STUDY OF THE MITOCHONDRIAL DNA OF SUNFLOWERS : COMPARISON BETWEEN MALE STERILE AND MALE FERTILE CYTOPLASMS.

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Abstract

Mitochondrial DNA (mt-DNA) from male fertile (F) and male sterile (S) Sunflower (**Helianthus annuus** L.) has been investigated within an isogenic couple HA89.

1. The mt-DNA from both cytoplasms exibit a buoyant density of 1.706.
2. The mt-DNA from F and S cytoplasms can be clearly distinguished :
   a. A 1.45 kilobase paires (kb) low molecular weight (LMW) mt-DNA supercoiled molecule is observed only in the F cytoplasm.
   b. Horizontal agarose gel electrophoresis of fragments obtained by using restriction endonucleases reveals differences between S and F cytoplasms.
3. A simplified, rapid technique for the extraction and analysis of sunflower mt-DNA makes it possible to characterize the mitochondrial genome of a single plant without destroying it.

Introduction

The first reliable cytoplasmic male sterile (CMS) sunflower from interspecific hybrids between the **Helianthus petiolaris** Nutt. and the cultivated sunflower was reported by Leclercq in 1969. CMS and fertility restoration system of sunflower, now widely used in oil seed industry, are based on a single source of CMS which makes a crop genetically vulnerable in terms of disease susceptibility (Leclercq, 1983).
Several distinct lines of evidence suggest that the mitochondria are carriers of genetic determinants conditioning CMS in plant. Since 1976 (Levings and Prings, 1976) it is possible, for different plants species, to clearly distinguish fertile from sterile cytoplasms using restriction endonucleases. It is also possible to characterize each cytoplasm, for some species, by studying the native mt-DNA.
In this report, we propose a simplified tool which makes it possible to investigate and characterize easily and rapidly the mitochondrial genome of a single plant. F and S cytoplasms of sunflower are analysed by using native mt-DNA or restriction endonucleases enzymes.

Materials and Methods

Plants were grown during at least 1 month in growth chambers. 6 g of leaves from a single plant were cut, washed and homogenized for 4 sec at low speed in a two speed waring blendor with buffer A (0.3 M Potassium chloride (KCL), 0.05 M Tris(hydroxymethyl) aminomethane (Tris) (pH 7.6), 0.02 M Ethylenediaminetetraacetic (EDTA), 0.3 % Bovine serum albumine (BSA), 0.02 M 2-mercaptoethanol (BME)). The preparation was filtered through two layers of Blutex (60 and 200 µm) priorly to centrifugation for 5 min at 4000 rpm in a Sorvall ss34 rotor. The supernatant was then centrifuged for 10 min at 10 000 rpm in the same rotor to pellet the mitochondria.
This pellet was resuspended with a small paint brush in 3 ml of buffer B (0.3 M KCL, 0.05 M Tris (pH 7.6), 0.I% BSA, 0.01 M Magnesium acetate (MgAc)) and treated with 60 µg/ml Bovine pancreatic DNAse. After 15 min at 37° C, the mitochondria were diluted with 10 ml of buffer C (0.3 M KCL, 0.02 M EDTA, 0.05 M Tris (pH 8.0)) and pelleted as described above.
The pellet of purified mitochondria was resuspended in 2 ml of buffer C and treated with 10 µg/ml of RNAse A and 150 u/ml of RNAse T1, in presence of 4 % sodium sarkosyl during 15 min at 37° C. The suspension was then extracted gently with phenol saturated chloroform during 10 sec. The upper phase was recovered after 5 min of centrifugation at 10 000 rpm in a Sorvall HB 4 rotor.
2 ml of a 7.5 M Ammonium Acetate (pH 7.5) were added to the mt-DNA solution and kept 10 min at room temperature. Then, two volumes of ethanol 95° were added. After 2 h incubation at -20 °C the mt-DNA was precipited 30 min at 10 000 rpm in a 15 ml siliconed corex tube. The mt-DNA pellet was finally resuspended, after it has been vacuum dried for 2 min, in 20 µl of TE buffer (0.001 M EDTA, 0.01 M Tris (pH 8.0)).

Results

BUOYANT DENSITY ANALYSIS

Mitochondrial DNA from S and F HA89 sunflower cytoplasms is observed as a single narrow and symetrical peak at 1.706 g/ml. The calculated GC % is 46.9. No contamination by nuclear or chloroplastic DNAs are detected by this method.

NATIVE MT-DNA ANALYSIS

In a 1 % horizontal agarose gel the native sunflower mt-DNAs give a broad band at the top of the gel. We observe that F mt-DNA contains an additional LMW band which migrates as a 1.0 kb linear band. Very faint bands about 1.50 and 1.70 kb are also observed.

Analysis with DNAse, RNAse and nuclease S1 suggest a supercoiled form (CCC) for the 1.0 kb band. Thus, the 1.50 kb band represent the linear form (L) while the 1.70 kb band represent the open circle form (OC), (Fig : 1). These results are confirmed by analysing the native F mt-DNA through an electron microscope. Open-circle molecules of approximatively 1.45 kb are observed as well as supercoiled molecules.

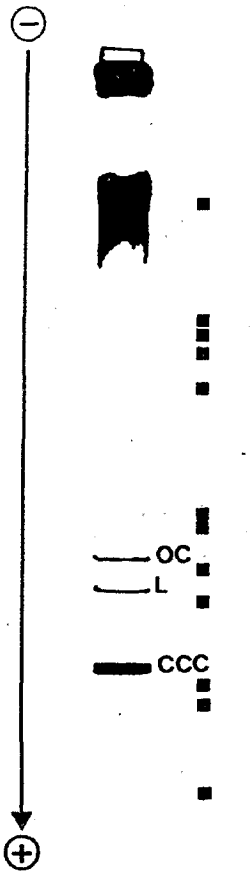

Fig : 1. Schematic diagrams of native mt-DNA from F cytoplasm prepared from photographs of a 1 % horizontal agarose gel. The arrow indicates the direction of the migration. CCC, OC and L indicate supercoil, open-circle and linear bands, respectively. For molecular weight references dots are placed beside the pattern(21.7/5.1/5.0/4.3/3.5/2.0/1.9 /1.6/1.4/0.9/0.8/0.6 kb). The extraction and purification of mt-DNA were carried out as described by Leroy **et al.**.

MT-DNA RESTRICTION ENDONUCLEASES ANALYSIS

The electrophoresis of restriction endonuclease fragments in horizontal 1 % agarose gel, reveals several differences between mt-DNA from F and S cytoplasms (Fig : 2). The examination of photographs and of corresponding densitometric tracings clearly shows that bands occur in nonstoichiometric amounts. It is unlikely that the observed lack of stoichiometry is caused by the presence of partial digestion products, since increasing amounts of enzyme and digestion time led to identical patterns. Furthermore, taking in consideration the buoyant density profiles of F and S mt-DNAs, we conclude that our preparation is not sufficiently contaminated with foreign DNA (chloroplast DNA and/or nuclear DNA) to account for the complexity and variability in stoichiometry of mt-DNA restriction patterns.

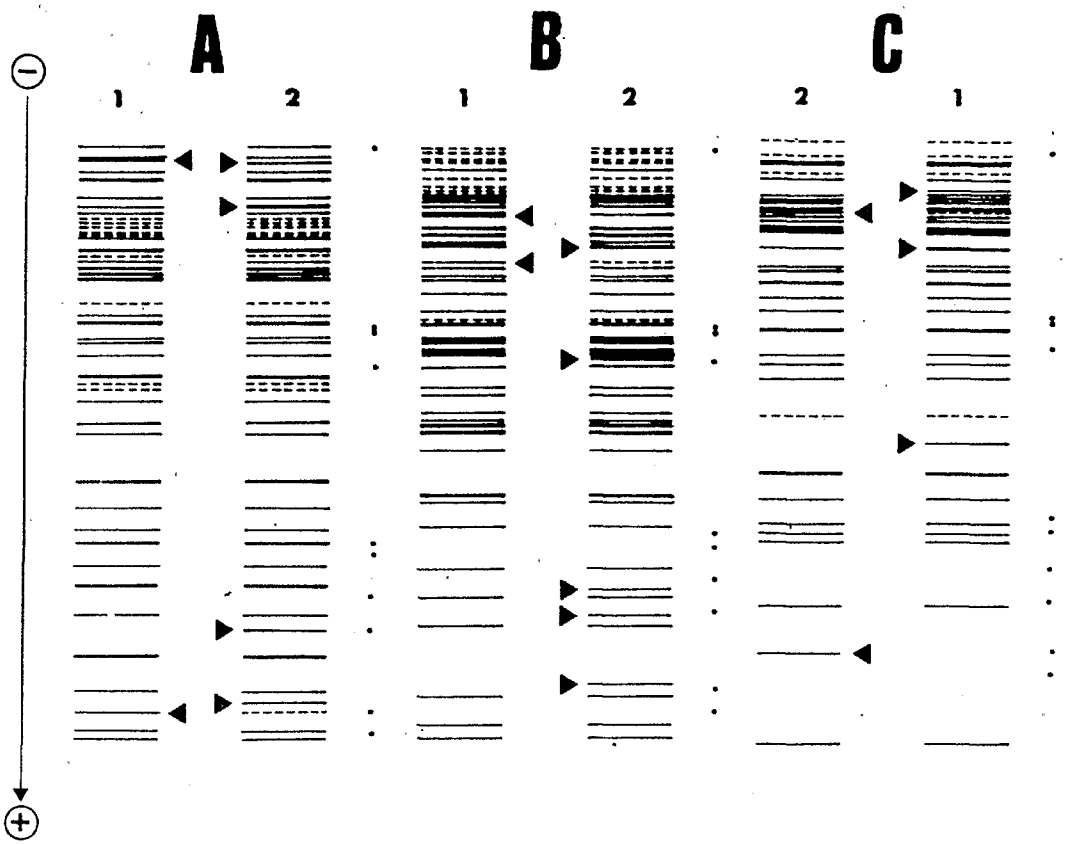

Fig : 2. Schematic diagrams of a Xhol (A), Sall (B) and Bgll (C) digests of mt-DNAs from HA89 S cytoplasm (1) and HA89 F cytoplasm (2) prepared from photographs of a 1 % horizontal agarose gel electrophoresis. For molecular weight references dots are placed beside each pattern (21.7/5.1/5.0/4.3/2.0/1.9/1.6/1.4/0.9/0.8 kb). The arrow indicates the direction of the migration. The thicker lines represent the more intense bands in the gel. The faint bands are represented by the dashed lines. Bands noted with an arrow are specific either to F and S or exhibit different intensities in F and S cytoplasms. The extraction and purification of mt-DNA were carried out as described by Leroy **et al.**.

A SIMPLE PROCEDURE TO EXTRACT MT-DNA FROM A SINGLE PLANT

Results given in Fig : 2 were obtained by using a classical mt-DNA extraction and purification protocol described by Leroy **et al.** (1984). Unfortunately the classical technique of mt-DNA extraction and study does not allow us to work easily and rapidely with a single plant. With the simpified protocol (given in Materials and Methods) we can obtained an identical restriction endonuclease pattern with Sall as we can see in Fig : 2. Thus it is possible to characterize the mitochondrial genome of a single plant (6 g of leaves) without destroying it. We can oberve, using the same protocol, the presence in F cytoplasm of the LMW mt-DNA which migrates as a 1.0 kb band in a 1 % horizontal agarose gel.

Discussion

The involvement of mt-DNA in plant CMS suggested by Duvick in 1965 was reinforced by the observation of specific differences in the restriction endonuclease fragment patterns and of the presence or absence of one or several LMW mt-DNA molecules from the fertile and the CMS line (Leaver and Gray 1982, see \for review). These kinds of differences are observed between S and F HA 89 sunflower mt-DNA, which seems to corroborate this relationship. Nevertheless, the correlation of these differences with the sunflower CMS trait remains to be demonstrated. It seems unlikely that the molecular mechanism responsible for the CMS phenotype will be the same for each CMS plant system : broad bean could be a good example in which the CMS factor seems to be "virus-like" (Scalla **et al.** 1981).
It must be emphasized that the S cytoplasm came from **H. petiolaris** while the F cytoplasm came from **H. annuus.** The observed differences in mt-DNA could be due to their different origins and might not, in fact, have a causal relationship with the CMS trait.
It is evidently impossible to analyse the **H. petiolaris** cytoplasm used in 1966 for the initial interspecific crossing. Nevertheless, a simple tool makes it now possible to characterize the mitochondrial genome of a single plant without destroying this plant. Such a tool will allow us to analyse the probable heterogeneity of the fertile cytoplasm of **H. petiolaris** with different nuclear background and compare it to the present day male sterile cytoplasm.

Conclusion

It is possible to clearly distinguish F and S cytoplasms of a HA89 isogenic couple by studying native mt-DNA or restriction endonuclease patterns of the mitochondrial genome. It is not known whether the differences observed between F and S mt-DNAs are correlated with the cytoplasmic male sterility phenotype.
A simplified technique of mt-DNA analysis will allow us to investigate the cytoplasm of wild sunflower populations. With such a tool we are planning to approach the interaction between the nucleus and the cytoplasm, within inbread sunflower and wild sunflower populations, in view to understand the cytoplasmic male sterility mechanism.

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