

Research paper

Nuclear-encoded mitochondrial complex I gene expression is restored to normal levels by inhibition of unedited *ATP9* transgene expression in *Arabidopsis thaliana*

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Abstract

Mitochondria play an important role during sporogenesis in plants. The steady state levels of the nuclear-encoded mitochondrial complex I (nCI), *PSST*, *TYKY* and *NADHBP* transcripts increase in flowers of male-sterile plants with impairment of mitochondrial function generated by the expression of the unedited version of *ATP9* (*u-ATP9*). This suggests a nuclear control of nCI genes in response to the mitochondrial flaw. To evaluate this hypothesis, transgenic plants carrying the GUS reporter gene, under the control of the *PSST*, *TYKY* and *NADHBP* promoters, were constructed. We present evidence that suppression by antisense strategy of the expression of *u-ATP9* restores the normal levels of three nCI transcripts, indicating that the increase in *PSST*, *TYKY* and *NADHBP* in plants with a mitochondrial flaw occurs at the transcriptional level. The data presented here support the hypothesis that a mitochondrial dysfunction triggers a retrograde signaling which induce some nuclear-encoded mitochondrial genes. Moreover, these results demonstrate that this is a valuable experimental model for studying nucleus–mitochondria cross-talk events.

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1. Introduction

Mitochondria are involved in different cellular processes, such as energy metabolism and respiration [10,21]. Sporogenesis, one of the most important processes in higher plants, requires a proper functioning of mitochondria. In fact, mutations in the mitochondrial genome affect the normal development of spores leading to a male-sterile phenotype [25]. However, little is known about the molecular mechanisms involved in mitochondrial pollen disruption. Previously, it has been reported

that a male-sterile phenotype can be induced in tobacco and *Arabidopsis* by expressing a wheat unedited *ATP9* gene (*u-ATP9*), fused to a gene fragment encoding a transit peptide of yeast *COXIV*. The expression of *u-ATP9* causes a mitochondrial dysfunction characterized by lower rates of respiration [9,13]. The expression of *u-ATP9* in *Arabidopsis* under the control of *CaMV35S* promoter (lines *CaMV35S::u-ATP9*) leads to abnormalities in vegetative development, while its specific floral expression when controlled by *APETALA3* promoter (lines *AP3::u-ATP9*), specific of petals and stamens [14] or the *A9* promoter (lines *A9::u-ATP9*) specific of tapetum [20], have little or no bearing on the major phenotypical characters, but a dramatic effect on the male reproductive organs [9]. This observation could be explained by an intense mitochondrial activity occurring in tapetal cells to support sporogenesis where any

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mitochondrial dysfunction disrupts pollen development, and is thus one of the major energy consuming processes in the whole plant [11,27]. Because cells can monitor and respond to changes in the state of their organelles, we postulate that a mitochondrial dysfunction could be sensed by the nuclear genome thus affecting the expression of nuclear-encoded mitochondrial genes. If this idea is correct, inhibition of the deleterious *u-ATP9* gene expression should restore the normal sporogenic function in male sterile plants. In the present work we report the specific restoration to fertility of the *AP3::u-ATP9* and *A9::u-ATP9* lines using *ATP9* antisense transcript (*AS-u-ATP9*) under the control of either *AP3* or *A9* promoters, respectively. This experimental model might be useful in developing a better understanding of nucleus–mitochondrial cross-talk, in particular the transcriptional events occurring during flower formation and differentiation, and also in assessing the role of mitochondria during different cellular processes such as sporogenesis. We further analyze the effect of restoring the mitochondrial dysfunction on the expression of three intrinsic subunits of *Arabidopsis thaliana* nuclear-encoded Complex I (nCI), NADH-ubiquinone oxidoreductase genes: the 55 kDa subunit NADH-binding protein (*NADHBP*) (At5g08530), *PSST* of 22 kDa (At5g11770) and *TYKY* of 28 kDa (At1g79010).

2. Results

2.1. Phenotypic analysis of plants carrying *AS-u-ATP9* constructs

Previously, we reported that the expression of *u-ATP9* gene induces a mitochondrial dysfunction in *Arabidopsis* leading to a male-sterile phenotype [9]. To determine whether this phenotype was indeed the effect of *u-ATP9* expression, we constructed different *Arabidopsis* lines by transformation with recombinant pZP212 plasmids, containing the antisense version of the *u-ATP9* transgene driven by three different promoters, *CaMV35S*, *A9* and *AP3* (Fig. 1). Three transgenic lines, *CAMV35S::AS-u-ATP9*, *AP3::ASu-ATP9* and *A9::AS-u-ATP9* plants were generated after transformation with *A. tumefaciens*. The presence of the transgene in transformed plants was verified by PCR analyses and the expression of the antisense gene was determined by RT-PCR. All *AS-u-ATP9* expressing plants were fertile and the vegetative and reproductive organs were normal (size, number and shape). Pollen grains were compared

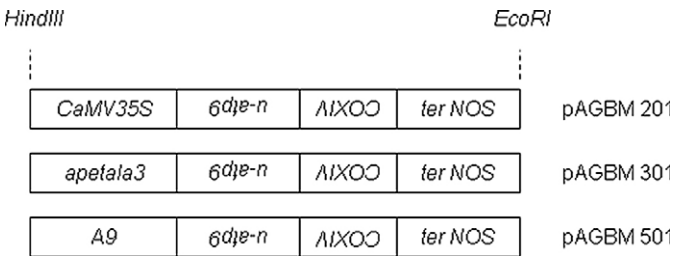


Fig. 1. Structure of the chimeric constructs of *u-ATP9* fused to *COXIV* pre-sequence. The expression of the antisense version of *u-ATP9* was driven by *CaMV35S*, *A9* and *AP3* promoters (pAGBM 201, 501 and 301, respectively).

to wild type plants and no significant differences were observed. *AS-u-ATP9* expression levels were determined by RT-PCR. Then, only plants showing higher levels of expression were used in crosses with male-sterile *u-ATP9* plants. The progeny of plants carrying both sense and antisense constructs (*AP3::u-ATP9* *x* *AP3::AS-u-ATP9* and *A9::u-ATP9* *x* *A9::AS-u-ATP9*) were identified by PCR using the primers TNOS, *u-atp9* and *u-atp9r* (see Section 4). In hybrid plants, the *u-ATP9* transcripts were not detected after RT-PCR (36 cycles), indicating that antisense specific inhibition of *u-ATP9* expression was successful (data not shown).

2.2. Expression of *AS-u-ATP9* gene restores pollen morphology and fertility

Pollen grains from *u-ATP9* plants showed an abnormal morphology, with differences in color, shape and size, and were deficient in germination with less than 1% of viable pollen (Table 1); while *AS-u-ATP9* expressing plants showed normal levels of pollen viability. Pollen grains of *AS-u-ATP9* plants under the control of either *AP3* or *A9* promoters were used to pollinate *u-ATP9* male-sterile plants. To maximize the chance of an antisense effect, the lines used in crosses expressed the sense and antisense transgenes under control of the same promoter. To evaluate the effects of the expression of *AS-u-ATP9* transgene in *u-ATP9* plants, the morphology, and the ability of pollen grains to germinate as well as the plants' capacity for self-pollination were analyzed (Fig. 2 and Table 1). Pollen grains from hybrid lines (*AP3::u-ATP9* *x* *AP3::AS-u-ATP9* and

Table 1
Restoration to fertility after crossing of *u-ATP9* with *AS-u-ATP9* lines

Line	Germinated pollen (%)
Wt	92 ± 4
<i>AP3::u-ATP9</i>	< 1
<i>A9::u-ATP9</i>	< 1
<i>AP3:: u-ATP9</i> <i>x</i> <i>AP3:: AS-u-ATP9</i>	86 ± 5
<i>A9::u-ATP9</i> <i>x</i> <i>A9::AS-u-ATP9</i>	82 ± 8

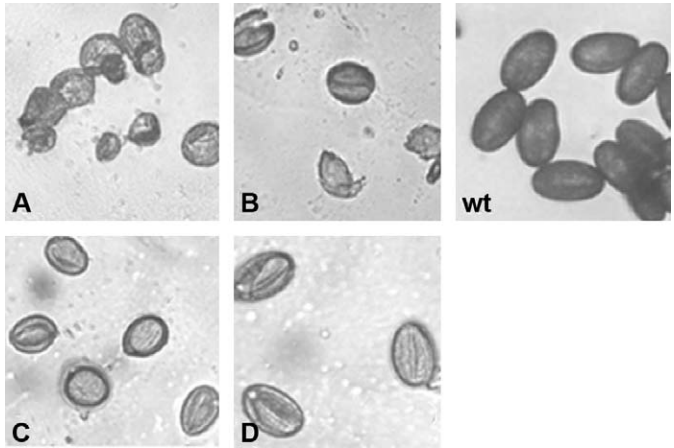


Fig. 2. Pollen grains from transgenic plants expressing *u-ATP9* under the control of *AP3*, and *A9* promoters (A and B, respectively) compared to wt. Figures C and D corresponds to pollen grains extracted from anthers of crossed lines (*AP3::u-ATP9* *x* *AP3::AS-u-ATP9* and *A9::u-ATP9* *x* *A9::AS-u-ATP9*, respectively).

A9::u-ATP9 xA9::AS-u-ATP9) were undistinguishable compared to those of wild-type ones. Indeed, while pollen from male-sterile, *AP3::u-ATP9* and *A9::u-ATP9* plants presented less than 1% germinated pollen grains, $86 \pm 5\%$ and $82 \pm 8\%$ of the spores from *AP3::u-ATP9 xAP3::AS-u-ATP9* and *A9::u-ATP9 xA9::AS-u-ATP9* lines germinated after 4 h of incubation, respectively. This result indicates that in these plants, the expression of the antisense transgene restores both the morphology and the functionality of pollen grains (Fig. 2 and Table 1).

2.3. Antisense *u-ATP9* restores normal nCI gene expression in *A. thaliana*

To evaluate the effect of *AS-u-ATP9* gene expression, the transcription levels of three nCI genes *PSST*, *NADHBP* and *TYKY* were analyzed in flowers from hybrid plants (*AP3::u-ATP9 xAP3::AS-u-ATP9* and *A9::u-ATP9 xA9::AS-u-ATP9*) compared to male sterile (*AP3::u-ATP9* and *A9::u-ATP9*) and wild type plants. Interestingly, in hybrid lines, the *PSST*, *TYKY* and *NADHBP* transcripts showed similar levels compared to wild type plants, either in *AP3* or *A9* promoter driven transgenes (Fig. 3, lanes 4 and 5, respectively). Control plants expressing *AS-u-ATP9* under the control of *AP3* or *A9* promoters (and also *CaMV35S*) did not show any difference compared with wild type (data not shown). These results demonstrate that the inhibition of *u-ATP9* transcript leads to the recovery of normal nCI expression.

2.4. Unedited-*ATP9* gene expression causes transcriptional induction of nCI genes

To investigate whether the variation of transcript levels of *PSST*, *TYKY* and *NADHBP* observed in *u-ATP9* and in *AP3::u-ATP9 xAP3::AS-u-ATP9* and *A9::u-ATP9 xA9::AS-u-ATP9* plants was the result of a transcriptional regulation process, quantitative analysis of *GUS* expression was performed in flowers of plants where the *GUS* reporter gene was placed under the control of the *PSST*, *TYKY* or *NADHBP* promoters (see Section 4). The activity of *GUS* in *PSST::GUS*, *TYKY::GUS*

and *NADHBP::GUS* plants were used as a control in each case (Fig. 4A–C) [28]. Indeed, hemizygous lines obtained by crossing the two ecotypes (Col-0 and C24) showed comparable transcription pattern, indicating that there are no effects on the promoter activities (data not shown). The *GUS* expressing plants were crossed with either *AP3::u-ATP9* or *A9::u-ATP9* male-sterile plants and the restored *u-ATP9::AS-u-ATP9* lines. All hybrid progeny gave male-sterile plants as expected, except when crossing were done with *u-ATP9::AS-u-ATP9* lines (fertile progeny). Flowers of *AP3::u-ATP9 xPSST::GUS*, *AP3::u-ATP9 xTYKY::GUS* and *AP3::u-ATP9 xNADHBP::GUS* hybrid lines were homogenized and assayed for *GUS* activity. The glucuronidase activity of *PSST::GUS*, *TYKY::GUS* and *NADHBP::GUS* lines was used as a reference value to evaluate the expression level of hybrid offspring. The same analysis was performed with the hybrids carrying the *A9::u-ATP9* construct. No significant changes were observed in *GUS* activity for the different *GUS::u-atp9::AS-u-ATP9* fertile lines (Fig. 4). Whereas, in all cases, plants containing *u-ATP9* showed 1.5–2.5-fold increased *GUS* activity (Fig. 4) indicating that the increase in these transcripts indeed operates at the transcriptional level.

3. Discussion

Cells modulate the expression of nuclear genes in response to alterations in mitochondrial function, a response termed retrograde regulation [17,19]. *Arabidopsis* plants expressing *u-ATP9* were found to exhibit a male-sterile phenotype associated to mitochondrial dysfunction. At the molecular level, these plants presented increased expression of three nuclear-encoded mitochondrial nCI genes [9] and other nuclear genes involved in mitochondrial biogenesis [1]. The male-sterile plants were restored to fertility using antisense technology. The restored lines presented a normal steady-state level for the three nuclear-encoded nCI transcripts suggesting that up-regulation of some nCI genes is triggered by mitochondrial dysfunction.

The nature of the retrograde signal and the corresponding factors involved is at present largely unknown in plants. Recently, Dojcinovic et al. [3] reported the induction of nuclear encoded alternative oxidase after the inhibition of the mitochondrial electron transport chain and identify a region in the *AOX* promoter necessary for mitochondrial retrograde regulation of expression. Signaling from mitochondria to the nucleus in yeast and animals usually involves metabolites and is likely associated with multiple signal transduction pathways [7,18].

Yeast mutants, depleted of mtDNA (*rho*), showed induction of several genes mediated by *RTG* genes which are central players in retrograde regulation [7,15]. In these mutants, proteins involved in peroxysomal activities and the anaplerotic pathways are induced, but a large proportion of down-regulated genes encode mitochondrial proteins of the oxidative phosphorylation apparatus. These data imply that yeast “petites” do not compensate for the respiratory-deficient state by up-regulating the expression of oxidative phosphorylation genes. This latter observation contrasts with the fact that some

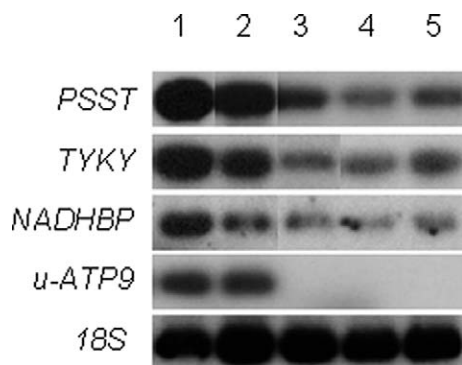


Fig. 3. Steady state levels of mRNA of nCI genes and *u-ATP9* in *Arabidopsis* plants: lane 1, *AP3::u-ATP9*; lane 2, *A9::u-ATP9*; lane 3, wild type; lane 4, *AP3::u-ATP9 xAP3::AS-u-ATP9*; lane 5, *A9::u-ATP9 xA9::AS-u-ATP9*. Total RNA from flowers was used. The housekeeping gene rRNA *18S* was used as internal control.

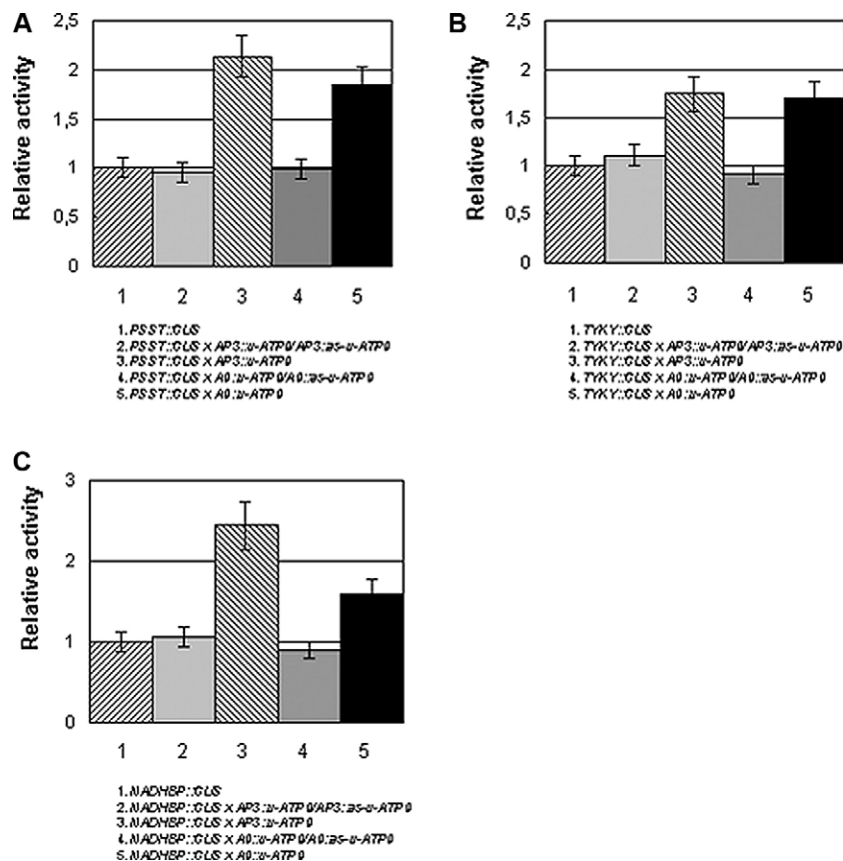


Fig. 4. Beta-glucuronidase activity in *u-ATP9* and hybrid lines. 0.3 μ g of total protein extracted from young flowers was incubated with the reaction medium for 30 min at 37 °C. The expression of GUS transcript were driven by *PSST* (A), *TYKY* (B) and *NADHBP* (C) promoters. The beta-glucuronidase activity of the different plants transformed with the *PSST::GUS*, *TYKY::GUS* and *NADHBP::GUS* constructs were used in each case as reference value.

oxidative phosphorylation genes are up-regulated in human cells harboring deleterious mtDNA mutations [12]. In plants, we observed up-regulation of at least three nCI genes [9, and this work], and also for the *Arabidopsis* frataxin homologous gene *AtFH* [1], suggesting activation of a general pathway destined to increase mitochondrial biogenesis so as to compensate for mitochondrial dysfunction. The retrograde pathway in yeast leads to the induction of genes involved in alternative metabolic pathways that appear to maintain vital components of the TCA cycle [7,26]. The reallocation of resources to the anaplerotic pathways makes sense for yeast, as it is able to survive on fermentable carbon sources in the absence of a functional organelle. However, this is not the case for animals or plants. Based on this situation, the yeast model which has been a paradigm for many different biological studies may not be a suitable model for gaining insight into mitochondria-nucleus cross-talk pathways in higher organisms. A profound difference in response to iron homeostasis signaling between *Candida albicans* and *S. cerevisiae* has been reported that can be attributed to the different life-styles of both yeast species [24].

The augmentation of steady-state levels of some mitochondrial genes may be explained either by transcription regulation or an increased stability of the mRNAs. The results obtained using *GUS* reporter gene under the control of the *TYKY*, *PSST* and *NADHBP* nCI promoters, clearly indicate that regulation

occurs at the transcriptional level. In fact, in double transgenic plants carrying *nCI::GUS* and *AP3* or *A9::u-ATP9* constructs, GUS activity was increased. The existence of transcriptional regulators, embedded in retrograde regulation in plants may be postulated. Accordingly, we propose that mitochondrial dysfunction triggers a retrograde signal which induces a number of nuclear-encoded mitochondrial proteins, including some nCI genes. However, the nuclear response is unable to restore full normal mitochondrial function and consequently, plants remain male-sterile.

While in anthers, respiration seems to be essential to sustain sporogenesis [9,13,27], in leaves the role of respiration is less clear because of the presence of chloroplasts, photosynthesis and the Glycine Cycle or photorespiration. Dutilleul et al. [6] have reported that leaf mitochondria modulate cellular redox homeostasis. A mutant lacking *NAD7*, a key component of complex I, presented disrupted photosynthesis suggesting that an active complex I is required for efficient redox exchange between compartments, connecting the energy producing processes, photosynthesis and respiration [5]. *nad7* mutant plants grow slowly but are able to complete a normal cycle and to gain normal biomass. In contrast, plants expressing *u-ATP9* transgene under *35SCaMV* promoter show a strong phenotype in vegetative as well as reproductive tissues [9]. This suggests that effective respiration, above a critical value, is required for proper growth

in photosynthetic tissues. Based on animal models, it has been put forward that phenotypic manifestations of mitochondrial defects occur only when a threshold level is exceeded [23].

Identifying the molecular signals involved in plant retrograde cross-talking pathways is a crucial issue. Some possible candidates are the ADP/ATP ratio, matrix pH imbalance or changes in redox homeostasis. It is tempting to postulate that during sporogenesis, besides the genetic developmental program, a transient imbalance produces a mitochondrial retrograde signaling that could account for anther specific induction of nCI genes in normal microsporophytic tissues compared to phototrophic ones.

Recently, Kuzmin et al. [16] reported that a permanent mitochondrial dysfunction found in maize and in *C. elegans* with a mitochondrial defect generated by RNAi of nuclear respiratory genes, induced the activation of *HSP* genes. This novel retrograde response to mitochondrial impairment, distinct from heat shock and not associated with oxidative stress, could then be a putative mechanism for facilitating cell defense and survival probably in order to stabilize complexes and/or prevent apoptosis [22]. Whether the retrograde response of nCI nuclear-encoded genes reported here is part of this mechanism remains to be determined.

4. Methods

4.1. Plant material, transformation and selection

A. thaliana plants (var. Columbia Col-0 and C24) were grown in a greenhouse at 22 °C under a 16 h light and 8 h dark cycle. Plant transformations were carried out using *Agrobacterium tumefaciens* as previously described [2] and selected in MS medium containing 30 µg/ml kanamycin and 100 µg/ml ampicillin and then transferred to a greenhouse.

4.2. Plasmid constructions

The *u-atp9* constructs contain a chimeric gene formed by the yeast *COXIV* presequence fused to the coding region of the unedited open reading frame from wheat *ATP9* [13]. The chimeric *u-ATP9* gene was cloned under the control of *CaMV35S*, *A9* or *AP3* promoters in the pPZP212 vector to generate pAGBM101, pAGBM401 and pAGBM001 recombinant plasmids, respectively, and used for agro-transformation of *Arabidopsis* plants as previously described [9]. Along with the sense construct, the antisense expressing plasmid vectors were constructed by insertion of the chimeric *u-ATP9* gene in inverted position (*AS-u-ATP9*), downstream of *CaMV35S* (pAGBM201), *A9* (pAGBM501) or *AP3* (pAGBM301) promoters. The different *GUS* constructs contain upstream sequences up to the following nucleotide positions: *PSST* promoter, -1700 (accession no. X96679); *NADHbp* promoter, -780 (accession no. AJ222638); *TYKY* promoter, -550 (accession no. AJ222639). Each promoter region is fused to the *GUS* gene in pBII01 vector as described [28]. All plasmids contain the promoter fragment and the 5' UTR up to and including the first

codon for the respective gene in frame with the β-glucuronidase gene. At least three independent *GUS* lines were crossed with at least two independent *A9::u-ATP9*, *A3::u-ATP9*, *AP3::u-ATP9* *xAP3::AS-u-ATP9* or *A9::u-ATP9* *xA9::AS-u-ATP9* lines. *GUS* activity was then measured in 10 individuals of each progeny.

4.3. Pollen viability and germination assay

The pollen viability was evaluated after incubation of pollen grains for 4 h at 25 °C as previously described [13]. Germination rate was evaluated on a population of approx. 400–500 grains. For all analyses, we collected the pollen of transgenic plants at the same flower opening period as for wild-type plants.

4.4. DNA extraction, purification and screening of the transformants

DNA was extracted from 20–40 mg of leaves or flowers using the CTAB method [4]. Screening of double transformants was performed by PCR, using the following primers: for *gus*: CTGCGTGATCGCGATCAACAGG and rev *gus*: AGGTACGGTAGGAGTTGGCCCC, to determine the presence of *GUS* transgene; *TNOS*: TCATCGCAAGACCGGCAACAGG, *u-atp9*: CACTACGTCAATCTATAAGA; and *u-atp9r*: ATCTCATTCGTTTTCCGATCG to determine the presence of *u-ATP9* and *AS-u-ATP9*, respectively.

4.5. RNA isolation, RT-PCR and blotting

Total RNA from growing plants was extracted using the RNA Plant Mini Kit (Qiagen). cDNA was synthesized using random hexamers as described in the Access RT-PCR system first strand protocol (Promega). Semi-quantitative analysis was performed on the RT-PCR amplification products after 16, 20, 24 and 28 PCR cycles. We determined that until 20 cycles the amplification reaction was still at the exponential phase for all genes tested. The following oligonucleotides were used: *psst*: GCCTAGTCCTCGCCAGTCTG, *GCTGGAGTAGTCCATAGAGC*; *tyky*: GATTTTGGCTCGCAGGTCAGT, *GCGGACATTCTGTGAGGAAC*; *nadhbp*: GAAAGCTGTGGGCAGTGCAC, *TCTCGAGCTCTGGCCTAAAG*; *u-atp9*: CACTACGTCAATCTATAAGA, *CGATCGGAAAACGAATGATCAG*. The control 18S (QuantumRNA 18S, Ambion) was used in a ratio of 2:9 18S primers: competitors, respectively, to modulate 18S amplification efficiency accordingly with manufacturer's protocol. After electrophoresis on 1.5% agarose gels, the PCR products were transferred onto Hybond N+ membranes (Amersham). Membranes were prehybridized for 1 h at 65 °C in 6X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured calf thymus DNA. Hybridization was carried out in the same solution for 18 h containing the [³²P]-labeled probes, *u-ATP9*, *AS-u-ATP9*, *TYKY*, *PSST*, *NADHBP* and the control 18S rRNA. Radioactive probes were generated using [³²P]dCTP (Ready-To-Go Labeling Beads

(-dCTP) (Amersham). After hybridization, the membranes were washed three times with 2X SSPE, 0.1% SDS at 60 °C for 30 min, three times with 0.2X SSPE, 0.1% SDS, at room temperature and then exposed to autoradiography films.

4.6. Quantitative fluorescence GUS assays

Young flowers from wild-type and transgenic plants were used for the determination of GUS activity using 4-methyl umbelliferyl glucuronide (MUG, Sigma-Aldrich) as previously described by Gallagher [8]. Floral tissues were homogenized in 50 mM Na₂HPO₄ pH 7.0 buffer containing 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine and 10 mM 2-mercaptoethanol. After centrifugation, different amounts of the supernatant (between 0.1 to 2 µg of total protein) were incubated in the presence of 1 mM MUG at 37 °C for 1 h. All incubations were carried out at 37 °C for between 30 min and 2 h by triplicate determination with deviations from the mean at around 10%. The reaction was stopped with 200 mM Na₂CO₃ and fluorescence was measured with excitation at 365 nm and emission at 455 nm.

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