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ISOLATION AND PRELIMINARY CHARACTERIZATION OF CRYPTIC SATELLITE DNA IN PEA

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Key Word Index—*Pisum sativum*; Leguminosae; pea; repetitive DNA; reassociation.

Abstract—Optimum conditions have been established for isolation of 'cryptic' satellite DNA from the genome of pea (*Pisum sativum*), using gradients of Cs_2SO_4 containing silver ions. At an Ag^+:DNA-P ratio (R_f) of 0.1, and at alkaline pH, four fractions are obtained: mainband (buoyant density 1.437 g cm^3; 67% of total DNA), satellite I (buoyant density 1.582 g/cm^3; 7% of total DNA), satellite II (buoyant density 1.520 g/cm^3, 11% of total) and satellite III (buoyant density variable between 1.45 and 1.51 g/cm^3; 15% of total). The reiterated DNA content of these four fractions has been investigated by reassociation experiments conducted over a Cot range of 1×10^{-5} to 2.0. All four fractions contain at least two kinetic components; each fraction, including the mainband, consists at least partly of highly reiterated DNA. Ribosomal RNA hybridizes only to the mainband.

INTRODUCTION

Satellite DNAs, that is DNA species which are separable from bulk or mainband DNA by centrifugation in CsCl, have been detected in a wide variety of higher plants [1]. A number of these satellite DNAs, including those from melon [2–4], tomato [5], flax [6], cucumber [6] and an orchid, *Cymbidium* [7] have been characterized in terms of denaturation and/or reassociation kinetics. All these higher plant satellite DNAs contain two types of sequence, one of which is a simple, very highly reiterated sequence, whilst the other is more complex and less highly reiterated.

Although satellite DNAs which are resolvable in CsCl gradients occur in plants, there are also many plants which do not contain such satellites. In this latter group of plants, however, satellite DNAs may often be resolved by centrifugation of DNA in the presence of silver ions in gradients of Cs_2SO_4. Such satellite DNAs are often referred to as 'cryptic' satellites. Cryptic satellite DNAs have been demonstrated in several plants, including broad bean, oat, rye [8], wheat, barley [9, 10] and mustard [11]. One of the two cryptic satellite DNAs from wheat and one of the two from barley have been characterized and shown to consist of the two types of sequence previously demonstrated in the satellite DNAs which are resolvable in CsCl [10]. The cryptic satellites from mustard are also heterogeneous [11].

In this paper, we report the isolation of cryptic satellite DNAs from pea (which does not have a CsCl-resolvable satellite). We also present a partial characterization of the cryptic satellites in terms of reassociation kinetics. The data obtained are consistent with those obtained by Thompson and his co-workers in their kinetic analysis of the total pea genome [12, 13].

RESULTS

$Cs_2SO_4 \cdot Ag^+$ *density gradients*

Centrifugation of DNA in gradients of Cs_2SO_4 has been carried out over a range of R_f values (i.e. Ag^+: DNA-P ratios) from 0.0 to 1.0. By far the most effective R_f value for resolution of cryptic satellite DNAs is 0.1 (Fig. 1).

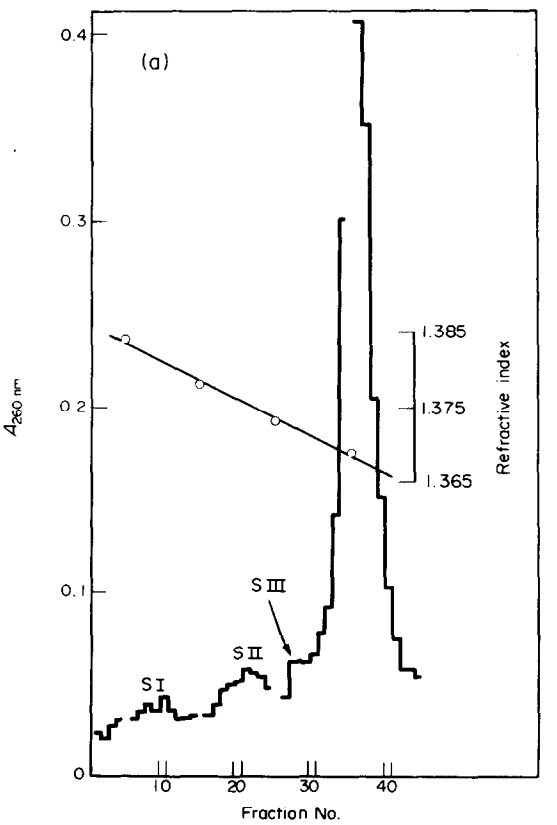

Fig. 1. Density gradient centrifugation of pea DNA in Cs_2SO_4 gradients containing Ag^+ ions at R_f 0.1.

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Indeed, only at this R_f is there clear resolution of any satellite components. At R_f 0.1, the mainband DNA sediments at 1.437 g/cm^3, and two heavy satellites, SI (1.582 g/cm^3) and SII (1.520 g/cm^3) are clearly resolved. The resolution of a third satellite, SIII, is more variable, both in position in the gradient and in the amount of satellite obtained. In most gradients, SIII appears as a heavy shoulder on the mainband, but in some gradients this satellite is almost completely resolved from the mainband, or, more rarely, overlaps with SII. The proportions of the four fractions, SI, SII, SIII and mainband, averaged from a large number of gradients, are shown in Table 1.

Reassociation of DNA at a fragment size of 750 base pairs

Reassociation of the standard DNAs from *Escherichia coli* and from 'phage T4 is illustrated in Fig. 2. The curves drawn are computed ideal second-order reassociation curves, and the data obtained fit the ideal curves very closely. The reassociation of the four fractions isolated from the pea genome is also illustrated in Fig. 2. All four exhibit heterogeneous reassociation kinetics. SI and SIII are very similar to each other, each containing a component (or group of components) with an average observed $Cot_{\frac{1}{2}}$ of $1–2 \times 10^{-2}$, and the remainder showing no reassociation at Cot values below 0.5×10^{-1}. The more rapidly reassociating components of SI and SIII are in fact heterogeneous, as judged from the reciprocal plots (not shown) and from the mismatch between the data and computed ideal second-order reassociation curves. However, the data cannot readily be resolved into a number of separate kinetic components. The rapidly reassociating components of SI and SIII are thus best regarded as groups of sequences exhibiting overall reassociation kinetics which approximate to, but do not fit exactly to an ideal second-order curve. The reassociation curve for SII is clearly biphasic, and fits almost exactly to two computed ideal second-order curves. The reciprocal plot (not shown) is also biphasic. The faster-reassociating component of SII exhibits an observed $Cot_{\frac{1}{2}}$ of 1×10^{-3}, and is clearly the simplest component isolated from the pea genome. The slower component of SII has an observed $Cot_{\frac{1}{2}}$ of 6×10^{-2}. Like the three satellites, the mainband also consists of at least two kinetic components, one with an observed $Cot_{\frac{1}{2}}$ of 2×10^{-1} and the remainder exhibiting no reassociation at Cot values below 5. As with the faster components of SI and SIII, the faster component of the mainband is best regarded as a group of sequences rather than a single kinetic component. The properties of the fractions isolated from the pea genome are summarized in Table 1.

Ribosomal RNA cistrons

Hybridization of ^{3}H-labelled pea rRNA to fractions obtained from $Cs_2SO_4 \cdot Ag^+$ gradients shows that none of the cryptic satellites contain the cistrons which code for rRNA. Instead, the rRNA hybridizes exclusively with the mainband.

DISCUSSION

In exhibiting more than one satellite resolvable in $Cs_2SO_4 \cdot Ag^+$, the pea genome resembles the genomes of barley, wheat [9, 10], melon, cucumber, marrow [8], mustard [11] and those of several mammals [14]. In pea, the resolution of satellite DNAs exhibits a very marked dependence on R_f, with no reproducible resolution occurring at any R_f value except 0.1. This extreme dependence on an R_f of 0.1 for resolution of cryptic satellites in our experiments may reflect the very complex sequence interspersion pattern exhibited by the pea genome [12, 13].

The reassociation data show clearly that all three cryptic satellites isolated from the pea genome consist of at least two, and for SI and SIII probably more, kinetic components, and thus resemble all other satellite DNAs which have been isolated from plants. In particular, satellites SI and SIII somewhat resemble the cryptic satellites isolated from wheat and barley [10] whilst SII clearly resembles the satellites isolated from tomato [5], cucumber [6] and mustard [11] in terms of complexity

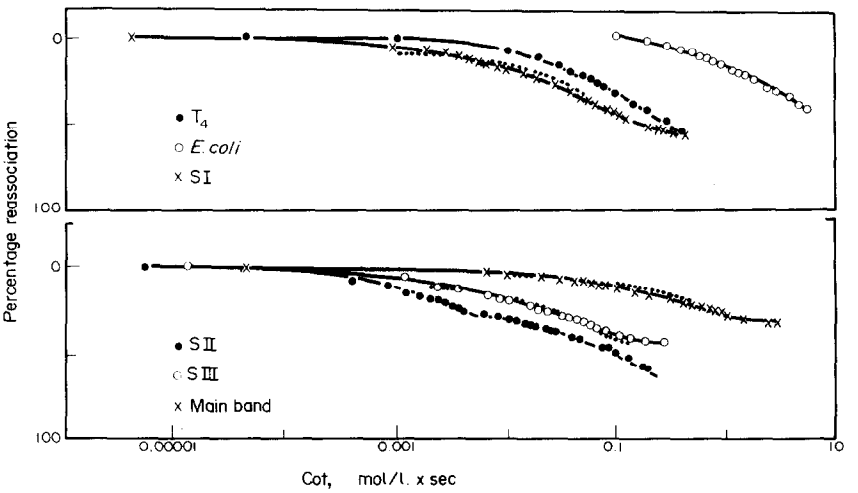

Fig. 2. Reassociation of pea genome components and of *E. coli* and T4 DNA. Each curve is based on data obtained with at least three different batches of DNA. The curves drawn for *E. coli* and T4 DNA are computer-fitted ideal second-order curves. The curve for SII is computer fitted, assuming the existence of two separate second-order components. The curves drawn for SI, SIII and mainband are best fits to the data. The nearest fit ideal second-order curves for SI, SIII and mainband are indicated by dotted lines.

Table 1. Properties of components isolated from the pea genome by centrifugation in $Cs_2SO_4.Ag^+$ gradients

Component	Density (g/cm³) at R_f 0.1	Proportion of total genome (%)	Kinetic analysis			
			Observed $Cot_{\frac{1}{2}}$ (mol/l.) × sec	'Pure' $Cot_{\frac{1}{2}}$* (mol/l.) × sec	Complexity, base pairs	Reiteration frequency per haploid genome
Satellite I	1.582	7	Fast fraction: 1.7×10^{-2} Slow fraction: not determined	7.8×10^{-3}	4.21×10^{3}	7.7×10^{4}
Satellite II	1.520	11	Fast fraction: 1.0×10^{-3} Slow fraction: 6.0×10^{-2}	3.0×10^{-4} 4.2×10^{-2}	1.61×10^{2} 2.26×10^{4}	9.3×10^{5} 55×10^{4}
Satellite III	1.45–1.51	15	Fast fraction: 1.03×10^{-2} Slow fraction: not determined	4.1×10^{-3}	2.21×10^{3}	3.22×10^{5}
Mainband	1.437	67	Fast fraction: 2.0×10^{-1} Slow fraction: not determined	6.0×10^{-2}	3.23×10^{4}	2.97×10^{4}

* Pure $Cot_{\frac{1}{2}}$ values are the $Cot_{\frac{1}{2}}$ values which would be exhibited if a particular component were present on its own. Thus, in satellite II, the faster component represents 30% of the reassociation curve, and the 'pure' $Cot_{\frac{1}{2}}$ value is obtained by correcting for the effective dilution of the faster component by the slower component (see ref. [5]).

and reiteration frequency. In the pea, the simpler component of SII has a complexity of 160 base pairs; in tomato, the equivalent component has a complexity of 150 base pairs [5], in cucumber, 150 base pairs [6] and in mustard, 165 base pairs [11] whilst highly reiterated DNA from plants of the genus *Brassica* has a complexity of 180 base pairs [15]. These complexities are remarkably similar to the size of the DNA fragment associated with the nucleosome [16] and indicate that the origin of repetitive DNA may have involved amplification of nucleosome-sized fragments of DNA.

Unlike a number of satellite DNAs isolated from plants [2, 3, 5], the satellite DNAs from pea do not contain the rRNA cistrons. The location of the rRNA cistrons in the mainband in $Cs_2SO_4 . Ag^+$ gradients contrasts with their clear resolution from the mainband in CsCl gradients [17]. A similar loss of resolution of the rRNA cistrons in $Cs_2SO_4 . Ag^+$ gradients occurs with mustard DNA [11]. The presence in the mainband of the rRNA cistrons clearly contributes to the more rapidly reassociating component of the mainband DNA seen in our experiments.

Our data may be directly compared with the data obtained by Thompson and his associates [12, 13] in their kinetic analysis of total pea DNA (i.e. pea DNA not fractionated into satellites and mainband). From our observations, 35–40% of the pea genome reassociates at Cot values below 2.0. The DNA involved in this rapid reassociation consists of several different types of sequence with complexities varying between 160 base pairs and several thousand base pairs, with a wide range of reiteration frequencies. In the experiments of Thompson and associates [12, 13], *ca* 45% of the pea reassociated at Cot values of less than 2.0, and could thus be classified as highly repetitive. Allowing for the difference in the techniques (hydroxyapatite vs optically-monitored reassociation; unfractionated vs fractioned DNA) our data are in good agreement with those of Thompson's group. Thompson and his colleagues also found that a further 30% of the genome reassociated at Cot values between 2.0 and 1×10^3 and could thus be classed as intermediate repetitive DNA, whilst the remainder behaved as unique sequences, although containing a very large proportion of diverged repeats [13]. Thompson *et al.* have suggested that both the highly repetitive and intermediate repetitive classes are very complex mixtures of components of different reiteration frequencies and kinetic complexities [13]. The number of different low Cot components seen in our experiments very much supports this view.

EXPERIMENTAL

Plants. Pea seeds (*Pisum sativum* L., var. Feltham First) were surface-sterilized for 20 min in sodium hypochlorite soln (2.5% available Cl_2), soaked for 4 hr in running tap H_2O, and planted in moist vermiculite in seed trays. The trays were kept in darkness at 22°. Shoot apices were harvested after 5 days.

DNA extraction. Shoot apices were homogenized in 4% (w/v) SDS. DNA was extracted from the homogenate as described previously [18].

$Cs_2SO_4 . Ag^+$ *density gradients.* DNA was dissolved in 50 mM Tris–acetate, pH 9.2. $AgNO_3$ was added to give the required R_f value (i.e. molar ratio of Ag^+:DNA-P). The soln was gently shaken, and then allowed to stand at 20° for 2 hr. Cs_2SO_4 was added to give a buoyant density of between 1.43 and 1.5 g/cm^3. Centrifugation was carried out at 44 000 rpm for 20 hr in a Type 65 rotor of a Beckman L5-65 preparative ultracentrifuge.

Fractionation of gradients. Five-drop fractions were collected from the gradients as described previously [19]. Every tenth fraction was used for measurement of refractive index, which in turn was used to calculate buoyant density. All the other fractions were diluted with 1 ml of 0.1 m NaCl and assayed for *A* at 260 nm.

Estimation of DNA. DNA in fractions obtained from density gradients was estimated from *A* at 260 nm and by the diphenylamine reaction [20].

Analysis of DNA by reassociation kinetics. Required fractions from density gradients were pooled and dialysed for 72 hr against several changes of 0.1 M NaCl. DNA was precipitated by addition of 2 vol. of EtOH, followed by storage at −20°, and then recovered by centrifugation. The DNA was dried, dissolved in 0.1 M NaCl and its absorbance spectrum was determined. The DNA was sheared by 20 passages through a fine syringe needle, to give a mean fragment length of 750 base pairs (see below). The DNA was then denatured by addition, with mixing, of 0.3 ml of 1 M NaOH to a 2.4 ml aliquot of DNA soln, after which the soln was allowed to stand for 20 min. Hyperchromicity was always in excess of 35%. The soln was then brought to 60°, neutralized by the addition, with mixing, of 0.3 ml of 2 M NaH_2PO_4, also at 60°, and rapidly transferred to a spectrophotometer cell. Reassociation was followed by continuous monitoring of *A* at 260 nm in a Pye–Unicam SP1800 recording spectrophotometer, with the cell compartment maintained at 60°. The *Escherichia coli* DNA and 'phage T4 DNA used as standards were treated in the same way.

Estimation of DNA fragment length. The fragment length of sheared DNA was estimated by electrophoresis in 1% agarose slab gels [21] using as markers a range of DNAs of known fragment length.

Hybridization of [^{3}H]*-rRNA to DNA.* [^{3}H]-rRNA (26S and 18S) from pea was hybridized to the DNA fractions obtained in density gradients as described in ref. [17].

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