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GENETIC TRANSFORMATION OF TOBACCO WITH orf522 MITOCHONDRIAL GENE FROM SUNFLOWER

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SUMMARY

Cytoplasmic male sterility (CMS) in sunflower has been correlated with the expression of orf522, a chimeric mitochondrial gene, generated by the insertion of exogenous genetic material, at 3' side of the atpA locus. In order to investigate the role of the orf522 in CMS, we constructed a recombinant plasmid, suitable for transforming tobacco plants by particle gun methodology. Several transformation events were obtained. Molecular analysis revealed that the orf522 gene is integrated into the nucleus. No integration events were observed in the mitochondrial (mt) genome. All the transgenic plants were fertile, suggesting that the orf522 from sunflower does not confer male sterility in transgenic tobacco when expressed at nuclear level.

Key words: *Helianthus annuus* L., cytoplasmic male sterility (CMS), particle bombardment, tobacco.

INTRODUCTION

Cytoplasmic male sterility (CMS) is caused by mutation(s) (alteration, rearrangement) of the mitochondrial genome (Bogorad, 1979; Hanson, 1991). They have been characterized in sunflower (Siculella and Palmer, 1988) as well as in several other species (Newton, 1988).

CMS plants show normal female fertility but they are not able to produce normal pollen (Laser and Lersten, 1972); the trait, as reported for plant organelles (Reboud and Zeyl, 1994), is maternally inherited. CMS is normaly used to obtain F_1 hybrid seed in such important crops as maize, rice, sorghum and sunflower (Kaul, 1988).

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In sunflower, several reports (Siculella and Palmer, 1988; Laver et al., 1991; Kohler et al., 1991) indicated that detectable alteration in the mitochondrial genome is limited to a 17 Kb region, and reflects as few as two mutations: a 12 Kb inversion and a 5 Kb insertion. One end point of both rearrangements is located within or near atpA, which is the only mitochondrial gene whose transcripts differ between the fertile and male sterile lines (Siculella and Palmer, 1988). Furthermore sequence analysis in the CMS 89 (D'Ambrosio et al., 1993), BASO (Horn et al., 1991) and RPA84A2 (Laver et al., 1991) sunflower male sterile lines, established that the insertion of exogenous genetic material at 3' side of the atpA locus generated a new gene of 522 bp, which is absent in the corresponding isonuclear fertile lines. Because of the heterogenous origin of its sequence, the orf522 has been considered a chimeric gene (D'Ambrosio et al., 1993).

Recently, the product (a 15 kDa protein) of this novel open reading frame (orf522) has demonstrated to be probably responsible for the CMS phenotype (Moneger et al., 1994; Smart et al., 1994).

In petunia and maize, CMS has been correlated with the expression of pcf and T-urf13 mitochondrial genes, respectively (Young and Hanson, 1987; Dewey et al., 1986).

In transformation experiments carried out on plant models the corresponding expression products have been driven in tobacco (Chaumont et al., 1995) and in petunia and tobacco (Wintz et al., 1995) mitochondria. Petunia and tobacco plants were transformed with constructs carrying T-urf13 or pcf genes, having at their 5' termini specific presenquences involved in the import in the mitochondrion of proteins encoded on nuclear genes. In every case no male sterile plants were obtained.

In order to transform directly the mitochondrial genome of a fertile plant and to study the modification(s) produced by the expression of the sunflower mitochondrial orf522 gene, the particle gun bombardment approach on a plant model (tobacco) were chosen. This species was chosen because, althought sunflower transformation has been reported (Everett et al., 1987; Schrammeijer et al., 1990; Knittel et al. 1994), there are several problem yet to regenerate easily the transformed plants.

MATERIAL AND METHODS

Construction of recombinant expression vector: The mtDNA region spanning atpA and orf522 genes is presented in Figure 1. The orf522 gene was inserted in the Xba I site of pRG2 plasmid using the following procedure. A DNA segment of about 1600 bp containing the orf522 gene was obtained by amplification of the sunflower mtDNA region bearing the gene (D'Ambrosio et al., 1993) using two primers having the following sequences:

5' TGCCTCTAGACAGAATTTCTCAATA 3'; 3' CTTCACTTGAGATCTGTTG 5'

identified on the sunflower mtDNA. They contain a single base pair change with respect to the Xba I site and were located 330 bp upstream the AUG codon and 740 bp downstream the TAA stop codon, respectively. After Xba I restriction digestion, the amplified product, obtained in presence of recombinant plasmid P91 as template, was cloned in the Xba I linearized pRG2 vector. Two recombinant clones, 53 and 59, were selected. The former contains the orf522 gene oriented in the same direction as 35S promoter. In the latter, the same regions had opposite directions. All the constructs were sequenced. For the biolistic experiments, a non recombinant pRG2 plasmid was utilized to examine the GUS activity.

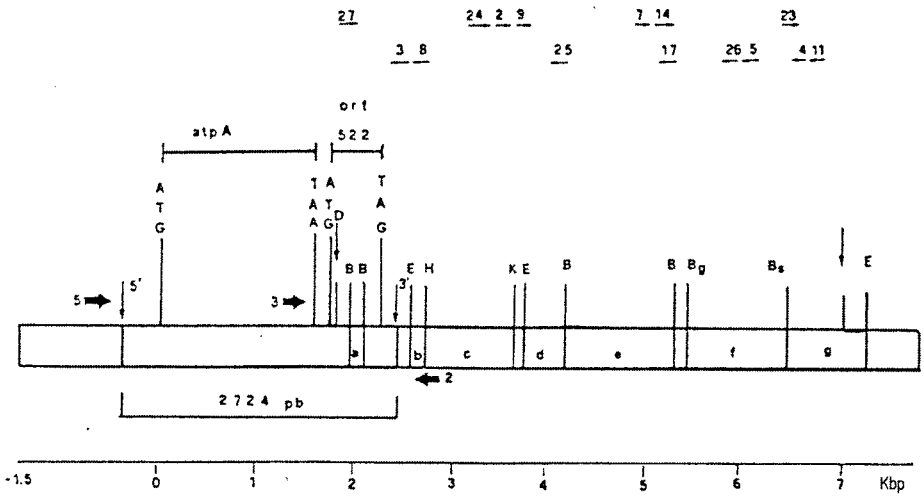

Figure 1: Schematic map of the CMS89 mtDNA region spanning the 5 kbp insert, the atpA and the orf522 genes (from D'Ambrosio et al., 1993)

Plant transformation: Plants were aseptically grown from seeds on MS0 medium containing MS salts + 100 mg/l myoinositol + 0.4 mg/l Thiamine + 30 g/l sucrose + 8.0 g/l agar, pH 5.7. Leaves were excised from plants and placed with abaxial side up on REG medium containing MS0 medium supplemented with 1.0 mg/l of NAA and 1.0 mg/l of BAP in Petri dishes. Plasmid DNA was purified by cesium chloride/etidium bromide density centrifugation. Plasmids were coated with 1 µm gold projectiles using calcium/spermidine precipitation as previously described.

DNA transfer was carried out using Biolistic TM particle delivery system, Du Pont PDS 1000/He Biolistic Gun (YE in Plant Mol. Biol. 15: 809-819, 1990). The

samples were placed at a distance of 6 cm between leaf and microprojectiles and at different pressures (1100, 1500 and 1800 p.s.i). Tissue was bombarded two times and then incubated at 25°C.

β-glucuronidase assays: Two days after bombardment, the leaves were assayed for GUS activity by adding a GUS hystochemical buffer (1mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 50mM sodium phosphate buffer (pH 7.0), 1mM EDTA and 3% Triton X-100) vacuumed for 1 minute and incubated at 37°C overnight in the dark.

Plant regeneration: Two days after bombardment the leaves were cut into sections and transferred to REG medium containing 100 mg/ml of kanamicin sulphate. Green calli which formed on the leaves after 2-3 weeks were subcultured onto the same selective medium. When the calli formed shoots, these were rooted on MS0 selective medium to obtain plants.

DNA isolation: Plant DNA was isolated from young leaves using the method reported by Dellaporta et al., 1983.

Polymerase Chain Reaction: To perform the PCR assay 50-100 ng of DNA was used. The primers position of the orf522 gene used are:

primer A (5' ATGGGGGGAATCCTTTAT 3') and primer B (5' GCACCTTCTAA-CATCTC 3'). The expected sizes of the amplified fragment was 550 bp. The reaction buffer contained: 500 mM KCl, 100 mM Tris-HCl; 15 mM MgCl2 and 0.1% gelatin. The concentration of the dNTPs used was 125 mM and primers were at 100 pMol each. 2.5 units of Taq polymerase (Boheringer Mannhein) were used.

The PCR cycle was: 5' at 95°C; 35 x (15" at 95°C, 1' at 60°C, 3' at 72°C); 10' at 72°C. PCR products (10 ul) were analyzed by electrophoresis.

Southern analysis: To perform Southern analysis 5 to 10 μg of genomic DNA was digested overnight with Xba I enzyme, electrophoresed on 0.8% agarose gel. The DNA was transferred by alkaline blotting to Hybond-N plus membrane (Amersham) as described by Maniatis et al., 1982.

The filter was probed with the 1600bp orf522 fragment obtained from PCR amplification of mtDNA as reported above. DNA probe was labelled with 32P (Amersham) by random primer DNA labelling kit (Boheringer Mannhein).

Mithocondrial DNA analysis: mtDNA was isolated from young green plants by the procedure of Wilson and Chourey (1984).

RESULTS AND DISCUSSION

The particle gun bombardment approach used in the experiments described in this paper was chosen for two consequent reasons:

i) the failure of the experiments in both tobacco and petunia plants with heterologous or homologous genes (Chaumont et al., 1995) suggested that the import in the mitochondria of a CMS related protein is not a sufficient event for the generation of this phenotypic trait;

ii) the expression of the same protein(s) into the mitochondria could, more likely, drive the protein on the specific site of the mitochondrion where it generates the phenotypic trait.

Since at our knowledge the only way to transform DNA segment directly into the mitochondria is the particle gun bombardment, this approach on tobacco plants has been used.

Several autonomous transformation events (~80) were obtained and evaluated for pollen and seed formation (Table 1).

Table 1: Transgenic tobacco plants obtained by particle bombardment and tested by PCR for orf522 presence

Pressure (p.s.i.)	N°expl.	N°calli	N°rooted plants	Viable pollen	PCR experiments			
					nDNA		mtDNA	
					T	P	T	P
1100	100	0	0	0	0	0	0	0
1500	100	60	75	75	15	15	75	0
1800	100	5	5	5	5	5	5	0

Note: T= tested, P= positive

All the transgenic plants have gone into flower with viable pollen production. Since a selective reliable protocol was used to obtain putative transgenic plants, only 20 out of 80 plants were tested by PCR and Southern experiments to verify the integration of transgene (Figure 2 and Figure 3) into the nuclei.

The results showed that all the plants screened were positive for orf522 mitochondrial gene presence (Table 1). The same couple of primers have amplified a 1100 bp fragment present both in transgenic and untransformed tobacco plant (Figure 2). Southern experiment with orf522 probe have not shown hybridization on the untransformed control (Figure 3). The sequence of primers have been compared to known tobacco genomic sequences (FASTA - Wisconsin) but no homology has been revealed. Sequencing experiments will carry out to study the origin and the function of 1100 bp fragment.

In order to investigate the presence of transgene at mitochondrial level, mtDNA was isolated from all the transgenic plants and PCR analysis showed in all cases the absence of the orf522 (Table 1).

These results all together demonstrated that the integration of the orf522 at nuclear level is possible using our experimental conditions. Nevertheless, this event does not affect either the mitochondrial protein syntesis pattern (data not shown), or the plant fertility. The hypothesis that the use of 1 mm projectiles and our specific experimental conditions made possible the cytoplasmic membrane penetration without lethal effect is very important. Nevertheless, no transfer of genetic material into the mitochondria was achieved for reasons which at the moment are completely unknown.

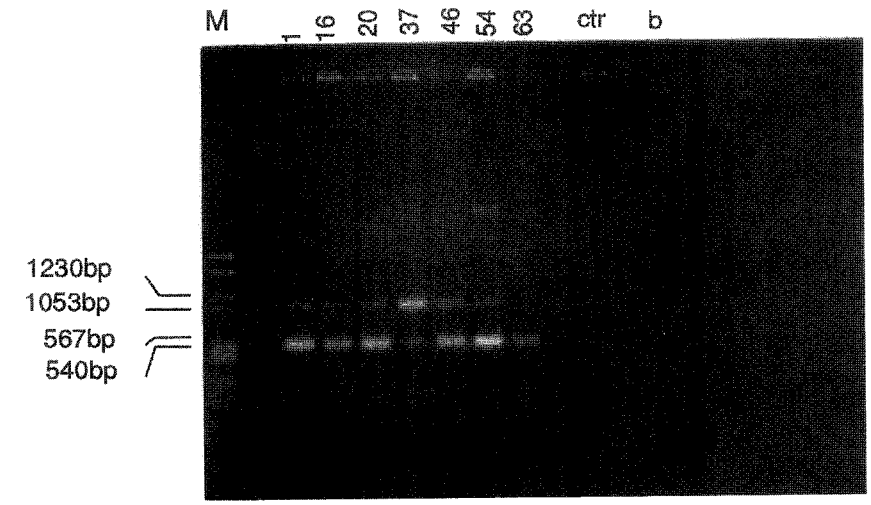

Figure 2 : PCR amplification from transformant tobacco DNA using Primers A and B. A 20th of PCR reaction was loaded on 1% agarose gel. The lane ctr contain untransformed tobacco DNA

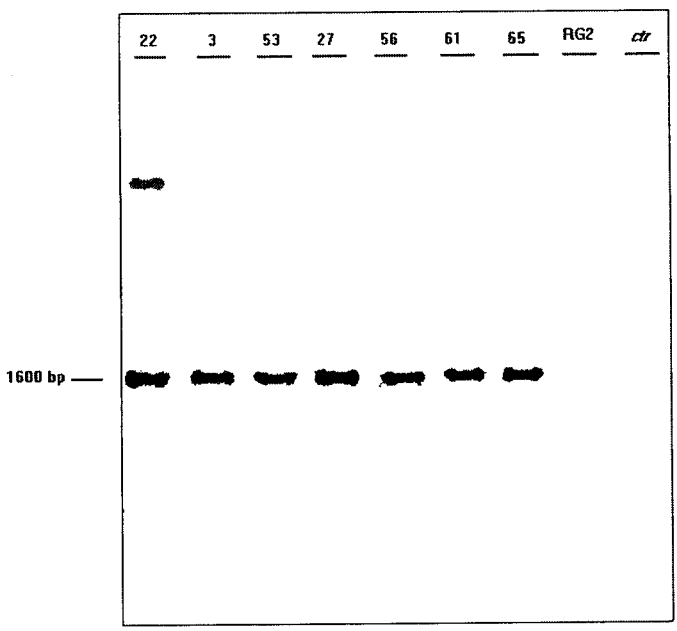

Figure 3 : Southern blot analisys of tobacco DNA. Total DNA extracted from transformant (22-65) and untransformant (ctr) lines was digested with Xba I

In order to improve the efficiency of delivering genetic material into intact mitochondria by particle gun, numerous parameters should be optimized:

particle size;

shape of the projectile;

distance between gun and tissue;

speed of projectile;

adsorbation of DNA to the particle.

We are currently investigating all these variants in our experiments, in order to optimize the direct transformation of tobacco plant mitochondrial genome.

On the other hand, since the molecular mechanism by which the specific sterility-associated mitochondrial genes cause male sterility, could be diversified in the various species, the unsuccessfull experiments made in tobacco and petunia do not prevent us from following the same strategy.

In order to transform tobacco and sunflower fertile plants, constructs carrying different promoters, targeting sequences and sunflower orf522 coding region, will use to demonstrate wheter and when the ORF522 protein acts to disrupt microsporogenesis.

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TRANSFORMACIÓN GENÉTICA DEL TABACO CON EL GEN MITOCONDRIA ORF522 DEL GIRASOL

RESUMEN

La androesterilidad citoplásmica (CMS) en girasol ha sido correlacionada con la expresión del gen quimérico mitocondrial orf522, generado por la inserción de material genético exógeno, en el lado 3' del locus atpA. Para investigar el papel del gen orf522 en la androesterilidad, se construyó un plasmido recombinante, adecuado para la transformación de plantas de tabaco por la metodologia del proyectil. Varias transformaciones fueron conseguidas. El análisis molecular reveló que el gen orf522 se integró en el núcleo. No se observó transformación en el genomio mitocondrial (mt). Todas las plantas transgénicas fueron fértiles, lo que sugiere que el gen orf522 del girasol no confiere androesterilidad en el tabaco transgénico cuando se expresa a nivel nuclear.

TRANSFORMATION GÉNÉTIQUE DU TABAC AVEC LE GÈNE MITOCHONDRIAL ORF522 DU TOURNESOL

RÉSUMÉ

La stérilité mâle cytoplasmique du tournesol (CMS) a été corrélée avec l'expression de l'orf522, un gène mitochondrial chimérique, résultant de l'insertion de matériel génétique exogène, à l'extrémité 3' du locus de l'atpA. Afin d'étudier le rôle de l'orf522 dans la CMS, on a construit un plasmide recombinant, adapté à la transformation des plantes de tabac par la méthode du canon à particules. Plusieurs cas de transformation ont été obtenus. L'analyse moléculaire a révélé que le gène orf522 était intégré dans le noyau. Aucun cas d'intégration n'a été observé dans le génome mitochondrial (mt). Toutes les plantes transgéniques étaient fertiles, suggérant que l'orf522 du tournesol ne confère pas la stérilité mâle chez le tabac lorsqu'il s'exprime au niveau du noyau.