

Fig. 2. Agarose slab gel electrophoresis of Kpn I digests of cp DNAs from: a oats; b barley; c rye; d wheat. Schematic representations of these Kpn I patterns are given in e-h

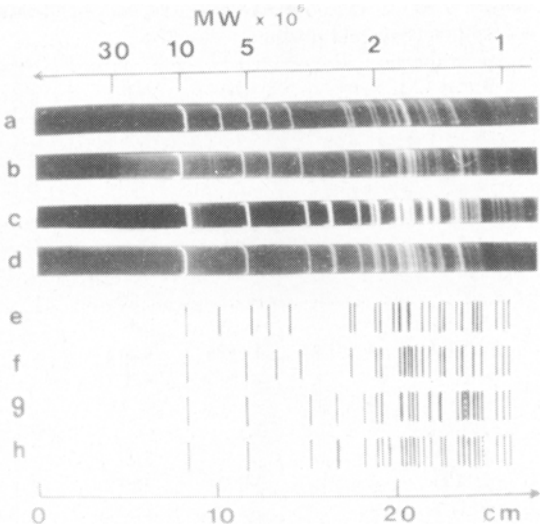

Fig. 4. Agarose slab gel electrophoresis of EcoR I digests of cp DNAs from: a oats; b barley; c rye; d wheat. Schematic representations of these EcoR I patterns are given in e-h

bands against nine, eight and seven common bands (Fig. 2). Similar degrees of species specific variability are obtained by comparison of Xho I and EcoR I cp DNA patterns. Xho 1 analysis indicated that rye, barley and oat cp DNAs are distinguished from wheat cp DNA by patterns with one, one and five specific bands and by 16, 15 and 10 common bands, respectively (Fig. 3). Rye, barley and oat cp DNAs contain respectively 1, 3 and 7 EcoR I specific fragments and 22, 20 and 17 EcoR I fragments in common with wheat cp DNA (Fig. 4).

A summary of these comparisons is presented in Table 1. Each of the four restriction enzyme analyses indicates that the number of common cp DNA fragments decreases and that the number of specific ones increases by following the cereal sequence from wheat to rye, barley and oats. More particularly, with the four enzymes used in this work, rye cp DNA appears to differ by 0 to 1 specific band from wheat cp DNA whereas barley and oat cp DNAs differ by 1 to 3 and by 2 to 7, respectively. The largest number of differences are apparent from EcoR I analysis. The patterns obtained with this enzyme contain also the greatest number of restriction fragments.

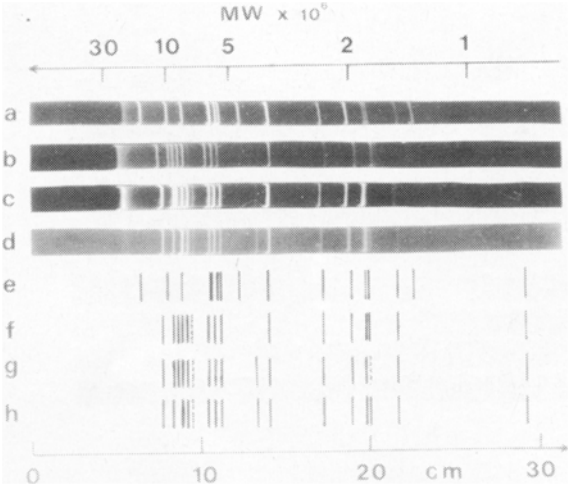

Fig. 3. Agarose slab gel electrophoresis of Xho I digests of cp DNAs from: a oats; b barley; c rye; d wheat. Schematic representations of these Xho I patterns are given in e-h

Molecular Weight of Cereal cp DNAs

The molecular weight of wheat, rye, barley and oat cp DNAs was determined on the Sal I and Kpn I patterns. The molecular weight of a given cp DNA was obtained by addition of the molecular weights of all the restriction fragments present on the corresponding pattern, as indicated under Methods. Results are shown in Table 2 and Table 3. The cp DNA molecular weights appear very similar, ranging from 79.7 to 82.5 $\times$ 10^6 d. In these determinations, the band multiplicity was taken into account. Restriction fragments corresponding to non-stoechiometric ratios can be easily evidenced by their greater fluorescence intensity than that expected from their molecular weight. Band multiplicity of the restriction fragments has been measured on the microdensitometric tracings of the u.v. photograph of each restriction pattern.

It is noticeable that prominent homologies occur in the distribution of the multiply represented restriction fragments among cereal cp DNAs patterns obtained with the same enzyme. In particular, the Sal I restriction fragments

Table 2. Molecular weight (x 10^6 d) of wheat, rye, barley and oats cp DNAs determined from Sal I restriction patterns

	Wheat	Rye	Barley	Oats
	15.1	15.1	15.1	15.1
	12.5	12.5	12.5	–
	8.6	8.6	8.6(2)	–
	8.35	8.35	8.35	8.35
	7.4	7.4	7.4	7.4
	–	–	–	5.6(2)
	–	–	–	5.3
	–	–	–	5.0
	4.5(3)	4.5(3)	4.5(2)	4.5(3)
	–	–	–	4.4
	4.3	4.3	–	–
	3.9	3.9	3.9	–
	–	–	–	3.1
	2.8(2)	2.8(2)	2.8(2)	2.8(2)
	–	–	–	2.5
	0.35	0.35	0.35	–
	0.3	0.3	0.3	–
Total	79.9	79.9	79.7	81.4

band multiplicity is indicated by numbers inside brackets

Table 3. Molecular weight (x 10^6 d) of wheat, barley and oats cp DNAs determined from Kpn I restriction patterns

	Wheat	Rye	Barley	Oats
	–	–	–	15
	–	–	–	13.3
	11.2	11.2	11.2	11.2
	9.8	9.8(2)	9.8(2)	–
	9.3	–	–	–
	–	–	7.8	–
	7.4	7.4	7.4	7.4
	6.8(3)	6.8(3)	6.8(2)	6.8(3)
	–	–	6.2	–
	5.5	5.5	5.5	5.5
	5.1	5.1	5.1	–
	4.7	4.7	–	4.7
	4.0	–	4.0	–
	3.0	3.0	–	3.0
	–	2.8	–	–
	1.4	1.4(2)	1.4	1.4
Total	81.8	82.5	81.8	81.9

band multiplicity is indicated by numbers inside brackets

of 4.5 x 10^6 d and 2.8 x 10^6 d and the Kpn I restriction fragment of 6.8 x 10^6 d containing double or triple amounts of DNA are common to wheat, rye, barley and oat cp DNAs. We have also shown recently (unpublished results) in the case of the wheat cp DNA, that the genes coding for the chloroplastic ribosomal RNAs were localized in these fragments.

Molecular weight determination of cereal cp DNAs has been restricted to Sal I and Kpn I patterns as difficulties were encountered with the Xho I and EcoR I patterns. In the case of Xho I patterns, measurements of cp DNA sizes were complicated by the occurence of some restriction fragments in less than stoechiometric amounts. These fragments marked in Fig. 3 by dotted lines were present whatever the conditions of hydrolysis. Their significance is presently unknown. This type of fragment is absent on the Xho I pattern of the oat cp DNA (Fig. 3a) and the size of the cp DNA obtained in this case is 79.5 x 10^6 d, a value very similar to those found with Sal I and Kpn I patterns.

The molecular weight of cereal cp DNAs as determined on EcoR I patterns without consideration of band multiplicity is about 50 x 10^6 d, a value seriously underestimating the true value. Unfortunately, determination of band multiplicity is hampered in patterns containing numerous and contiguous fragments. It seems also that a non-negligible part of cp DNA is lost because low molecular weight EcoR I fragments migrate out of the gel when low agarose concentrations are used.

Cereal mt DNA Analysis by Restriction Endonucleases

Comparison of the Sal I, Kpn I, Xho I and EcoR I patterns of the mt DNAs isolated from wheat, rye, barley and oats revealed that each cereal contains a specific mt DNA (Figs. 5, 6). The mt DNA patterns contain a greater number of restriction fragments, about three times more, than the corresponding cp DNA patterns (involving the same cereal and the same enzyme). The complexity of the patterns precludes accurate determination of restriction fragment

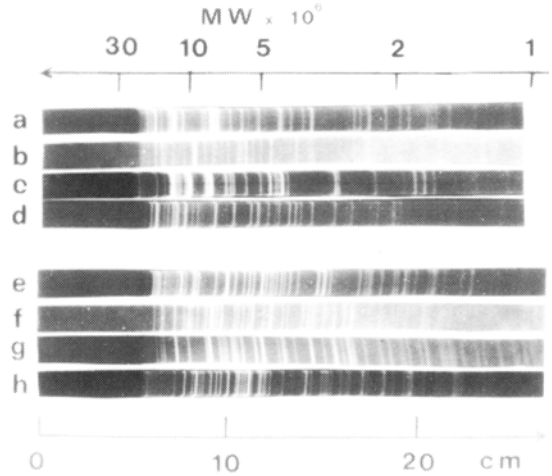

Fig. 5. Agarose slab gel electrophoresis of Sal I digests of mt DNAs from: a oats; b barley; c rye; d wheat and of Kpn I digests of mt DNAs from: e oats; f barley; g rye; h wheat

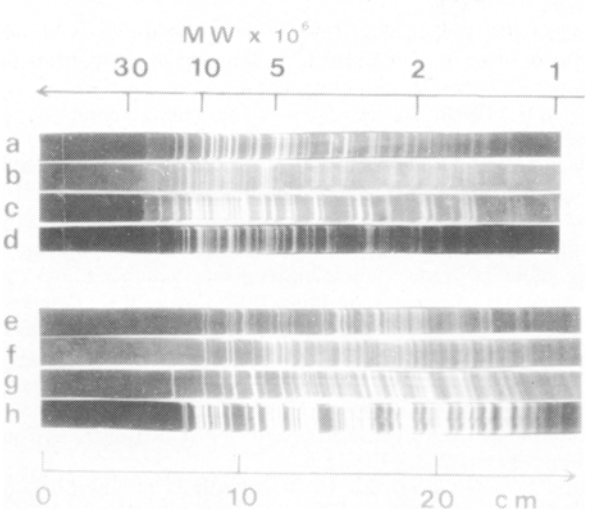

Fig. 6. Agarose slab gel electrophoresis of Xho I digests of mt DNAs from: a oats; b barley; c rye; d wheat and of EcoR I digests of mt DNAs from: e oats; f barley; g rye; h wheat

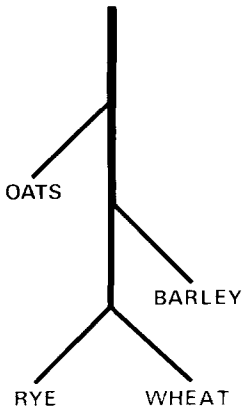

Fig. 7. Phylogenetic tree of cereal evolution based on cp DNA restriction patterns homology

homologies between mt DNAs of the four cultivated cereals. Another disadvantage is the difficulty in calculating mt DNA sizes because of imprecisions in molecular weight determinations with fragments larger than 15×10^6 d. The molecular weight of the four mt DNAs estimated by summing the molecular weight of all the restriction fragments results in a value greater than 150×10^6 d, in all restriction patterns. This value does not take into account band multiplicity.

Conclusions

The specific cleavage of cytoplasmic DNAs by restriction endonucleases has been used recently to establish phylogenetic relationships in animals (Hayashi et al. 1979; Brown and Wright 1979; Avise et al. 1979) and in higher plants (Vedel et al. 1978; Timothy et al. 1979) belonging either to the same genus or to different genera. In the present work, we have applied this useful tool to study evolutionary relationships between cultivated wheat, rye, barley and oats. Restriction endonuclease analyses of cp and mt DNAs show that each cereal contains specific cytoplasmic DNAs. Comparison of the cp DNA restriction patterns indicates that cp DNA homologies follow the sequence: wheat, rye, barley, oats. A phylogenetic tree of cereal evolution has been deduced (Fig. 7), suggesting that wheat and rye diverged after their common ancestor had diverged from the barley ancestor. This was preceded by the divergence of the common ancestor of wheat, rye and barley and the oats ancestor. It appears that the phylogenetic tree of cereal evolution established from restriction nuclease analysis of the chloroplastic genomes is the same as that found previously by comparing renaturation kinetics of the nuclear genomes (Flavell et al 1977). On the other hand, the cereal cp DNAs cannot be distinguished by their molecular weight as calculated from the Sal I and Kpn I restriction patterns. The molecular weight of 80-82 $\times 10^6$ d found with all cereal cp DNA is in agreement with values obtained previously by renaturation kinetics and electron microscopic analysis of oats cp DNA (Kolodner and Tewari 1975). These results support the idea that all of the circular cp DNA molecules in a given plant have the same sequence. The cp DNAs of the cultivated wheat, rye, barley and oats reveal an interesting homology of multiple occuring fragments, namely, the 4.5 and 2.8 $\times 10^6$ d Sal I fragments and the 6.8 $\times 10^6$ d Kpn I fragment. The localization of the wheat chloroplastic rDNA genes in these fragments (Quétier and Vedel, unpublished results) suggests that the chloroplastic rDNA genes of rye, barley and oats are also localized in these fragments.

mt DNA cereal genomes appear more diversified than the corresponding cp DNAs, but this is based on the highly complex patterns obtained with the restriction nucleases used, signifying many restriction sites for these particular enzymes. Because of this, mt DNA restriction analysis appeared more useful than cp DNA restriction analysis in distinguishing between species belonging to the same genera (Vedel et al. 1978; Timothy et al. 1979; Belliard et al. 1979).

Complexity of the mt DNA restriction patterns does not allow us to determine their molecular weight.

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