MOLECULAR APPROACH OF CYTOPLASMIC MALE STERILITY IN SUNFLOWER

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INTRODUCTION

The first cytoplasmic male sterility (CMS) of the sunflower originated from the interspecific cross between a *Helianthus petiolaris* form used as female and the sunflower (Leclercq, 1969). As described by the author the CMS form has been obtained in the BC_1 generation. Since a lot of nuclear background have been transferred to Leclercq's CMS forms, couples of lines carrying either the normal cytoplasm (*H. annuus*) or the CMS cytoplasm are available for a large scale hybrid seed production .The major drawback to the use of this CMS in such a way is that it can lead to a high degree of cytoplasmic genetic uniformity which in turn might make the crops genetically susceptible to specific diseases such as *Helminthosporium maydis* race T on Texas maize cytoplasm (Ullstrup, 1972). New CMS have been obtained by Serieys in interspecific crosses, one of which using *Helianthus petiolaris fallax* as female parent and the sunflower line CC 60 as male parent (Serieys, 1986). We called it "fallax CMS" and it appeared particularly fruitfull both for a practical use and for basic studies. As already described by Serieys the CMS was induced as early as in the F_1 generation. Although the fertility of the cross was very low two other F_1 male fertile plants led no longer to female progenies. Thus only one plant gave rise to the CMS called "fallax".

The empiric knowledge of the CMS genetic mechanisms is indeed a disadvantage. A best understanding of this phenomenon will help in its use for plant improvement. The nuclear-cytoplasmic determinism of CMS led us to study the mitochondrial and the chloroplastic DNAs of both the wild forms, the CMS forms and their respective maintainer forms. The Leclercq's CMS had been first investigated but as the wild species ecotype of the initial cross has been lost and the CMS trait didn't appear at the F_1 generation we looked more markedly at fallax CMS which is an original system. In order to determine the cytoplasmic diversity we tested several *Helianthus petiolaris* ecotypes to check the identification of potentially useful alternative cytoplasm sources.

The first step of our work had been to set up protocols for extracting mitochondrial and chloroplastic DNAs from green leaves of the wild forms. These preparations revealed more difficulties than expected. So the main results exposed below concerned first a method to obtain relatively well hydrolysable ct and mt DNA from leaves of sunflower and wild species. Then we determined that the ct DNA of the Leclercq's CMS forms and their respective maintainers were not different for at least four restriction endoncleases. On an other hand the fallax CMS form and the maintainer were different. The unrestricted mt DNA displayed a set of low molecular weight (LMW) molecules in Leclercq's CMS maintainer lines that is not detected in the CMS form. Furthermore the *Helianthus petiolaris* mt DNA displayed another set of LMW molecules and the fallax CMS had the same set while there was no plasmid in the maintainer form. Concerning the restriction mt DNA diagrams of the Leclercq's CMS and their maintainers no difference or an undecided difference has been revealed by ECO RI restriction endonuclease while Leroy *et al.* (1985) showed more differences using SAL I, XHO I and BGL I enzymes. For the fallax CMS and its maintainer the ECO RI mt DNA diagrams were different. On the contrary we could not distinguish between the wild form from the fallax CMS which looked like similar.

MATERIALS AND METHODS

Seeds of the sunflower lines were sown in trays (100 seeds per tray) and grokn up in a greenhouse for 5 to 6 weeks (16 hours of light

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per day) at 20°C until use. The plants were close-cut for the preparations of organelles. The seeds of the wild species were sown by Serieys in Montpellier in small rabble pots and then transferred in large pots in Dijon. The leaves of the plants were picked up three to four times before they died.

Several methods were used to prepare either chloroplasts or mitochondria from sunflower lines but for the wild species the fresh weight was so limiting that we prepared successively the chloroplasts, and then the mitochondria.

The techniques to isolate the organelles has been adapted from Bookjans *et al.* (1984) for the high ionic strength grinding medium with DNAs treatment for the mitochondria and modified from Vedel et Quetier (1976) for the DNA preparation. The Cesium chloride gradient step was omitted and most of the proteins were eliminated by SDS and potassium acetate precipitation before phenol-chloroform treatment. All the materials for DNA restriction analysis were purchased from Boehringer or B.R.L.

RESULTS AND DISCUSSION

Unrestricted mitochondrial DNA run on agarose horizontal gels resolved in a main band of high molecular mt DNA called major mt DNA plus faint bands (or not) of low molecular weight mt DNAs. We summarize in Table 1 the presence or the absence of these LMW molecules.

All the B type male fertile inbreds exhibited the same unrestricted mt DNA profile whereas the A type Leclercq's male sterile ones did not display any LMW DNA band. Leroy *et al.* showed for HA 89 that these 1.9 and 1.0 kb DNA species corresponded respectively to the open-circle and the supercoiled form of a 1.45 kb mt DNA molecule. *H. petiolaris* different subspecies revealed different LMW DNA profiles ; the same set for the subspecies 199 as *H. annuus* B types, no LMW for the subspecies 91 and a new set of three LMW DNA bands for the subspecies 200. Furthermore the fallax CMS mt DNA displayed the same set of bands as 200 while its RHA 274 maintainer had no LMW DNA bands. So we could conclude that the set of LMW DNA molecules is maternally inherited. Thus it is likely that the *H. petiolaris* 6392 used by Leclercq for the cross by the sunflower did not carry any LMW mt DNA molecule.

The LMW mt DNA species are variable in their absence or presence and in their sizes. What does this situation mean ? Is it related to any agronomic trait ? How did originally this diversity appear and by which mechanisms is it maintained along the crosses ? Which nuclear or (and) mitochondrial genes are involved ? We are presently unable to answer these questions but the LMW DNAs revealed to be practical markers of the cytoplasms.

Table 1

Presence or absence of bands of low molecular weight mt DNAs

Types of cytoplasm	Number of LMW bands	Position on a 1% agarose gel	Length
1. *Helianthus annuus* cytoplasm cultivated forms which are maintainers of Leclercq's CMS (B type of breeders) nuclear background : HA 89	2	1.8/1.0	1.45 kb
CC 60	2	1.8/1.0	ND
CANP 3	2	1.8/1.0	ND
CVH 11	2	1.8/1.0	ND
CCM 61	2	1.8/1.0	ND
CIC 61	2	1.8/1.0	ND
2603	2	1.8/1.0	ND
CC 40	2	1.8/1.0	ND
CA 4	2	1.8/1.0	ND
2. Leclercq's CMS cytoplasm (A type of breeders) nuclear background : HA 89	0		
CC 60	0		
CVH 11	0		
CANP 3	0		
3. *Helianthus petiolaris* species No 91 (*Helianthus petiolaris*)	0		
No 199 (*Helianthus petiolaris petiolaris*)	2	1.8/1.0	ND
No 200 (*Helianthus petiolaris fallax*)	3	3.0/1.6/0.8	ND
4. Fallax MS cytoplasm	3	3.0/1.6/0.8	ND
5. Maintainer of fallax CMS, on Leclercq's cytoplasm : RHA 274	0		

Are the plasmid homologies a way to understand the phylogeny of the *Helianthus petiolaris* species ? This way could lead to a first estimation of the intraspecific cytoplasmic diversity. Hybridization experiments will bring more details about the origins of these plasmid-like molecules. At present time, two situations are observed in plants : first there is an homology with the main mt DNA so the small DNA molecules could originate from it by excision or recombination events ; second there is no homology and their origins become more putative by total excision from the main mt DNA or through an extramitochondria import.

The biological role of the mitochondrial small DNA molecules remains unknown. In sunflower their participation in CMS mechanism had to be ruled out of the original mechanism of the CMS. In some plants, sorghum,

sugarbeet, maize, homologies with mt mRNA had been set up but no evidence for a protein translation have been pointed out (for review see Chase and Pring, 1985).

Concerning the ECO RI restriction patterns of mt DNA, seven B types of sunflower displayed the same restriction pattern (2603, CANP3, CC40, CIC61, CCM61, CVH11, CA4). The profile is complex with more than fifty fragments some of which were not in the same stoechiometry. The A types of sunflower (CANP3A, CC60A) exhibited an identical diagram except for two bands which appeared less intense as in the B types. No marked difference had been detected with the ECO RI endonuclease.

In order to compare the *H. petiolaris fallax* species, its CMS form and its RHA 274 backcross parent we hydrolysed the respective mitochondrial DNAs with ECO RI. We pointed out that RHA 274 mt DNA was different for several mt DNA fragments from the fallax CMS mt DNA. Unfortunately we failed to obtain a well restricted mt DNA of *H. petiolaris fallax* and rough comparison with the fallax CMS mt DNA did not displayed difference. Further studies are in progress to look for any difference with other restriction endonucleases.

The ct DNA of different isogenic couples (A and B types) have been compared after hydrolysis by ECO RI, XHO I, Bam Hl and Pst 1 restriction endonucleases. These four enzymes failed to detect differences between the *H. annuus* cytoplasm and the Leclercq's one. At present time other restriction enzymes are being tried.

For *Helianthus petiolaris* ct DNA studies most of the preparations were unrestrictable so we had to overcome a lot of difficulties to obtain fine preparation of that DNA. The comparison of ECO RI restricted ct DNA of *Helianthus petiolaris fallax* and the sunflower pointed out two differences : the presence of a 6.8 kb fragment for the wild species instead of a 6.2 kb one for the sunflower and a new fragment of 3.8 kb for *Helianthus petiolaris fallax*. The ct DNA profile of the fallax CMS form displayed differences in regard to the wild species whereas it looked like the *H. annuus* cytoplasm ct DNA diagram. By analysing several preparations of these DNA forms we observed that the concerning fragments were more or less intense or even absent depending on the preparation. Other *Helianthus petiolaris* ecotypes have been investigated. In spite of the fact that the restricted ct DNA profiles were not as suitable as we hoped they permitted us to recognize differences between the different ecotypes. In regard to that first results the situation of the fallax CMS was attractive since we could identify the mt and the ct DNA of the wild species of the cross and the CMS maintainer. So in order to study more precisely the differences we isolated and then cloned the concerning fragments. Now we try to identify the fragment of 6.8 kb as this of 3.8 kb. We suppose they could be contaminant nuclear DNA which we have to characterize further.

CONCLUSIONS

The sunflower mt DNA appeared stable since all the B forms of isogenic couples did not differ with ECO RI endonuclease. Moreover since the ECO RI mt DNA profiles of the A and the B lines are very similar the *Helianthus annuus* species and the *H. petiolaris* species are closely related. The A type mt DNA did not display LMW DNA species so it is likely the *H. petiolaris* form used by Leclercq was lacking these small DNA molecules. Since the original cross the lines used as male parent have not transmitted their own LMW DNA species.

To detect possible mt DNA differences other restriction enzymes have to be assayed.

The *Helianthus petiolaris* species display a cytoplasmic diversity for the LMW mt DNA molecules while this variability is not observed for the cultivated forms of the *H. annuus* species.

An extensive characterization of the ct DNA and mt DNA of the wild forms of *H. petiolaris* remains to be done. On the same way the characterization of the ct DNA and the mt DNA of the sunflower is now more developped.

The model of CMS induction is very unique. We are preparing the molecular tools for following both the ct DNA and mt DNA through the interspecific cross leading to the cytoplasmic male-sterile phenotype.

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APPROCHE MOLÉCULAIRE DE LA STÉRILITÉ MÂLE CYTOPLASMIQUE CHEZ LE TOURNESOL

Résumé

La 1ère SMC du tournesol a été obtenue dans un croisement interspécifique (Leclercq, 1969) dont la forme sauvage a été perdue. D'autres SMC ont depuis été obtenues. L'une d'entre elles (Serieys, 1986) peut servir comme modèle d'étude pour comprendre le mécanisme d'induction de la SMC et en particulier si les ADN cytoplasmiques peuvent être modifiés lors du croisement.

La première étape de notre travail a été de mettre au point des protocoles pour extraire les ADN chloroplastiques et mitochondriaux des feuilles adultes des formes sauvages. Ces préparations se sont révélées beaucoup plus difficiles à réaliser que prévu. Ainsi les résultats exposés concernent d'abord une méthode pour obtenir des ADN mt et ct bien digérables à partir de feuilles des espèces sauvages. Ensuite nous avons déterminé que l'ADN ct des lignées SMC (Leclercq) et de leurs mainteneurs respectifs ne sont pas différents pour au moins 4 endonucléases de restriction différentes. Par ailleurs, la forme SMC fallax et le mainteneur sont différents. L'ADN mt non hydrolysé montre un ensemble de molécules de faibles poids moléculaires, pour les lignées mainteneuses du cytoplasme Leclercq, qui ne sont pas détectées dans la forme SMC. De plus, l'ADN mt de *H. petiolaris fallax* montre un autre ensemble de molécules de faibles P.M. tandis qu'aucune molécule n'est détectée pour le mainteneur RHA 274.

En ce qui concerne les diagrammes de digestion de l'ADN mt par Eco RI, ils ne semblent pas différents pour les couples SMC Leclercq, mainteneur alors que le mâle-stérile fallax et son mainteneur sont différents. En revanche, nous ne pouvons pas encore conclure entre la forme sauvage et le mâle-stérile fallax qui de toute façon se ressemblent beaucoup.

ENFOQUE MOLECULAR DE LA ANDROESTERILIDAD CITOPLASMATICA DEL GIRASOL

Resúmen

La primera androesterilidad citoplasmática (CMS) del girasol se ha obtenido en un cruce interspecifico (Leclercq, 1969) cuya forma silvestre se ha perdido. Otras CMS se han obtenido desde entonces. Una de ellas (Serieys, 1986) puede servir de modelo de estudio para comprender el mecanismo de inducción de la CMS y en particular si los ADN citoplasmáticos pueden ser modificados durante el cruce.

La primera etapa de nostro trabajo consistio en éllevar a su punto protocolos para extraer los ADN cloroplásticos (ct) y mitocondriales (mt) de las hojas adultas de las formas silvestres. Estas preparaciones se revelaron mucho más difíciles de realizar que previsto. Así los resultados expuestos conciernen ante todo un método para obtener ADN mt y ct bien digeribles a partir de las hojas de especies silvestres. Luego hemos determinado que el ADN ct de los linajes CMS (Leclercq) y sus mantenedores respectivos no son diferentes al menos para 4 endonucleasas de restriccíon distintas. Por otra parte, la forma CMS fallax y el mantenedor son diferentes. El ADN mt no hidrolizado presenta un conjunto de moleculas de reducido PM, para los linajes mantenedores del citoplasma Leclercq, que no se han hallado en la forma CMS. Además, el ADN mt de *H. petiolaris fallax* presentera un cunjunto de moleculas de reducido PM mientras no se ha hallado molécula alguna en el mantenedor RHA 274.

En lo que concierne los diagramas de digestión del ADN mt por Eco R 1, no parecen diferentes en las parejas CMS Leclercq mantenedor mientras el androestéril fallax y su mantenedor son diferentes. En cambio, todavía no podemos concluir entre la forma silvestre y el androesteril que de todos modos se parecen mucho.