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Nuclear effect on mitochondrial protein expression of the CMS *Owen* cytoplasm in sugar beet

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Abstract In sugar beet, cytoplasmic male sterility (CMS) is conferred by the *Owen* mitochondrion (*Svulg*). In order to find polypeptides specific to this cytoplasm and putatively involved in CMS, we assessed the protein expressions of *Svulg* and a non-sterilizing mitochondrion (*Nvulg*) by *in organello* protein synthesis of mitochondria isolated from leaves. Given the hydrophobicity of mitochondrial translation products, we compared the *in organello* synthesis polypeptides of both cytoplasms with an acid-base two-dimensional electrophoresis adapted to hydrophobic protein separation. To evaluate the possible effect of nuclear background, we assessed the mitochondrial protein expression in three different nuclear backgrounds by using three near-isogenic-line pairs. While three to four variant polypeptides were revealed for each nuclear context, each variant polypeptide was specific to a nuclear-cytoplasmic context. Although this study did not enable us to unambiguously find any variant polypeptide related to CMS, we did observe an effect of the nucleus on mitochondrial gene expression.

Keywords Nuclear-cytoplasmic male sterility · Plant mitochondria · In organello synthesis · Sugar beet · Acid-base electrophoresis

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Introduction

The regulation of mitochondrial gene expression by nuclear genes is the key means by which the activities of the nuclear and organelle genetic systems are coordinated. In yeast, a large number of nuclear genes mediating the expression of specific mitochondrial genes have been identified (Costanzo and Fox 1990; Grivell 1995). However, in plants the mechanisms through which nuclear genes regulate mitochondrial gene expression are much less well understood. Mitochondrial variation is phenotypically characterized by plants that are unable to produce functional pollen grains (CMS, cytoplasmic male sterility). CMS has been described in numerous higher plant species and has been used as a model to study nuclear-cytoplasmic interactions (see review by Schnable and Wise 1998). Indeed, plants with ''sterilizing" mitochondria can produce pollen grains due to to the ''counteraction" of nuclear genes that restore male fertility, the so-called restorer loci. CMS was first introduced into sugar beet (*Beta vulgaris* ssp. *vulgaris*) by F.V. Owen (1945). This so-called *Owen* cytoplasm is currently used worldwide for hybrid seed production. Despite extensive efforts, up to now the relationship between mitochondrial gene expression and male sterility has not been documented in beet. At the DNA level, data provided by restriction and hybridization analysis suggest that considerable rearrangements have occurred in the *Owen* mitochondrial genome with reference to a given non-sterilizing cytoplasm (Brears and Lonsdale 1988; Kubo *et al.* 1999). A random amplified polymorphic DNA (RAPD) analysis between the *Owen* mitochondrion and a non-sterilizing mitochondrion recently revealed so many differences that it was impossible to ascribe any to CMS (Lorenz et al. 1997). The same phenomenon was encountered at the RNA level (Saumitou-Laprade et al. 1993a; Xue et al. 1994). In addition, a nuclear effect was suspected in several cases at the transcript level (Saumitou-Laprade et al. 1993a). Conversely, one-dimensional electrophoresis analysis of *in organello* labeled polypeptides did not reveal any polymorphism be-

tween a given non-sterilizing cytoplasm and the *Owen* cytoplasm (Halldén et al. 1992).

Within this framework, the investigation reported here used the following strategies:

1) the protein expression of both mitochondrial types was assessed by *in organello* synthesis;
2) given the hydrophobicity of polypeptides encoded by mitochondria, the *in organello* synthesis products of both cytoplasms were compared using an acid-base two-dimensional electrophoresis adapted to hydrophobic protein separation;
3) in order to take into account the putative effect of nuclear background, the mitochondrial protein expression was assessed in three different nuclear backgrounds using three near-isogenic line (NIL) pairs.

Material and methods

Plant material

Three pairs of sugar beet were used in this study: INRA418 (INRA, France), FD33 (the Florimond Desprez Company, France) and FC603 (Fort Collins 603, USDA). They were kindly provided by INRA (Dijon, France) and the Florimond Desprez Company (France). A NIL pair is composed of two lines sharing approximately the same nuclear genome through recurrent backcrosses, but differing by their cytoplasm (*Owen versus* non-sterilizing type). The following notation will be used; for example, in the *INRA418* NIL pair, INRA418*s* is the line with the sterilizing cytoplasm, while INRA418*n* is the line with the 'normal' one. The lines bearing the sterilizing cytoplasm are male-sterile, whereas the ones bearing the 'normal' mitotype are male-fertile, i.e. hermaphrodite. It must be noted that INRA418*s*, FD33*s* and FC603*s* exhibit the identical male-sterile phenotype.

Oligonucleotide fingerprinting

In order to estimate the nuclear similarity within and between NIL pairs, total DNA was digested by *Hae*III or *Eco*RI and hybridized with a $(AAC)_5$ or a $(GATA)_4$ oligonucleotide probe, respectively, as described in the procedure of Weising et al. (1991) and Saumitou-Laprade et al. (1999).

Computation of nuclear similarity

Estimates of nuclear genetic similarity were based on the calculation of Jaccard's coefficient between two individuals, *i* and *j* (Jaccard 1908): $S_{ij}=a/(a+b+c)$ where *a* is the number of common bands between *i* and *j*, *b* the number of bands present in *i* and absent in *j* and *c* is the number of bands present in *j* and absent in *i*. This coefficient was computed between lines within a NIL pair. It provides an estimate of the nuclear isogenicity of the NIL pair, but also of the nuclear isogenicity between lines from different NIL pairs, thereby assessing the nuclear diversity among NILs.

Mitotyping

The mitochondrial type (mitotype) of each analyzed plant was determined by means of a restriction fragment length polymorphism (RFLP) procedure using three mitochondrial probes as described by Saumitou-Laprade et al. (1993b). Accordingly, both mitotypes were defined among 20 previously identified mitotypes (Desplanque et al. 1999): *Svulg* (the *Owen* CMS mitotype; also called *S1* by Halldén et al. 1990) and *Nvulg*, the reference non-sterilizing mitotype in this study, which is the most frequently used mitotype in breeding programs.

In organello protein synthesis

Mitochondria were isolated from 2 g of young beet leaves and purified by ultracentrifugation on a discontinuous Percoll gradient (13.5%/26%/45%). Mitochondria were collected at the 26%/45% Percoll interface and incubated for 60 min at 25°C in a protein synthesis medium for *in vitro* [^{35}S]-methionine labeling (Boutry et al. 1984).

Two-dimensional acid-base electrophoresis

The first-dimension electrophoresis consisted of a standard 12–17% SDS-PAGE with 100,000–120,000 cpm of *in organello* synthesized proteins per lane. Strips 5- to 6-mm wide were cut in the lane of interest and incubated 4×15 min with the cationic detergent in denaturing buffer (1% tetradecyltrimethylammonium bromide (TDAB), 250 m*M* sucrose, 100 m*M* KH_2PO_4, pH 4.0). The second dimension involved a 10%–16% discontinuous TDAB-PAGE (pH 3.0) with a 0.8% agarose acidic stacking gel (Pennin et al. 1984). Electrophoreses were conducted for 16 h at 50 V. The proteins were electroblotted on an Immobilon-P membrane in acidic buffer (85 m*M* glycine, 20% methanol, 0.03% TDAB, pH 3.0). The membranes were then exposed 2–3 weeks on a BioMaxMR film for autoradiography.

Molecular mass was determined using the Pharmacia low-molecular-weight electrophoresis calibration kit.

Results

Nuclear similarity within and between NIL pairs

Nuclear similarity within and between NIL pairs was estimated by oligonucleotide fingerprinting (Table 1). With a total number of 51 polymorphic bands, isogenicity within NIL pairs averaged 81.3%. A large nuclear diversity was found between NIL pairs (less than or equal to 38% common bands, with an average of 30.4%). This material was therefore adapted to study the specific

Table 1 Estimation of the nuclear similarity within and among NIL pairs by oligonucleotide fingerprinting. For each line to line comparison, the ratio of the number of common bands over the total number of bands is given.

Genotype	FD33*s*	FD33*n*	INRA418*s*	INRA418*n*	FC603*s*	FC603*n*
FD33*s*	1 (26/26)	0.96 (25/26)	0.36 (14/39)	0.37 (14/38)	0.29 (12/42)	0.26 (9/35)
FD33*n*		1 (25/25)	0.37 (14/38)	0.38 (14/37)	0.27 (11/42)	0.26 (9/34)
INRA418*s*			1 (27/27)	0.89 (25/28)	0.31 (13/42)	0.22 (8/37)
INRA418*n*				1 (26/26)	0.35 (14/40)	0.22 (8/36)
FC603*s*					1 (28/28)	0.59 (17/29)
FC603*n*						1 (18/18)

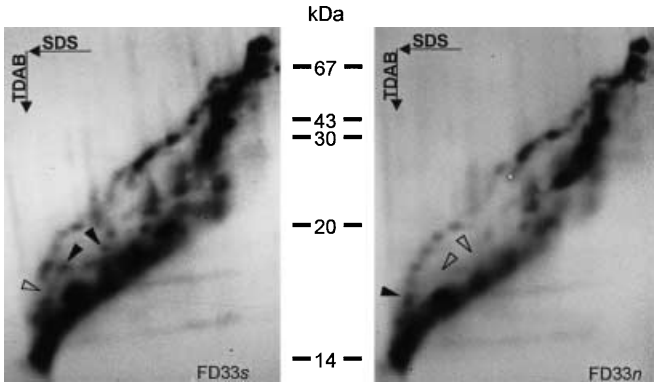

Fig. 1 Autoradiograms of the acid-base electrophoresis gels. Comparison of mitochondrial polypeptide expression within the NIL FD33 pair from young leaves. Variant spots are indicated by *black arrowheads*; their position is indicated by *white arrowheads* in the line where they are absent.

mitochondrial expression within a given nuclear background (intra-NIL pair comparison) as well as the influence of the nuclear genome on the mitochondrial expression.

Two-dimensional electrophoresis analysis

To analyze the protein expression of the mitochondrial genome, we analyzed the *in organello* synthesized products of mitochondria isolated from young leaves by two-dimensional electrophoresis.

An acid-base electrophoresis was chosen to tentatively separate hydrophobic polypeptides and reveal any polymorphism between the *Svulg* and *Nvulg* mitochondrial protein expression patterns. This particular two-dimensional electrophoresis takes advantage of the fact that some polypeptides migrate differently when two different detergents are used, i.e. an anionic detergent (SDS) and a cationic one (TDAB). It is particularly appropriate for hydrophobic proteins that are barely separated by the classical first dimensional isoelectric focusing. Approximately 55 reproducible mitochondrial labeled products were revealed by the acid-base two-dimensional electrophoresis autoradiogram (Fig. 1).

Comparison of mitochondrial polypeptide expression within and between NIL pairs

The protein expression of both mitochondria was compared within each NIL pair. In order to only take into account the reproducible variations, we conducted three *in organello* syntheses for NIL pairs INRA418 and FD33 and two for the NIL pair FC603. In each case, the same plant was used for all experiments.

Considering each NIL separately, NIL FD33 revealed three variants, two of which were specific to *Svulg*; NIL INRA418 revealed four variants, three of which were specific to *Svulg* and NIL FC603 revealed three variants, two of which were specific to *Svulg*. Thus, for each nuclear context, reproducible polypeptides specific to a given cytoplasm, whether *Svulg* or *Nvulg*, were revealed.

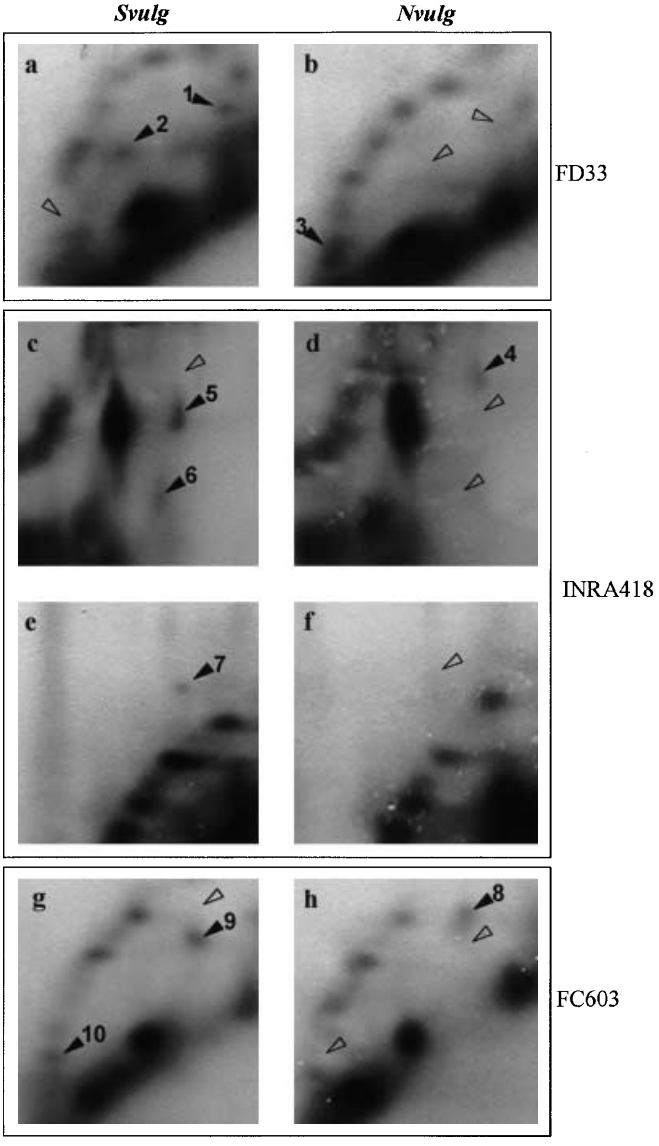

Fig. 2a–h Polypeptide variants between *Svulg* and *Nvulg* revealed by acid-base electrophoresis. Focus on autoradiograms are presented for: **a** FD33*s*, **b** FD33*n* **c, e** INRA418*s*, **d, f** INRA418*n*, **g** FC603*s*, **h** FC603*n*. Variant spots are indicated by *black arrowheads*; their position is indicated by *white arrowheads* in the line where they are absent

Autoradiograms of NIL FD33 revealed the presence of spots 1 and 2 only in the sterile plant (Fig. 2a), whereas spot 3 only appeared in the fertile plant (Fig. 2b). Four variant spots were found for NIL INRA418: spots 5, 6 and 7 were present in the sterile plant (Fig. 2c, e); spot 4, which was specific to the fertile plant, was found within the area of spot 5 (Fig. 2c, d). In NIL FC603, close variant spots 8 and 9 also migrated in the same area (Fig. 2g, h) in fertile and sterile plants, respectively. Spot 10 was only detected in the fertile plant (Fig. 2g). Inter-

Table 2 Comparison of the mitochondrial expression of the three NIL pairs as revealed by acid-base two-dimensional electrophoresis. The nucleo-cytoplasmic context in which the spot is present is indicated by a cross

Genotypes	Spot identification (spot size kDa)									
	1 (20)	2 (19)	3 (17)	4 (46)	5 (39)	6 (29)	7 (21)	8 (24)	9 (22)	10 (17)
FD33*s*	X	X								
FD33*n*			X							
INRA418*s*					X	X	X			
INRA418*n*				X						
FC603*s*									X	X
FC603*n*								X		

estingly, close spots 4, 5 and 8, 9 were only separated by the second dimension of the acid-base electrophoresis. These variations in electrophoretic mobility may reflect a small variation in size or in amino acid composition, resulting in modifications in the charge and/or weight of these polypeptides (Noel et al. 1979).

When all three NILs were considered, no variant was found, regardless of the nuclear background. Therefore, the polypeptide variants revealed were specific to a given nuclear-cytoplasmic context. Results are summarized in Table 2.

Discussion

Total *in organello* synthesis products revealed by autoradiography

Recent sequencing of the *Arabidopsis thaliana* mitochondrial genome revealed that it potentially codes for 57 known genes that code for three ribosomal RNAs, 22 transfer RNAs but only 32 polypeptides. The presence of 85 additional open reading frames (ORFs) has also been reported (Unseld et al. 1997; Marienfeld et al. 1997). Moreover, about 40 *in organello* synthesized polypeptides from tobacco leaves were separated by standard two-dimensional electrophoresis with isoelectric focusing gels as the first dimension (De Paepe et al. 1993). It therefore seems reasonable to suppose that the average of 55 mitochondrial labeled products separated by acid-base electrophoreses is representative of the beet mitochondrial protein expression.

Nuclear-cytoplasm interaction

When the polypeptide pattern of the *Nvulg* and *Svulg* mitotypes of the three NIL pairs was revealed on SDS-PAGE, 20 bands were observed, none of which was specific to a given cytoplasm. No differential polypeptide expression was revealed for both cytoplasms, whether with Tricine-SDS-PAGE [which improved separation of polypeptides below 30 kDa (Schägger and von Jagow 1987)] or with TDAB-PAGE (data not shown). Similar results were described by Halldén et al. (1992) when they compared *in organello* syntheses of a non-sterilizing mitochondrion and *Svulg*.

Conversely, when polymorphism within a NIL pair was examined, consistent differences could be detected by acid-base two-dimensional electrophoresis. Three to four variants were revealed for each nuclear background. We chose to monitor the mitochondrial protein expression of three NIL pairs representing a large nuclear diversity, as revealed by DNA fingerprinting and postulated that only the variant spots, which were present irrespective of the nuclear background, could be considered as authentic cytoplasm-specific spots. With this criterion, not one spot was selected.

Consequently, the present study illustrates the necessity to be particularly cautious when comparing mitochondrial expression to detect gene products related to a specific cytoplasm. Since strict isogenicity is never achieved within a NIL pair, the conclusions which can be drawn from the study of a single NIL are limited.

In addition, we report variations in *in organello* synthesized products based on a comparison of six different nucleo-cytoplasmic contexts. These contexts seem to modify the expression of at least ten *in organello* synthesized polypeptides.

The results described in this paper on a few polypeptides could reflect a more general feature. In plants, the effect of the nucleus on mitochondrial gene expression has been documented by studying mechanisms of male-fertility restoration. Restorer loci, with alternative alleles, may also affect mitochondrial transcripts non-related to CMS (e.g. in rapeseed, Singh et al. 1996). Moreover, in a few cases, the action of non-restorer genes on specific mitochondrial gene expression has also been documented. For example the nuclear *Mct* gene alters the pattern of *cox2* transcripts of teosinte mitochondria within a maize nuclear background (alloplasmic effect) (Cooper et al. 1990). Rocheford and Pring (1994) also reported an extensive nuclear effect on *orf221* transcript patterns in maize. The originality of the present results is that they reflect nuclear-cytoplasmic interactions that are not the product of alloplasmy. Indeed, the mitochondrial protein expression is monitored within the genetic diversity of a single subspecies. In addition, the observed nuclear effects are not due to the segregation of restorer loci, since each nuclear background is a male-sterility maintainer, i.e. lacks restorer alleles.

The origins of the variations observed in this study may be diverse. Differential editing influenced by the nuclear-cytoplasmic context has been reported in *Petunia*, where differentially edited transcripts of the ribosomal protein S12 gene (*rps12*) could be translated to give several forms of RPS12 (Lu *et al.* 1996). In addition, some mitochondrial gene products are translated into a premature form, as is the case for the COX2 subunit (Pratje *et al*. 1983) and PCF, a chimeric gene product related to CMS in petunia (Nivison *et al*. 1994). It must be noted that *in organello* synthesis might favor the obser-

vation of such nuclear effects by revealing transient products, such as premature polypeptide forms.

Searching for mitochondrial sterilizing factors

Despite extensive efforts, no candidate mitochondrial gene inducing *Owen* CMS in sugar beet has been isolated. Even though the two-dimensional electrophoresis used in our study provided a good representation of the protein mitochondrial expression and polymorphism between both cytoplasms (*Nvulg* and *Owen*), no variant could be strictly correlated to one mitochondrial genotype over all three nuclear backgrounds. Various explanations can be proposed:

1) the resolution of the acid-base electrophoresis was not sufficient to detect the expression of sterilizing factors, particularly when the differences between male and fertile phenotypes were caused by a quantitative variation in proteins;
2) *in organello* synthesis patterns may remain unmodified in the *Owen* CMS. In rice, duplicated and rearranged *atp6* related to CMS may only affect the transcript level (Iwabuchi et al. 1993; Akagi et al. 1994);
3) sterilizing factors may only occur in the reproductive tissues, as described in common bean in the CMS-associated *orf239* (Abad *et al*. 1995) and in sorghum for the *atp6* altered transcript (Howad and Kempken 1997). Moreover, Lind *et al*. (1991) reported differences between polypeptide patterns of mitochondria isolated from roots, leaves and flowers in beet plants that could not be related to the *Owen* CMS.

Recently, an original report by Kubo et al. (1999) described a tissue-specific modification in the transcript pattern of *coxI* within a single NIL pair between *Svulg* and a non-sterilizing mitotype in flower buds. Moreover, the transcript pattern of the flower buds from the restored plants was identical to the non-sterilizing cytoplasm. They consequently proposed *coxI* as a candidate gene for causing CMS. However, we do not know whether the modification observed on the 5′ untranslated sequence might have an effect on translated products or could be detected by acid-base two-dimensional electrophoresis. Nevertheless, our results indicate that the highest caution should be taken when studying mitochondrial gene expression and show that it should be assessed on a large scale of nuclear diversity.

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