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Differential targeting of VDAC3 mRNA isoforms influences mitochondria morphology

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Intracellular targeting of mRNAs has recently emerged as a prevalent mechanism to control protein localization. For mitochondria, a cotranslational model of protein import is now proposed in parallel to the conventional posttranslational model, and mitochondrial targeting of mRNAs has been demonstrated in various organisms. Voltage-dependent anion channels (VDACs) are the most abundant proteins in the outer mitochondrial membrane and the major transport pathway for numerous metabolites. Four nucleus-encoded VDACs have been identified in *Arabidopsis thaliana*. Alternative cleavage and polyadenylation generate two VDAC3 mRNA isoforms differing by their 3' UTR. By using quantitative RT-PCR and in vivo mRNA visualization approaches, the two mRNA variants were shown differentially associated with mitochondria. The longest mRNA presents a 3' extension named alternative UTR (aUTR) that is necessary and sufficient to target VDAC3 mRNA to the mitochondrial surface. Moreover, aUTR is sufficient for the mitochondrial targeting of a reporter transcript, and can be used as a tool to target an unrelated mRNA to the mitochondrial surface. Finally, VDAC3-aUTR mRNA variant impacts mitochondria morphology and size, demonstrating the role of mRNA targeting in mitochondria biogenesis.

mRNA localization | mRNA sorting | green RNA | porin | plant

Eukaryotic cells are characterized by compartmentalization of functions in complex organelles, and cell activity depends on synthesis and precise localization of proteins. The mechanisms of protein sorting are the subject of intense research, and models are often reevaluated (1). The earliest models for protein localization were based on the presence of targeting signals in the polypeptide sequences. However, the targeting of mRNAs to various subcellular regions has recently emerged as an essential regulatory step for protein localization (2).

Mitochondria contain hundreds of proteins, but only a few are encoded by the mitochondrial genome. The other proteins are nucleus-encoded and imported into the organelle. Protein import into mitochondria is controlled by peptide sequences that are recognized by mitochondrial receptors (3, 4). In parallel with this posttranslational model, a cotranslational model is now emerging (2, 5). A microarray analysis in *Saccharomyces cerevisiae* has shown that approximately half of the mRNAs encoding mitochondrial proteins are found in the vicinity of mitochondria (6). Mitochondrial targeting of mRNA was also demonstrated in mammals and plants (7, 8).

Mechanisms of mitochondrial targeting of mRNAs were essentially studied in yeast. In this species, three factors have been clearly identified to influence mRNA targeting to the mitochondrial surface. (i) Targeting of many but not all mRNAs appears dependent on translation (9, 10). (ii) The RNA-binding protein Puf3p is essential for mitochondrial targeting of a subset of mRNAs (9). (iii) The translocase of the outer mitochondrial membrane (TOM complex) is also involved in mRNA binding (10–12). In addition, some mRNAs are targeted to mitochondria without the need of the TOM complex, Puf3p, or translation, suggesting alternative routes, and other mRNAs need at least

two of the above factors. These results suggest that there is not one unique pathway, but, rather, different, overlapping routes for mRNA localization to the mitochondrial surface (2, 5). Furthermore, *cis* elements in the mRNA sequence have been identified. Most of them were found in the 3' UTR, but some were also identified in the coding sequence (CDS) (6, 9, 11, 13, 14). For example, the N-terminal mitochondrial targeting sequence (MTS) and 3' UTR as well as internal signals in CDS appear involved in mitochondrial targeting of the highly studied yeast ATP2 mRNA (11, 13, 15).

Cleavage and polyadenylation are essential for maturation of the 3' ends of pre-mRNAs. Recent analyses show that most eukaryotic genes have multiple cleavage and polyadenylation sites, leading to transcript isoforms with different CDS and/or variable 3' UTRs. Alternative cleavage and polyadenylation (APA) has been found in three-quarters of mammalian mRNA genes and half of zebrafish and drosophila genes (16). Seventy percent of *Arabidopsis thaliana* (*At*) genes use more than one poly(A) site (17). The 3' UTRs contain *cis* elements that play key roles in mRNA metabolism, in particular in mRNA localization, stability, and translation. Therefore, APA isoforms with different 3' UTR can have distinct properties.

Voltage-dependent anion channels (VDACs) are present in all eukaryotic cells. They are the most abundant proteins in the

Significance

Eukaryotic cells have developed a variety of mechanisms to sort proteins into distinct intracellular compartments, using protein signals and potentially mRNA localization. Most mitochondrial proteins are nucleus-encoded and imported into mitochondria. The amino acid signals and receptors involved in import are now well characterized. However, a new model is emerging, involving mRNA targeting in mitochondrial protein import. In this work, we show that two mRNA isoforms encoding a mitochondrial porin are differentially targeted to the mitochondrial surface. The mRNA sorting signal has been identified and can be used as a tool to target a reporter RNA to mitochondria. Moreover, the differential mRNA targeting impacts mitochondria morphology and size, demonstrating the role of mRNA targeting in mitochondria biogenesis.

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outer mitochondrial membrane (OM) and the major transport pathway for numerous metabolites. They are also involved in programmed cell death. In plants, they play important roles in developmental processes and in environmental stress responses. Moreover, they are found in various membranes—in particular, in nonmitochondrial ones (18, 19). Four nucleus-encoded VDACs (VDAC1–4) have been identified in *At*, and the specific function of each isoform is still poorly understood (20, 21). In this study, we show that APA produces two major isoforms of VDAC3 transcripts (At5g15090) that differ by the length of their 3' UTR, one being 140 nt longer than the other. Using quantitative RT-PCR (RT-qPCR) and “green RNA” approaches, we show that the longest mRNA is targeted to the mitochondrial surface and that the 140-nt extension (named alternative UTR; aUTR) is necessary for mitochondrial targeting. Moreover, we demonstrate that aUTR is sufficient to target an unrelated mRNA to the mitochondrial surface. Finally, we show that VDAC3 mRNA targeting to the mitochondrial surface modifies mitochondria size and number.

Results

APA Generates Different VDAC3 mRNA Isoforms. The *At* gene model At5g15090.1 proposed for VDAC3 in TAIR10 (<http://arabidopsis.org/>) corresponds to a Riken full-length cDNA (AY063891) with a 346-nt 3' UTR (Fig. 1A). However, alignment of VDAC3 ESTs (UniGene database, cluster At.24654) with this 3' sequence reveals that most ESTs end before position 220, with a hot spot at position 206. Only a few ESTs have a longer 3' UTR corresponding to At5g15090.1 (Fig. S1). Both short and long isoforms are confirmed by RT-PCR and RT-qPCR (Fig. 1B and C). In accordance with EST analysis, the long isoform is minor and represents 2–4% of total VDAC3 mRNAs in seedlings and leaves. APA thus leads to a major isoform ending around position 206 and to a minor one ending around position 346. According to Tian and Manley (16), we named the most abundant 3' UTR the constitutive UTR (cUTR) and the downstream region the aUTR. Therefore, the long 3' UTR is named caUTR.

VDAC3 mRNA Isoforms Are Differentially Associated with Mitochondria. We previously showed by RT-qPCR that some cytosolic mRNAs

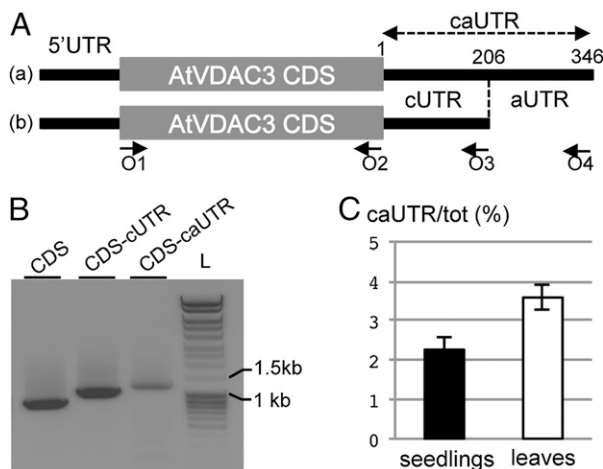


Fig. 1. VDAC3 APA variants are detected in *At*. (A) At5g15090.1 transcript model (a) has a 346-nt 3' UTR (caUTR). However, most of VDAC3 transcripts (b) have a shorter 3' UTR that ends around position 206 (cUTR). The downstream region (positions 206–346) is named the aUTR. O1–O4 correspond to oligonucleotides used in B and listed in Table S1. (B) RT-PCR allows the detection of VDAC3 mRNA isoforms. PCR products corresponding to CDS, CDS–cUTR, and CDS–caUTR were obtained, respectively, with O1–O2, O1–O3, and O1–O4 primers. L, DNA ladder. (C) The percentage of the long transcript vs. total transcripts (caUTR/tot) is determined by RT-qPCR in seedlings and leaves. The qPCR results are normalized with GAPDH as reference. Error bars represent SEM ($n = 3$).

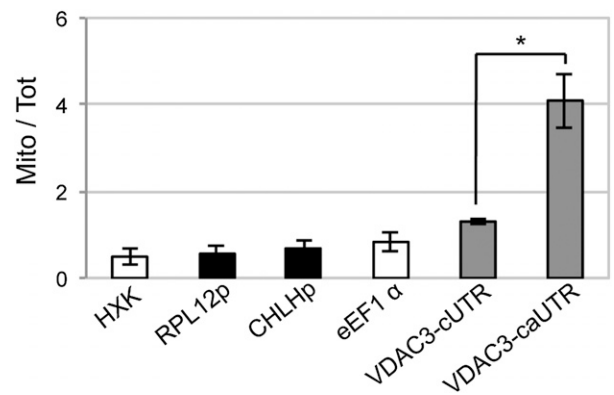


Fig. 2. VDAC3 isoforms are differentially associated with mitochondria. mRNAs are quantified by RT-qPCR in mitochondrial (Mito) and total (Tot) extracts from *At* cell culture. The quantity of each mRNA in the mitochondrial fraction (Mito) is normalized by its quantity in the total fraction (Tot) to take into account the expression level of the gene. Mito/Tot ratios are expressed relatively to GAPDH ratio. VDAC3-caUTR Mito/Tot ratio is 3.1 higher than VDAC3-cUTR Mito/Tot ratio. As controls, mRNAs encoding plastidial proteins (in black) or cytosolic proteins (in white) are used and give the contamination background of mitochondrial extracts. Error bars represent SEM ($n = 3$). * $P = 0.012$ (Student test). CHLHp, plastidial Mg chelatase; eEF1 α , elongation factor 1 α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HXK, hexokinase; RPL12p, plastidial ribosomal protein L12.

are targeted to mitochondria in potato. These mRNAs are removed under stringent washing of mitochondria, indicating that they are associated with the mitochondrial surface (8). We investigated mRNA association with the mitochondrial surface in *At* cell cultures and showed that VDAC3 mRNA isoforms are differentially associated with mitochondria (Fig. 2). VDAC3-caUTR appears three times more associated with mitochondria than VDAC3-cUTR.

To confirm these results, a *vdac3* transfer DNA (T-DNA) insertion line (SALK_127899.41.10.x) was stably transformed with a VDAC3 transgene corresponding to At5g15090.1 cDNA (VDAC3-caUTR; Fig. 3A, construction 1). The insertion mutant is a *vdac3* knockdown mutant that has no phenotype under normal growth conditions (22). In the VDAC3-caUTR transgenic line, VDAC3 mRNA isoforms were quantified by RT-qPCR in both total and mitochondrial extracts, using primers hybridizing with cUTR or aUTR sequences. In the total extract VDAC3-caUTR isoform represents 6% of VDAC3 mRNAs, in accordance with APA observed in wild-type (WT) plants. The mitochondrial association for VDAC3 mRNAs was then determined. VDAC3-caUTR isoform is enriched by a 2.7 factor in the mitochondrial fraction compared with VDAC3-cUTR (Fig. 3B, construct 1).

These results show that the VDAC3-caUTR transcript is more associated with mitochondria than VDAC3-cUTR in both cell cultures (Fig. 2) and seedlings (Fig. 3). This finding suggests that aUTR contains a mitochondrion-targeting *cis*-element.

aUTR Is Necessary and Sufficient to Target mRNA to the Mitochondrial Surface. To investigate the role of aUTR in VDAC3 mRNA targeting to mitochondria, additional transgenic lines corresponding to *vdac3* mutant transformed with VDAC3 variants were established (Fig. 3, constructs 2–4). mRNAs corresponding to VDAC3 variants, RPL12p and CHLHp were quantified in both mitochondrial and total extracts. The mitochondrial/total (Mito/Tot) ratios for VDAC3-cUTR or -CDS mRNAs appear similar to the Mito/Tot ratios for RPL12p and CHLHp mRNAs (Fig. 3, constructs 2 and 4), demonstrating that neither VDAC3-CDS nor VDAC3-cUTR transcripts are associated with mitochondria. By contrast, the VDAC3-aUTR transcript is found enriched in the mitochondrial fraction (Fig. 3, construct 3). These results strengthen the idea that aUTR contains a *cis*

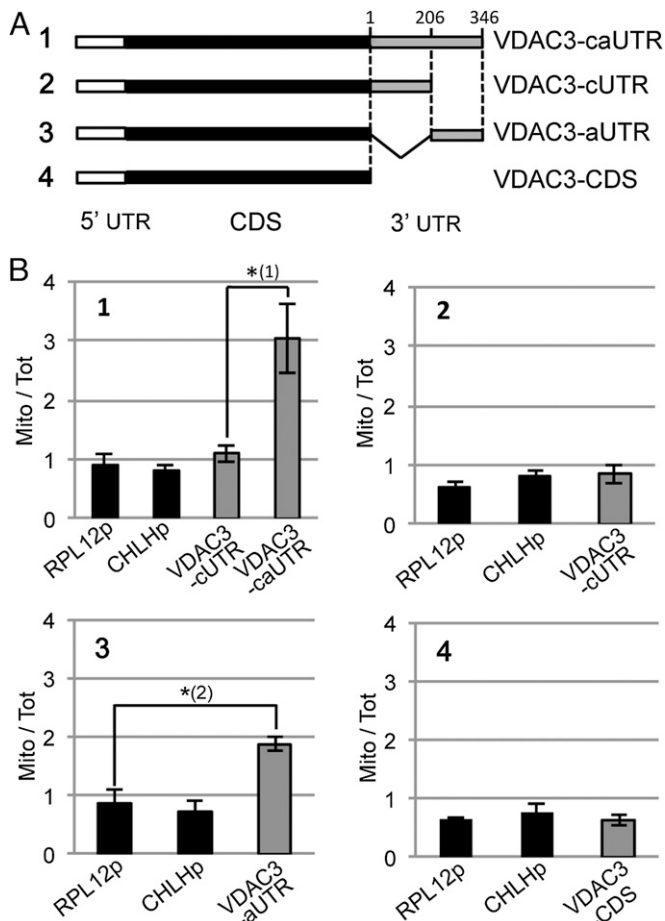


Fig. 3. VDAC3 mRNA variants are differentially associated with mitochondria in *vdac3* transgenic lines. (A) VDAC3 mRNA variants (constructs 1–4) expressed in *vdac3* transgenic lines. (B) mRNA levels are determined by RT-qPCR in mitochondrial (Mito) and total (Tot) extracts from 8-d water-cultured seedlings. Mito/Tot ratios are expressed relative to GAPDH ratio. RPL12p and CHLHp (in black) give the contamination background of mitochondria preparations. Error bars represent SEM ($n = 3$). Student test: $^{*1}P = 0.032$; $^{*2}P = 0.015$.

element necessary and sufficient to target VDAC3 mRNA to the mitochondrial surface.

To confirm these results, the green RNA strategy (23, 24) was adapted in *Nicotiana benthamiana* (*Nb*) to visualize mRNA association with mitochondria in living cells. Briefly, 24 stem-loop (SL) sequences of MS2 phage were inserted between the CDS and 3' sequences of interest (Fig. 4A). The constructs were transiently coexpressed in *Nb* leaves with MS2 coat protein fused to GFP. This protein specifically recognizes and associates with SL in mRNAs, allowing the indirect labeling of mRNAs by GFP. Plant mitochondria are physically discrete organelles and are not organized in network as in mammals and yeast (25). When labeled by pSu9-RFP (RFP fused with the first 69 amino acids of ATP synthase subunit 9), they appear as very mobile structures in confocal microscopy (Fig. S2B and C). mRNAs fused to SL and labeled by GFP appear as green mobile particles in the cytosol, some of them interacting with mitochondria (Fig. S2B and C). These particles cannot be detected when mRNAs are not fused to SL (Fig. S2A).

We set up the green RNA strategy in *Nb* with two control potato mRNAs that were previously reported as mitochondrion-targeted for mitochondrial malate dehydrogenase (MDH) or not targeted for RPL12p (8) (Fig. 4, constructs 3 and 4). We first determined that, for ~100 mRNA particles per construct, the

proportion of green particles associated with a mitochondrion. They represent 36% and 51% of green particles for RPL12p- and MDH-SL, respectively (Fig. 4A and B). These distributions appear different according to the χ^2 test (probability 0.035). To take into account the dynamics of both mitochondria and green particles, we then measured the distance between each mRNA particle and the nearest mitochondrion, from center to center (Fig. S2D). For the MDH-SL transcript, most of the particle centers are at <1 μm from the mitochondrion center. For the RPL12p-SL transcript, they are distributed between 0.5 and 2.5 μm from the mitochondrion center. Thus, the density of green particles very close to mitochondria is clearly higher for MDH-SL than for RPL12p-SL (Fig. 4C, constructs 3 and 4). These results (i) confirm the differential association of MDH and RPL12p mRNAs with mitochondria in *Nb* living cells, as previously observed by RT-qPCR in potato (8); (ii) show that the targeting signals are conserved between *Nb* and potato; and (iii) validate our strategy to study mitochondrial association of mRNAs in vivo.

To use the green RNA strategy to investigate the role of 3' UTRs in the mitochondrial targeting of VDAC3 mRNAs, we constructed VDAC3-SL variants with aUTR or cUTR sequences (Fig. 4, constructs 1 or 2, respectively). These constructs were transiently expressed in *Nb*. Using 3' RACE, we verified that mRNAs corresponding to constructs 1 and 2 contained the full-length aUTR and cUTR, respectively. Another VDAC3-SL variant with caUTR sequence was also tested. Using both 3' RACE and RT-qPCR, we showed that >90% of mRNAs corresponding to this construct were polyadenylated around position 206 and were undistinguishable from those of construct 2. These results were in accordance with APA observed in *At* (see above). Because it would not be possible to assign a specific green RNA particle to a specific mRNA isoform, this construct was not further used.

With the green RNA approach (Fig. 4, constructs 1 and 2), VDAC3-SL-cUTR gives similar results to RPL12p-SL, and VDAC3-SL-aUTR gives similar results to MDH-SL. This finding shows that aUTR allows an efficient targeting of VDAC3 mRNA to mitochondria compared with cUTR. These in vivo results are in accordance with RT-qPCR data (Fig. 3). Last, when the aUTR sequence is fused to the RPL12p sequence (Fig. 4A, construct 5), it increases both the proportion of RPL12p mRNA associated with mitochondria and the density of green particles close to mitochondria (Fig. 4, constructs 4 and 5), showing that aUTR is sufficient to target an unrelated mRNA to mitochondria.

The results obtained by RT-qPCR (Fig. 3) and with the green RNA approach (Fig. 4) clearly show that aUTR is necessary and sufficient to target VDAC3 mRNA to mitochondria. Moreover the *cis* element in VDAC3 aUTR is functional in different plants—in *At* (Fig. 3) and in *Nb* (Fig. 4)—and can be used as a tool to target an unrelated mRNA to the mitochondrial surface.

VDAC3-cUTR and VDAC3-aUTR Differentially Impact the Mitochondria

Size and Number. Although many of the nucleus-encoded proteins can be imported into mitochondria in a posttranslational process, a cotranslational import is also proposed (2). So the simplest hypothesis for VDAC3 mRNA targeting is that it allows a more efficient import of VDAC3 protein into mitochondria. The *vdac3* mutants have no phenotype under normal growth conditions (20–22), so they cannot be used for complementation analyses. Because VDAC1 is the closest VDAC3 paralog (18), we transformed a *vdac1* T-DNA insertion line (SALK_034653) with two VDAC3 transgenes harboring a HA tag at the N terminus of CDS and differing by their 3' extensions (cUTR or aUTR sequences; Fig. 5 and Fig. S3A and B). *Vdac1* mutant shows phenotypes associated with mitochondrial dysfunctions, with distorted rosette and cauline leaves, delayed development, and reduced fertility (21) (Fig. 5A). Moreover, they present a reduced number of mitochondria, which are abnormally enlarged (21) (Fig. 5B–D and Fig. S4). For each construct (with cUTR or

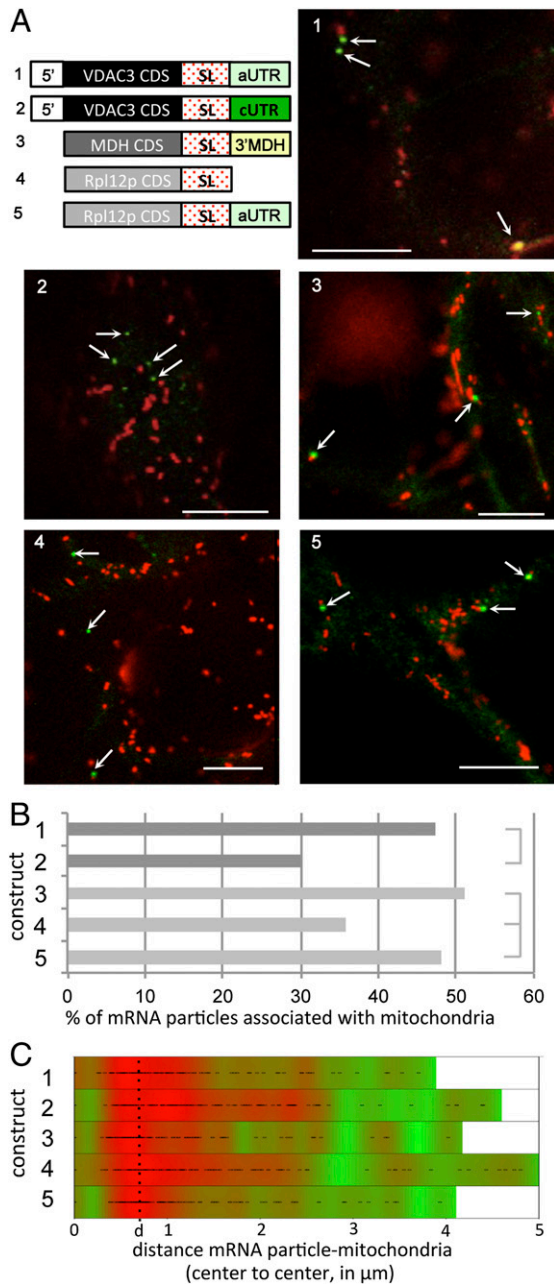


Fig. 4. In *Nb* living cells, differential mitochondrial targeting of mRNAs is detected with the green RNA strategy. (A) mRNA constructs 1–5 and examples of confocal images. Constructs 3 and 4 correspond to control mRNAs previously shown as targeted (MDH; construct 3) or not (RPL12p; construct 4) to the mitochondrial surface in potato (8). Constructs 1, 2, and 5 contain 3' extensions corresponding to VDAC3–cUTR or –aUTR. SL, MS2 stem-loops; 5' and 3', 5' UTR and 3' UTR sequences, respectively. Mitochondria are labeled by pSu9–RFP (in red). Arrows show GFP-labeled mRNA particles (in green). (Scale bars: 10 μm.) (B) Percentage of mRNA particles associated with mitochondria—that is, overlapping or in contact with mitochondria. According to a χ^2 test ($d_f = 1$), the distribution is different for VDAC3–aUTR compared with VDAC3–cUTR (constructs 1 and 2; probability 0.005) and for MDH and RPL12–aUTR compared with RPL12 (constructs 3 and 5 compared with construct 4; probability 0.035 and 0.017, respectively). n , number of particles for each construct: 1, $n = 93$; 2, $n = 110$; 3, $n = 86$; 4, $n = 103$; 5, $n = 106$. (C) Density of mRNA particles around mitochondria. To obtain this scatterplot with smoothed color density representation, distances from the center of each mRNA particle to the center of the closest mitochondrion are first measured. Then, the number of particles at a given mitochondrion distance is calculated and represented as a density obtained through a kernel density estimate (R software). Red, high density of RNA

aUTR sequences), three independent transgenic lines expressing similar levels of HA–VDAC3 protein were further analyzed (Fig. S3C). All were able to restore a WT phenotype at the level of the plant (Fig. 5A). All also induced a reduction of mitochondria size and an increase in mitochondria number (Fig. 5B–D). In plants expressing HA–VDAC3–cUTR, the mitochondria morphology and number were indistinguishable from that of Col-0 plants. However, plants expressing HA–VDAC3–aUTR presented a higher number of mitochondria, which were smaller than those in Col-0, suggesting a differential impact of cUTR and aUTR on mitochondria size and number. The quantity of HA–VDAC3 protein in mitochondria was evaluated by Western blot (Fig. S3D) and, surprisingly, appeared similar in plants expressing either cUTR or aUTR constructs. Moreover, mitochondria extracted from both transgenic lines were Proteinase K-treated (Fig. S3E). This treatment degrades TOM20 that presents a cytosolic domain, but does not affect HA–VDAC3 that is an integral OM protein, thus showing that HA–VDAC3 from both transgenic lines is correctly inserted into the OM. Finally, the quantity of HA–VDAC3 proteins in peripheral membranes was determined on electron microscopy images (Fig. 5E) and appeared similar with both constructs. Consequently the differential effect of VDAC3–cUTR and –aUTR constructs on mitochondria size and number is not due to differences in VDAC3 protein import efficiency.

Discussion

In this work we show that one *At* VDAC gene, *VDAC3*, is transcribed and matured into two mRNA isoforms differing in their 3' UTR. By using RT-qPCR and in vivo visualization of mRNAs, the longest isoform was shown to be strongly associated with the mitochondrial surface. The *cis* element responsible for this targeting is localized in the 140-nt most distal 3' sequence, called aUTR. The aUTR sequence is necessary and sufficient to target VDAC3 mRNA to the mitochondrial surface, but also to target an unrelated mRNA to mitochondria. Moreover, aUTR is functional in different plant species. We also show that VDAC3 proteins expressed from VDAC3–cUTR or –aUTR are present in equal amount in OM and are able to complement the macroscopic phenotype of *vdac1* mutants. However, the expression of VDAC3–aUTR induces stronger mitochondrial modifications, leading to mitochondria of reduced size and higher number.

Four *VDAC* genes are ubiquitously expressed in *At*, even if their expression patterns present clear differences (Genevestigator) (20, 21). Apart from their conserved role in metabolite exchanges, the specific role of each VDAC is still unknown. The four proteins are found in mitochondria, and three of the four are also detected in the plasma membrane (21). KO mutants for *VDAC1*, *VDAC2*, and *VDAC4* present growth-delay phenotypes, distorted leaves and reduce fertility. By contrast, *vdac3* KO mutant has no phenotype in classical growth conditions. However, *VDAC3* is probably involved in response to pathogens (26) and in seed germination (22). *VDAC4* was shown to complement *vdac1* mutant (21), and our work shows that *VDAC3* is also able to complement *vdac1* mutation, suggesting that *At*–VDACs have partially redundant functions. Depending on the *VDAC3* mRNA isoform (cUTR or aUTR), however, differences in *vdac1* complementation are observed at the mitochondrial level. Moreover, *VDAC3* is detected in both mitochondria and plasma membrane (21). It is possible that mRNA isoforms play different roles regarding the localization of the protein and that the mRNA variant with cUTR is more implicated in the plasma localization of *VDAC3*. Our finding suggests that the role of *VDAC3* is linked to the intracellular localization of its mRNA. Such a

particles; green, low density; white, no particle. Zero represents the mitochondrion's center. When an mRNA particle and a mitochondrion are in contact without overlapping, the average distance between the particle and the mitochondrion centers is 0.65 μm (d).

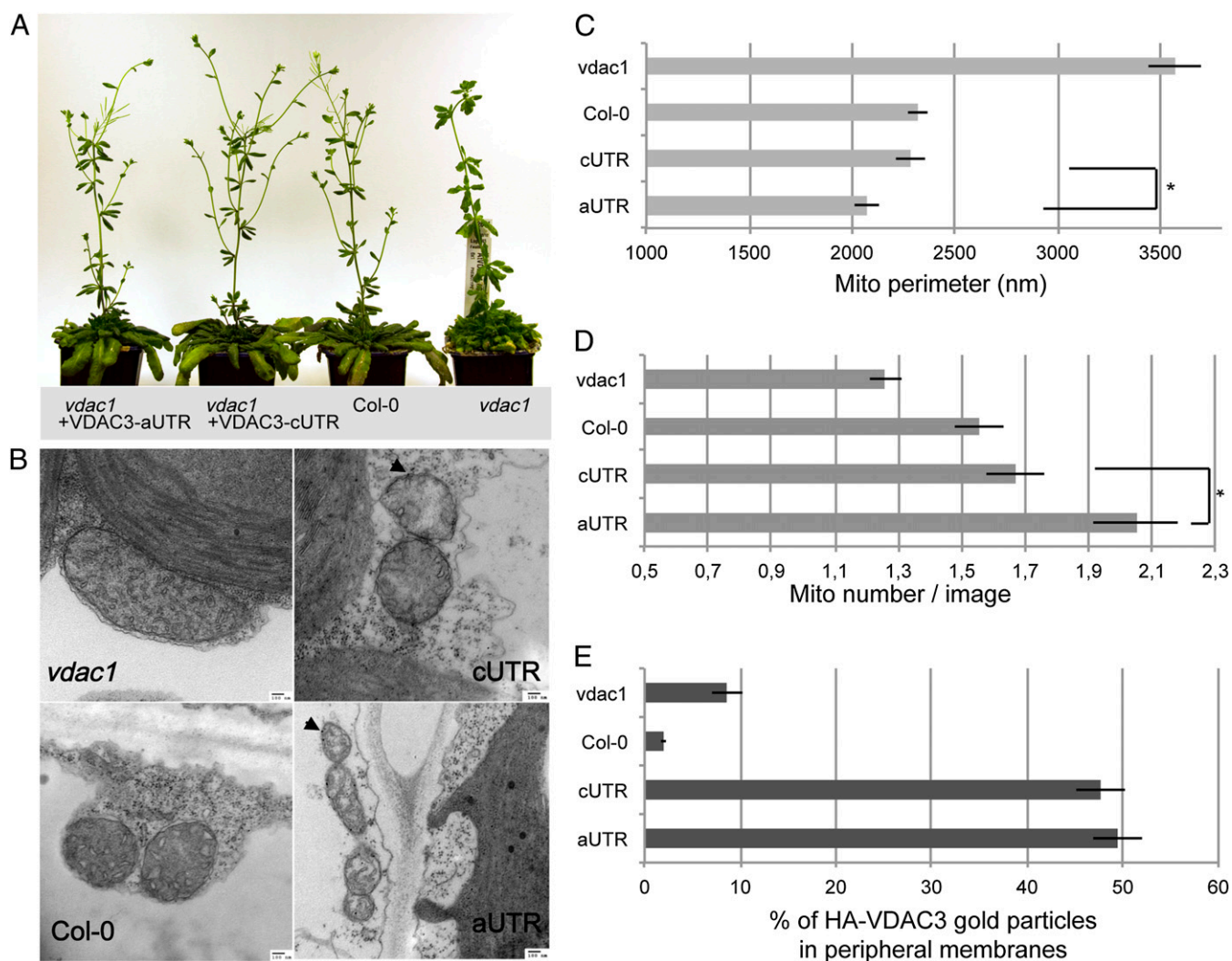


Fig. 5. In *vdac1* transgenic lines, VDAC3 mRNA variants restore a WT phenotype but have different impacts on mitochondria size and number. *Vdac1* mutant is stably transformed with either HA-VDAC3-cUTR (cUTR) or HA-VDAC3-aUTR (aUTR) constructs. For each construct, three independent transformed lines are analyzed at the level of the plant (A) and by electron microscopy (C-E). At least 25 images of leaf sections per transgenic line are analyzed. (A) The transgenic lines, with cUTR or aUTR sequences, are able to restore a WT phenotype at the plant level. (B) Electron microscopy images of leaf sections from Col-0, *vdac1* mutant, and *vdac1* complemented with cUTR or aUTR constructs. Immunolocalization of HA-VDAC3 is performed with HA antibody and secondary antibody-gold particles. VDAC3 protein is normally located in the OM. Arrowheads indicate examples of particles associated with peripheral mitochondrial membranes. (Scale bars: 100 nm.) (C) Mean mitochondria perimeter determined on electron microscopy images. Number of mitochondria analyzed per construct are as follows: aUTR, $n = 163$; cUTR, $n = 137$; Col-0, $n = 147$; *vdac1*, $n = 96$. $*P = 0.034$ (Student test). (D) Mean mitochondria number per image ($4 \mu\text{m}^2$). Number of images analyzed per construct are as follows: aUTR, $n = 81$; cUTR, $n = 85$; Col-0, $n = 110$; *vdac1*, $n = 81$. $*P = 0.018$ (Student test). (E) Immunolocalization of HA-VDAC3 in leaf sections. At least 100 gold particles for each transgenic line ($n = 3$) are analyzed, and the percentage of particles in peripheral membranes is calculated. Error bars represent SEM.

result points out the complexity and sophistication of VDAC's functions.

mRNA targeting the mitochondrial surface is clearly established in different organisms (2, 5). Approximately half of the mRNAs encoding mitochondrial proteins are found in the vicinity of yeast mitochondria, suggesting a link between mRNA localization and protein translocation (6). A few studies directly show that mitochondrial targeting of mRNA can improve protein import and rescue respiratory defects (27-29). Here we show that differences in VDAC3 mRNA targeting impact mitochondria morphology and size but do not cause differences in VDAC3 protein import efficiency. A hypothesis for VDAC3 mRNA localization at the mitochondrial surface is that it could allow a specific localization of the protein into particular regions of OM, as has been already demonstrated for the endoplasmic reticulum (1, 30, 31).

Finally we demonstrate in this work that the 140-nt sequence corresponding to aUTR is sufficient to target an unrelated RNA to the mitochondrial surface and that this sequence is functional in different plants. Many approaches try to develop systems associating an efficient RNA targeting to the mitochondrial surface and an efficient RNA import into mitochondria (32-35). It should be noted that, if natural import of mRNA into chloroplasts has been shown (36), natural mRNA import into mitochondria is never demonstrated. Moreover, allotropic expression of nucleus-encoded mitochondrial genes can be unsuccessful due to incorrect localization of the protein into mitochondria (37, 38). In these contexts, aUTR sequence offers a tool to target RNA and proteins to/into mitochondria.

Materials and Methods

See *SI Materials and Methods* for details on methods.

Plant Material. The insertion mutants SALK_034653 for *vdac1* (21) and SALK_127899.41.10.x for *vdac3* (22) were from Columbia ecotype. *At* cell culture was Landsberg ecotype (39).

VDAC3 Constructs. The VDAC3 cDNA was obtained from S. Filleul (Institut des Sciences du Végétal, Gif sur Yvette, France) (21). MDH and RPL12p cDNA were amplified by RT-PCR from potato total RNA. All VDAC3 constructs were performed in Gateway binary vectors and used the same 3' extensions containing caUTR, cUTR, or aUTR sequences. For transformation of the *vdac3* mutant, VDAC3 sequences were under the control of VDAC3 endogenous promoter (Fig. 3). For the green RNA approach, the VDAC3 5' UTR-CDS sequence and the variable 3' sequences were cloned, respectively, upstream and downstream from the 24 MS2 SL sequence. The constructs were under the control of the constitutive 35S promoter (Fig. 4). Similar constructs were done for MDH and RPL12p (Fig. 4). For transformation of *vdac1* mutant, the VDAC3 genomic sequence (with VDAC3 promoter) was first cloned. A HA sequence was added at the beginning of CDS, because a tag at the C terminus of VDAC protein is known to alter its integration into OM (40). Two variants were then generated, with cUTR or aUTR sequence in 3' (Fig. 5).

Mitochondria Preparations. Mitochondria were extracted from 4-d cell culture (41) or from 8-d water-cultured seedlings (42).

- Hermesh O, Jansen RP (2013) Take the (RN)A-train: Localization of mRNA to the endoplasmic reticulum. *Biochim Biophys Acta* 1833(11):2519–2525.
- Weis BL, Schleiff E, Zerges W (2013) Protein targeting to subcellular organelles via mRNA localization. *Biochim Biophys Acta* 1833(2):260–273.
- Harbauer AB, Zahedi RP, Sickmann A, Pfanner N, Meisinger C (2014) The protein import machinery of mitochondria—a regulatory hub in metabolism, stress, and disease. *Cell Metab* 19(3):357–372.
- Murcha MW, Wang Y, Narsai R, Whelan J (2014) The plant mitochondrial protein import apparatus - the differences make it interesting. *Biochim Biophys Acta* 1840(4):1233–1245.
- Michaud M, Duchene AM (2012) Macromolecules trafficking to plant mitochondria. *Mitochondrial Genome Evolution*, ed Maréchal-Drouard L (Academic, New York), pp 347–421.
- Marc P, et al. (2002) Genome-wide analysis of mRNAs targeted to yeast mitochondria. *EMBO Rep* 3(2):159–164.
- Matsumoto S, et al. (2012) Localization of mRNAs encoding human mitochondrial oxidative phosphorylation proteins. *Mitochondrion* 12(3):391–398.
- Michaud M, Maréchal-Drouard L, Duchène AM (2010) RNA trafficking in plant cells: Targeting of cytosolic mRNAs to the mitochondrial surface. *Plant Mol Biol* 73(6):697–704.
- Saint-Georges Y, et al. (2008) Yeast mitochondrial biogenesis: A role for the PUF RNA-binding protein Puf3p in mRNA localization. *PLoS ONE* 3(6):e2293.
- Eliyahu E, et al. (2010) Tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner. *Mol Cell Biol* 30(1):284–294.
- Gadir N, Haim-Vilmovsky L, Kraut-Cohen J, Gerst JE (2011) Localization of mRNAs coding for mitochondrial proteins in the yeast *Saccharomyces cerevisiae*. *RNA* 17(8):1551–1565.
- Michaud M, Maréchal-Drouard L, Duchène AM (2014) Targeting of cytosolic mRNA to mitochondria: Naked RNA can bind to the mitochondrial surface. *Biochimie* 100:159–166.
- Margeot A, et al. (2002) In *Saccharomyces cerevisiae*, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function. *EMBO J* 21(24):6893–6904.
- Sylvestre J, Vialette S, Corral Debrinski M, Jacq C (2003) Long mRNAs coding for yeast mitochondrial proteins of prokaryotic origin preferentially localize to the vicinity of mitochondria. *Genome Biol* 4(7):R44.
- García M, Delaveau T, Goussard S, Jacq C (2010) Mitochondrial presequence and open reading frame mediate asymmetric localization of messenger RNA. *EMBO Rep* 11(4):285–291.
- Tian B, Manley JL (2013) Alternative cleavage and polyadenylation: The long and short of it. *Trends Biochem Sci* 38(6):312–320.
- Wu X, et al. (2011) Genome-wide landscape of polyadenylation in *Arabidopsis* provides evidence for extensive alternative polyadenylation. *Proc Natl Acad Sci USA* 108(30):12533–12538.
- Homblé F, Krammer EM, Prévost M (2012) Plant VDAC: Facts and speculations. *Biochim Biophys Acta* 1818(6):1486–1501.
- Takahashi Y, Tateda C (2013) The functions of voltage-dependent anion channels in plants. *Apoptosis* 18(8):917–924.
- Tateda C, Watanabe K, Kusano T, Takahashi Y (2011) Molecular and genetic characterization of the gene family encoding the voltage-dependent anion channel in *Arabidopsis*. *J Exp Bot* 62(14):4773–4785.
- Robert N, et al. (2012) Voltage-dependent-anion-channels (VDACs) in *Arabidopsis* have a dual localization in the cell but show a distinct role in mitochondria. *Plant Mol Biol* 78(4–5):431–436.

RNA Extraction, Reverse Transcription, and RT-qPCR. They were performed according to ref. 8. For RT-qPCR, three biological replicates, corresponding to mitochondrial and total RNA extracted from the same material, were prepared. For each extract, three technical replicates were done. The qPCR efficiency for each primer's couple was determined by serial dilutions of cDNA. The qPCR results were normalized with the mitochondrial RPL5 mRNA.

Confocal Microscopy. *Nb* leaves were directly observed 2–4 d after agro-infiltration by using a LSM700 confocal microscope (Zeiss). GFP and RFP fluorophores were excited at 488 and 555 nm, respectively, and emission signals were simultaneously collected at 488–560 nm for GFP and at long-pass 560 nm for RFP.

Electron Microscopy and Immuno-Cytolocalization. Ultrathin sections of leaves and immunolocalization with HA antibody (Sigma H9658) were performed according to Robert et al. (21). At least 25 images of leaf sections were analyzed for each transgenic line.

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- Yang XY, et al. (2011) *Arabidopsis* kinesin KP1 specifically interacts with VDAC3, a mitochondrial protein, and regulates respiration during seed germination at low temperature. *Plant Cell* 23(3):1093–1106.
- Bertrand E, et al. (1998) Localization of ASH1 mRNA particles in living yeast. *Mol Cell* 2(4):437–445.
- Querido E, Chartrand P (2008) Using fluorescent proteins to study mRNA trafficking in living cells. *Methods Cell Biol* 85:273–292.
- Scott I, Logan DC (2011) Mitochondrial dynamics. *Plant Mitochondria*, ed Kempken F (Springer, Berlin), pp 31–63.
- Lee SM, et al. (2009) Pathogen inducible voltage-dependent anion channel (AtVDAC) isoforms are localized to mitochondria membrane in *Arabidopsis*. *Mol Cells* 27(3):321–327.
- Ginsberg MD, Feliciello A, Jones JK, Avvedimento EV, Gottesman ME (2003) PKA-dependent binding of mRNA to the mitochondrial AKAP121 protein. *J Mol Biol* 327(4):885–897.
- Kaltimbacher V, et al. (2006) mRNA localization to the mitochondrial surface allows the efficient translocation inside the organelle of a nuclear recoded ATP6 protein. *RNA* 12(7):1408–1417.
- Bonnet C, et al. (2007) Allotopic mRNA localization to the mitochondrial surface rescues respiratory chain defects in fibroblasts harboring mitochondrial DNA mutations affecting complex I or v subunits. *Rejuvenation Res* 10(2):127–144.
- Crofts AJ, et al. (2005) The role of mRNA and protein sorting in seed storage protein synthesis, transport, and deposition. *Biochem Cell Biol* 83(6):728–737.
- Washida H, et al. (2012) RNA targeting to a specific ER sub-domain is required for efficient transport and packaging of α -globulins to the protein storage vacuole in developing rice endosperm. *Plant J* 70(3):471–479.
- Niaz AK, et al. (2013) Targeting nucleic acids into mitochondria: Progress and prospects. *Mitochondrion* 13(5):548–558.
- Tarassov I, et al. (2013) Mitochondrial targeting of RNA and mitochondrial translation. *Translation in Mitochondria and Other Organelles*, ed Duchene AM (Springer, Berlin).
- Wang G, et al. (2012) Correcting human mitochondrial mutations with targeted RNA import. *Proc Natl Acad Sci USA* 109(13):4840–4845.
- Val R, et al. (2011) Organelle trafficking of chimeric ribozymes and genetic manipulation of mitochondria. *Nucleic Acids Res* 39(21):9262–9274.
- Nicolaï M, et al. (2007) Higher plant chloroplasts import the mRNA coding for the eucaryotic translation initiation factor 4E. *FEBS Lett* 581(21):3921–3926.
- Perales-Clemente E, Fernández-Silva P, Acín-Pérez R, Pérez-Martos A, Enriquez JA (2011) Allotopic expression of mitochondrial-encoded genes in mammals: Achieved goal, undemonstrated mechanism or impossible task? *Nucleic Acids Res* 39(1):225–234.
- Figuerola-Martínez F, et al. (2011) What limits the allotopic expression of nucleus-encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes. *Mitochondrion* 11(1):147–154.
- Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat Protoc* 2(7):1565–1572.
- Kozjak-Pavlovic V, Ross K, Götz M, Goosmann C, Rudel T (2010) A tag at the carboxy terminus prevents membrane integration of VDAC1 in mammalian mitochondria. *J Mol Biol* 397(1):219–232.
- Pujol C, et al. (2008) Dual-targeted tRNA-dependent amidotransferase ensures both mitochondrial and chloroplastic Gln-tRNA_{Gln} synthesis in plants. *Proc Natl Acad Sci USA* 105(17):6481–6485.
- Day DA, Neuberger M, Douce R (1985) Biochemical characterization of chlorophyll-free mitochondria from pea leaves. *Aust J Plant Physiol* 12(3):219–228.