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A mitochondrial dysfunction induces the expression of nuclear-encoded complex I genes in engineered male sterile *Arabidopsis thaliana*

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Abstract To study the effect of a mitochondrial dysfunction induced by the expression of the unedited form of the subunit 9 of ATP synthase gene (*u-atp9*) in *Arabidopsis*, we constructed transgenic plants expressing *u-atp9* under the control of three different promoters: CaMV 35S, apetala 3 and A9. The size and shape of transgenic plants bearing the *apetala3::u-atp9* and *A9::u-atp9* genes looked normal while the *35S::u-atp9* transformed plants showed a dwarf morphology. All *u-atp9* expressing plants, independent of the promoter used, exhibited a male sterile phenotype. Molecular analysis of male sterile plants revealed the induction of the mitochondrial nuclear complex I (nCI) genes, *psst*, *tyky* and *nadh binding protein* (*nadhbp*), associated with a mitochondrial dysfunction. These results support the hypothesis that the expression of *u-atp9* can induce male sterility and reveal that the *apetala3::u-atp9* and *A9::u-atp9* plants induced the sterile phenotype without affecting the vegetative development of *Arabidopsis* plants. Moreover, male sterile plants produced by this procedure are an interesting model to study the global changes generated by an engineered mitochondrial dysfunction at the transcriptome and proteome levels in *Arabidopsis* plants.

Key words: Male sterility; Plant mitochondrion; Nuclear complex I gene; *Arabidopsis thaliana*

1. Introduction

Previously, we reported the induction of male sterile phenotype in tobacco plants expressing a wheat unedited atp9 gene (*u-atp9*) [1]. The import of U-ATP9 protein into mitochondria induced mitochondrial dysfunction since a lower rate of respiration was observed correlating with a male sterile phenotype [1–3]. Moreover, the fertility of male sterile lines was restored by crossing with transgenic tobacco plants expressing an antisense *u-atp9* gene used as male parents [4]. Tobacco plants expressing *u-atp9* gene revealed abnormal sporogenesis, whereas vegetative development was less or not affected. These results suggest that full mitochondrial function is required in anther for normal sporogenesis and that RNA editing of mitochondrial mRNAs is necessary to obtain fully active proteins within the organelles [1,2,4].

Mitochondria are generally thought to have arisen from an intracellular bacterial symbiont of an ancestral eukaryotic cell [5]. After that, most of the genetic information from the mitochondrial genome was transferred to the nuclear genome. During evolution, the mitochondrial genome has been reduced and now encodes genes for a restricted number of proteins [6,7]. Most of them are critical components of the electron transport complexes.

The nuclear genes coding for the respiratory complex I (CI) subunits in plant mitochondria are of special interest since they contribute to the assembling of mitochondrial CI. It has been demonstrated that this complex is the major shuttling route of electrons from reduced intermediates produced in the Krebs cycle to the ubiquinone pool. This function is essential for normal respiratory function and efficient ATP generation during plant development [8]. Moreover, all the genes needed for the biogenesis, maintenance and regulation of mitochondria are, at the present, encoded in the nucleus [7]. This situation suggests that a coordinate expression of nuclear and organellar genomes is required for normal plant development.

With the aim to study the nucleo-mitochondrial interplay, we decided to analyze the consequences of a mitochondrial dysfunction on the nuclear-encoded mitochondrial genes required for organelle biogenesis. In fact, our knowledge of the regulation of nuclear-encoded mitochondrial proteins in plants is scarce in contrast with the well-documented mammalian and yeast systems [9,10]. In particular, little is known about the interorganellar signals that induce transcription during flower formation and differentiation.

In the present work, we report the effect of the expression of a wheat unedited gene in *Arabidopsis* using three different promoters: the constitutive CaMV 35S and the tissue specific promoters apetala 3 (ap3) and A9. These three constructs render a male sterile phenotype when expressed in *Arabidopsis* plants. The specific expression of the transgene in floral organs under the control of ap3 and A9 promoters does not affect the vegetative development.

Our results provide evidence that this model may be useful

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to develop male sterile lines to be used for creating new hybrid lines. Moreover, the mitochondrial dysfunction model proposed here might be useful to better understand the nucleo-mitochondrial signalling and consequently, the role of mitochondria during sporogenesis and plant development. Indeed, we show that in *u-atp9* expressing plants, the expression of nCI genes is strongly affected.

2. Materials and methods

2.1. Plant material and plant transformation

Arabidopsis thaliana (var. Col-0) plants were used in these experiments. The plants were transformed with *Agrobacterium tumefaciens* as previously described [11]. The plants were cultivated in a greenhouse. Selection of transgenic plants was performed in Murashige–Skoog agar medium (Sigma Chemical Co.) containing 30 μg/ml of kanamycin. Plant transformation was carried out using the *u-atp9* gene from wheat, which only shows a few differences in amino acids with respect to the *Arabidopsis* ATP9 protein (Fig. 1).

2.2. Vectors

A *Hin*dIII–*Xba*I fragment containing 930 bp of the *A. thaliana* anther specific promoter was obtained from plasmid pWP80 (a gift of Dr. P. Wyatt, Leicester University, UK). 1.8 kb of ap3 promoter was obtained by polymerase chain reaction (PCR) on *A. thaliana* total DNA using primers AP3a: TCTTAAGAATTATAGTAGCAC and AP3c: GTTGAAGAGATTTTGTGGAGA. The PCR product was cloned in the pTBlue vector (Novagen). The *u-atp9* constructs driven by A9 and ap3 promoters were obtained by replacing the CaMV 35S promoter in plasmid pH2 [1] by either ap3 or A9 sequences. The promoter–*u-atp9*–terminator cassettes were then transferred to pPZP212 vector to agrotransformation of *Arabidopsis* plants. The plasmids pAGB 101, pAGBM 401 and pAGBM 001 containing the CaMV 35S, A9 and ap3 promoters respectively were used in this work. Along with the sense construct, a series of antisense expressing *u-atp9* genes under the control of the different promoters were obtained.

2.3. Pollen germination

To evaluate the fertility of the different lines, we determined the ability of the pollen grains to germinate. For the wild-type plants, pollen was collected from up to five just opened flowers. In contrast, the flowers of male sterile plants remain closed. Therefore, to analyze the pollen in these plants, we collected the flourish at the same period of flower opening than the wild-type plants.

2.4. DNA isolation

DNA was extracted from 20–40 mg of plant tissues using the CTAB method [12].

2.5. RNA isolation, reverse transcriptase (RT)-PCR analysis and blotting

Total RNA was extracted from leaves and flowers using the RNA plant mini kit (Qiagen). cDNA was synthesized on poly(A)+ RNA with an oligo(dT) primer using superscript reverse transcriptase (Life Technologies). cDNA was submitted to 20 cycles of PCR amplification using the following primers: *eif4A*: 5′-TGTTTGATATTCAGAAATTCTAC-3′, 5′-ATACTCCATATGACTTAAACTTA-3′; *psst*: 5′-GCCTAGTCCTCGCCAGTCTG-3′, 5′-GCTGGAGTAGTCCATAGAGC-3′; *tyky*: 5′-GATTTTGGCTCGCAGGTCACTG-3′, 5′-GCGGACCATTTCTGTGAGGAAC-3′; *nadhbp*: 5′-GAAAGCTGTGGGCAGTGCAC-3′, 5′-TCTCGAGCTCTGGCCTAAAG-3′; *u-atp9*: 5′-CACTACGTCAATCTATAAGA-3′, 5′-CGATCGGAAAACGAATGAGATCAG-3′. The PCR products were electrophoresed on 1.5% agarose gels and transferred onto Hybond N+ membranes (Amersham Pharmacia). Blots were prehybridized for 1 h at 65°C in 6× SSPE (1.08 M NaCl, 0.06 M Na_2HPO_4, 6 mM ethylenediamine tetraacetic acid (EDTA), pH 7.7), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml denatured DNA from calf thymus. Hybridization was carried out in the same solution for 18 h containing the [^{32}P]-labeled probes. The radioactive probes *u-atp9*, *tyky*, *psst, nadhbp* and the control *eif4A* were synthesized using [^{32}P]CTP (ready-to-go labeling beads (-dCTP) (Amersham Pharmacia). After hybridization, the membranes were washed three times with 2× SSPE, 0.1% SDS at 60°C for 30 min and then three times with 0.2× SSPE, 0.1% SDS, at room temperature. After that, the membranes were dried and exposed to X-ray films.

3. Results

3.1. Transgenic lines of A. thaliana

Plants were transformed with recombinant pPZP212 plasmids [13] carrying the *u-atp9* gene under the control of the following promoters: 35S from CaMV, ap3 and A9. Mitochondrial targeting was accomplished by adding a specific target transit peptide, the yeast cytochrome oxidase subunit 4 (COXIV) presequence, to the *u-atp9* coding region [1]. The wheat and *Arabidopsis atp9* genes are very similar with a few differences in amino acids (Fig. 1). Wheat mitochondrial *atp9* mRNA undergoes eight editing C–U transitions to produce a functional protein containing five different residues compared with the predicted protein from the gene sequence. One editing event creates a stop codon that eliminates six C-terminal residues of the predicted protein [14]. The *Arabidopsis* gene is very similar to the wheat counterpart with four differences in residues at positions 17, 22, 28 and 71; the stop codon at position 75 is already present in the gene sequence and thus is not created by editing in this plant. The expression of a transgenic wheat *u-atp9* in *Arabidopsis* should generate a protein that differs by five internal amino acids and an extension of six C-terminal residues to the endogenous *A. thaliana* mitochondrial ATP9 protein. In transgenic tobacco plants, the expression of *u-atp9* induces a mitochondrial dysfunction [1]. We decided to use this approach to create male sterile lines in *Arabidopsis* excepting that we included the tissue specific promoters ap3 and A9 besides the CaMV 35S promoter.

3.2. The expression of u-atp9 gene leads to male sterile phenotypes

The phenotypic characteristics of the transformed plants were analyzed. The size, number and shape of different plant organs, especially the floral organs and the pollen grains, were determined after 30 days of development. The sizes of the *apetala3::u-atp9* and *A9::u-atp9* transformed plants (18.0 ± 2.5 cm and 18.3 ± 3.5 cm, respectively) were very similar to the wild-type (18.4 ± 3.0 cm). However, the size of the *35S::u-atp9* bearing plants was significantly affected showing

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                          10        20        30        40        50        60        70
wheat u-atp9 MLEGAKSIGAGAATIALAGAAVGIGNVLSSLIHSVARNPSLAKQSFGYAILGFALTEAIALFAPMMAFLISFVFRSHKKS
A.t. e-atp9  MLEGAKLIGAGAATIALAGAAIGIGNVFSSLIHSVARNPSLAKQLFGYAILGFALTEAIALFALMMAFLILFVF
A.t. u-atp9  MLEGAKSIGAGAATIASAGAAIGIGNVFSSLIHSVARNPSLAKQSFGYAILGFALTEAIALFAPMMAFLILFVF
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Fig. 1. Alignment of *atp9* sequences from wheat unedited *atp9*, edited *Arabidopsis atp9*, and *Arabidopsis* unedited *atp9*. Underlined residues represent residues that are changed by editing process of the transcript.

a dwarf phenotype (3.9 ± 1.5 cm, Fig. 2). Furthermore, there were no significative differences in wild-type, *apetala3::u-atp9* and *A9::u-atp9* plants in the number of leaves (19 ± 3, 19 ± 3 and 20 ± 5, leaves/plant respectively) or flowers (26 ± 4, 27 ± 5 and 27 ± 9 flowers/plant, respectively). In contrast, the size of the *35S::u-atp9* plants was strongly affected; the morphology of the leaves was normal but their number (9 ± 2 leaves/plant) and also the number of flowers per plant (7 ± 3) were drastically reduced.

Transgenic *Arabidopsis* plants were defective in flowering and fructification. Plants carrying the *apetala3::u-atp9* gene did not show differences in the vegetative development (Fig. 2). By contrast, they showed small and brown anthers and the petals were less developed than the wild-type (Fig. 3). Moreover, fructification was aborted whereas the morphology of the pistils was unaffected except that they were longer than the wild-type.

The transgenic *A9::u-atp9* plants were defective in anther development presenting brown coloration and a smaller size than the wild-type (Fig. 3). The morphology of the female organ was not affected but the pistils showed longer size as observed for *apetala3::u-atp9* plants. In this line, the size and shape of the petals were also affected (Fig. 3).

The morphology, color, size and shape of anthers, pistils and petals of *35S::u-atp9* plants were affected (Fig. 3).

In all three transgenic lines presented here, the morphology of the pollen grains was abnormal, with differences in color, shape and size (Fig. 4).

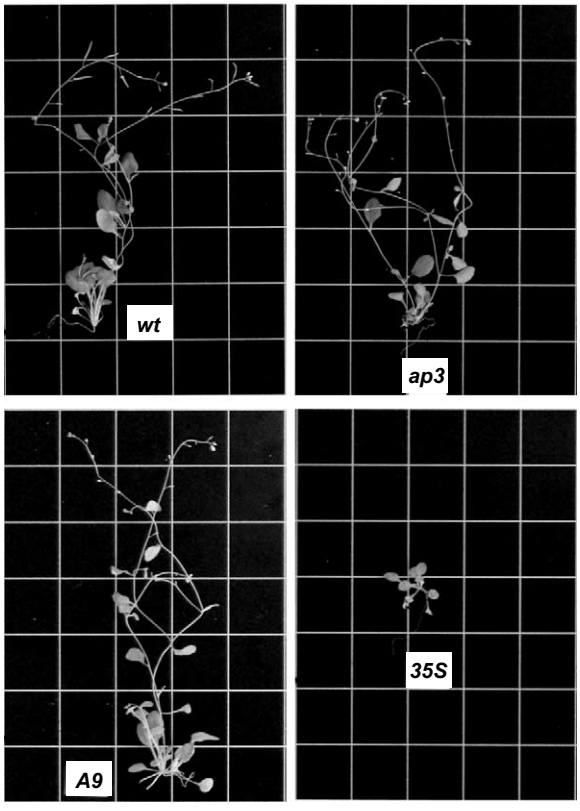

Fig. 2. Plants expressing *u-atp9* under the control of three different promoters. Transgenic and wild-type *Arabidopsis* plants were photographed at 30 days of development during blossom. wt, wild-type; ap3, apetala 3 promoter driven *u-atp9* plant; A9, tapetum specific A9 promoter driven *u-atp9* plant; 35S, CaMV 35S promoter driven *u-atp9* plant.

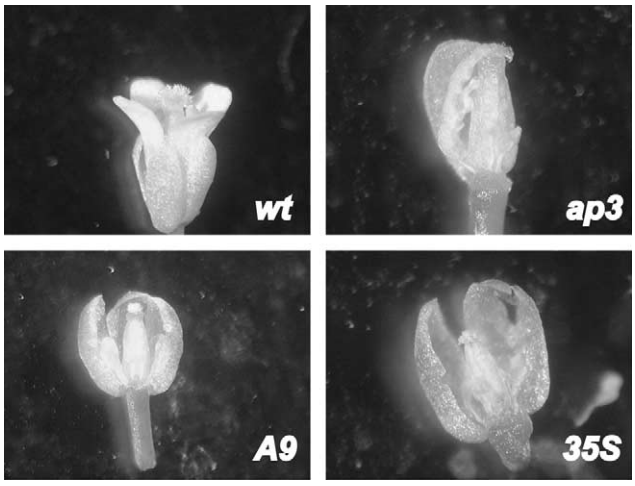

Fig. 3. Flowers from transgenic plants expressing *u-atp9*. Flowers from plants carrying *apetala3::u-atp9*, *A9::u-atp9* and *CaMV35S::u-atp9* genes at the same developmental stage were photographed under binocular and compared to wild-type flowers.

To evaluate male fertility, we analyzed the ability of pollen grains to germinate after 2–4 h in the germination media. More than 95% of the pollen grains from wild-type plants germinated, whereas the *35S::u-atp9*, *apetala3::u-atp9* and *A9::u-atp9* pollen presented only <1, <1 and 5–10% of germination, respectively.

The female fertility of the male sterile plants was estimated by measuring seed production after backcrossing with wild-type plants as the male parent. All backcrossed *apetala3* and *A9::u-atp9* plants were female fertile, whereas only 35% of the *35S::u-atp9* were female fertile.

3.3. Molecular analysis of the male sterile plants

To determine the presence of the *u-atp9* transgene, Southern blot analyses on PCR products from total DNA were performed with a [^{32}P]-*u-atp9* probe. Two primers located at

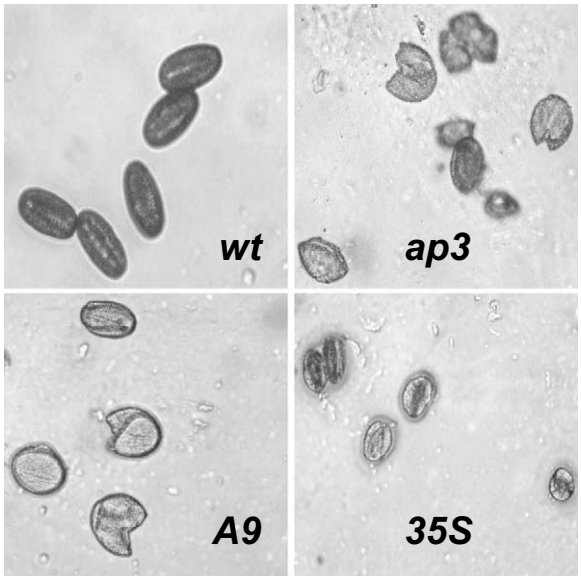

Fig. 4. Pollen grains from transgenic plants expressing *u-atp9*. Pollen grains were extracted from anthers of wt, *apetala3::u-atp9*, *A9::u-atp9* and *CaMV35S::u-atp9 Arabidopsis* plants and visualized under light microscope.

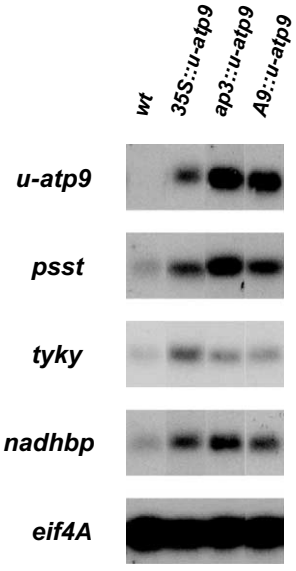

Fig. 5. Plants expressing *u-atp9* under different promoters show increases in steady-state of nCI mRNAs. 1 μg of total RNA from young flowers was reverse transcribed using oligo(dT) primer and then amplified with specific primers (see Section 2). Samples were separated by electrophoresis and blotted onto membranes. Membranes were hybridized with specific probes corresponding to *u-atp9* and the *psst*, *tyky*, *nadhbp* nCI genes. The housekeeping gene *eif4A* was used as a control.

the COXIV presequence and the 3′ of the transgene in the NOS terminator region were used. These primers specifically amplify the transgene but not the mitochondrial *atp9* gene. All the transgenic plants tested carried the *u-atp9* gene, whereas the untransformed wild-type plants gave no signal (data not shown).

3.4. Transgenic plants show increased expression of nCI genes

To evaluate whether nCI genes respond to the induced mitochondrial dysfunction, we analyzed the expression of *psst*, *tyky* and *nadhbp* genes in flowers of plants expressing *u-atp9* by semiquantitative RT-PCR. We used flowers at an early stage of development because of the precocious expression of these genes [15], and to avoid the degenerative changes at the late stage of flower development observed in *u-atp9* transgenic plants.

A high level expression of *u-atp9* was found in flowers of plants transformed with the transgene controlled by the A9 and ap3 promoters (Fig. 5, lanes 3 and 4). Unexpectedly, lower expression levels were detected in transgenic plants in which *u-atp9* was controlled by the constitutive 35S driven promoter. One possibility is that premature degeneration of flower tissues is responsible for this situation. However, the expression of the housekeeping gene *eif4A* indicates that this is not the case and suggests that CaMV is poorly expressed in flower tissues in *Arabidopsis*. Interestingly, three nCI genes, *psst*, *nadhbp* and at a lesser extent *tyky*, are strongly expressed in male sterile plants (Fig. 5). It should be noted that in all cases, the expression of *eif4A* was not affected indicating that the signal obtained represents the steady-state abundance of the target transcripts.

4. Discussion

The effect of the specific expression of the *u-atp9* gene in floral organs using ap3 and A9 promoters was studied along with control plants bearing *u-atp9* driven by the CaMV 35S constitutive promoter. Previously, we described the induction of a male sterile trait and their restoration to fertility in tobacco plants expressing *35S::u-atp9* [1,4].

The unedited *atp9* transcript codes for a protein with 80 residues. The edited mRNA codes for a more hydrophobic protein differing in five amino acids and that is shortened by six residues producing a different C-terminus [14,16] (Fig. 1).

Phenotypic analysis of the *apetala3::u-atp9* and *A9::u-atp9* plants indicates that transgene expression had no effect on plant growth but they show dramatic changes in the morphology of floral organs. Specific *u-atp9* expression under the control of A9 or ap3 promoters leads to pollen abortion and induction of a male sterile phenotype as expected [17]. We also observed phenotypic effects in petals of *A9::u-atp9* expressing plants. This result indicates that the 930 bp fragment containing the A9 promoter used was able to drive the expression of the transgene in other tissues besides tapetum.

The expression of *u-atp9* seems to have light or no effects on the major phenotypic characters, but a dramatic effect on the male reproductive organs. In contrast, *35S::u-atp9* expression showed a remarkable effect in the size of plants. This severe effect on plant morphology was not observed previously on transformed tobacco plants [1,2]. Although the reason is not clear, this situation might reflect the different nuclear background of *Arabidopsis* (diploid) and *Nicotiana tabacum* (allotetraploid).

The effect in male fertility trait was due to the transgene expression since the backcrossing of male sterile lines with wild-type pollen produced seeds in almost all plants tested. We postulate that a mitochondrial flaw could be sensed by the nuclear genome, and affected the expression of nuclear-encoded mitochondrial genes. To test this hypothesis, we analyzed the expression of some of the nCI genes, *tyky*, *psst* and *nadhbp*. It was reported that the steady-state levels of the transcripts from nCI genes are 6–10 times higher in flowers compared to leaves or roots [18,19]. Moreover, the expression of antisense *nadhbp* gene induced a male sterile phenotype in transgenic potato [20] suggesting the importance of the mitochondrial function during sporogenesis.

Recently, an essential region for anther pollen specific expression has been reported in the promoters of the nCI *psst*, *tyky* and *nadhb* genes [15]. This region might respond to physiological demands of the mitochondria during sporogenesis [15]. In anthers, particularly in tapetal cells, an intense mitochondrial activity occurs during sporogenesis, one of the major energy consuming processes in the whole plant. The results shown in this work indicated that the tissue specific expression of *u-atp9* is suitable to induce a male sterile phenotype. In fact, the use of a mitochondrial dysfunction should be carefully controlled to avoid secondary effects on the whole plant, thus justifying the choice of specific promoters. Moreover, these results revealed the consequences of the existence of a mitochondrial dysfunction. Interestingly, we found an induction of three nuclear-encoded constituents of the electron transport complex I in male sterile plants. We interpret these results as a compensatory response of the nucleus, which might sense the affected mitochondria reacting by increasing mitochondriogenesis.

It should be interesting to extend these studies to other

nuclear-encoded mitochondrial genes to ascertain if we are observing a general response in male sterile plants or it concerns only some nCI genes. The analysis of nCI promoters to search for specific sequences responsible of the sensing response is also in progress.

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