Natural Variation in Arabidopsis Leads to the Identification of REME1, a Pentatricopeptide Repeat-DYW Protein Controlling the Editing of Mitochondrial Transcripts[1][W][OA]

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In vascular plants, organelle RNAs are edited by C-to-U base modification. Hundreds of mitochondrial C residues are targeted for editing in flowering plants. In this study, we exploited naturally occurring variation in editing extent to identify *Required for Efficiency of Mitochondrial Editing1* (*REME1*), an Arabidopsis (*Arabidopsis thaliana*) pentatricopeptide repeat protein-encoding gene belonging to the DYW subclass that promotes editing of at least two C residues on different mitochondrial transcripts. Positional cloning identified *REME1* unambiguously as the gene controlling editing of *nad2*-558. Virus-induced gene silencing of *REME1* confirmed its role in editing of *nad2*-558 and allowed us to identify *orfX*-552 as a second C whose editing is positively controlled by *REME1*. An unexpected outcome of *REME1* silencing was the finding of a number of mitochondrial C targets whose editing extent exhibits a significant and reproducible increase in silenced tissues. That increase was shown to be partly due to the virus inoculation and partly to *REME1*-specific silencing. Analysis of an insertional T-DNA mutant within the *REME1* coding sequence confirmed the findings of the virus-induced gene silencing experiments: decrease in editing extent of *nad2*-558 and *orfX*-552 and increase in editing extent of two sites, *matR*-1771 and *rpl5*-92. Transgenic complementation of the low-edited accession (Landsberg *erecta*) restored the editing of *nad2*-558 and *orfX*-552 to high-edited accession (Columbia)-type levels or to even higher levels than Columbia. There was no effect of the transgene on editing extent of *matR*-1771 and *rpl5*-92. The strategy and tools used in this report can be applied to identify additional genes that affect editing extent in plant mitochondria.

RNA editing is a process that alters the genetic information at specific sites on RNA molecules. Editing has been described in a wide range of organisms from viruses to animals and plants. Several systems involving unrelated mechanisms seem to have arisen separately during evolution (for review, see Gott and Emeson, 2000). In vascular plants, organelle transcripts are modified posttranscriptionally by C-to-U editing. While 30 to 40 C-to-U editing events are typically found in vascular plant chloroplast transcriptomes (Maier et al., 1995; Tsudzuki et al., 2001; Tillich et al., 2005), 1 order of magnitude more cytosine residues are subject to editing in the mitochondria of flowering plants: 427, more than 500, 491, and 357 sites have been reported, respectively, in rapeseed (*Brassica napus*), Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and sugar beet (*Beta vulgaris*; Notsu et al., 2002; Handa, 2003; Mower and Palmer, 2006; Bentolila et al., 2008). Conversion of a C to a U in a mitochondrial RNA usually results in a codon encoding an amino acid similar to that found in other plants or microorganisms at the homologous amino acid position, although silent editing events also occur (Covello and Gray, 1990; Gray and Covello, 1993). The main purpose of RNA editing has been thought to be the correction of deleterious mutations that would otherwise hamper the proper function of the encoded product. Indeed, an unedited ATP9 protein variant was shown to be not functional in tobacco (*Nicotiana tabacum*) because its presence disturbed mitochondrial function, resulting in a male sterility phenotype (Hernould et al., 1993).

The first trans-acting factor involved in plant organelle editing was found by screening Arabidopsis mutants defective in NADH dehydrogenase activity (Kotera et al., 2005). CHLORORESPIRATORY REDUCTION4 (CRR4) is required for editing of the start codon of the Arabidopsis chloroplast *ndhD* mRNA, which codes for a subunit of the chloroplast NAD dehydrogenase. CRR4 is a member of the pentatricopeptide repeat (PPR) protein family in plants (Small and Peeters, 2000) that comprises 450 members in Arabidopsis and 477 in rice (O'Toole et al., 2008), most of which are predicted to be targeted to either mitochondria or plastids (Lurin et al., 2004). Plant PPR proteins can be

[1] This work was supported by the National Science Foundation (grant nos. DBI–0421799 and MCB–0929423 to M.H. and MCB–1020636 to S.B.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.165969

separated into two major subfamilies based on the nature of their PPR motifs, the P and PLS subfamilies. The P subfamily contains the canonical motif of 35 amino acids, while the PLS subfamily is characterized by the presence of longer (L) or shorter (S) variant PPR motifs within the tandem arrays. The PLS subfamily, which is specific to the plant kingdom, can be further separated into two groups, a subclass containing the DYW domain and a subclass lacking this domain (Lurin et al., 2004). All the chloroplast PPR motif-containing trans-factors identified so far belong to the PLS-E subgroup (Kotera et al., 2005; Okuda et al., 2007; Chateigner-Boutin et al., 2008; Hammani et al., 2009) or the PLS-E-DYW subgroup (Zhou et al., 2008; Cai et al., 2009; Hammani et al., 2009; Okuda et al., 2009, 2010; Robbins et al., 2009).

Similarly, the vast majority of the mitochondrial PPR motif-containing trans-factors belong to the PLS subfamily. MEF1, the first mitochondrial editing factor identified, is involved in editing of three specific C residues in different mitochondrial genes of Arabidopsis (Zehrmann et al., 2009). MEF1 belongs to the DYW subclass of PPR proteins, as does rice OGR1 (Kim et al., 2009). Mutants in the *OGR1* gene fail to modify seven C residues edited in the wild type and exhibit various morphological phenotypes, including delayed seed germination, retarded growth, dwarfism, and sterility (Kim et al., 2009). Recently, a moss PPR protein with a DYW domain was shown to be responsible for editing of mitochondrial *ccmFc* transcript (Tasaki et al., 2010). Like chloroplast editing trans-factors, members of the PLS-E subgroup that lack the DYW motif have also been shown to be necessary for mitochondrial editing (Sung et al., 2010; Takenaka, 2010; Takenaka et al., 2010). PPR596 is the only example so far of a member of the P subfamily that is involved in mitochondrial editing (Doniwa et al., 2010).

Rather than analysis of mutants, our work on mitochondrial RNA editing relies on the use of natural variation in Arabidopsis coupled with a genetic approach to identify components of the RNA/protein complex responsible for the C-to-U modification (Bentolila et al., 2005, 2008). This strategy can potentially allow the identification of factors whose disruption would result in embryo lethality. We report here the identification of the gene *Required for Efficiency of Mitochondrial Editing1* (*REME1*), which belongs to the DYW subclass of PPR-containing genes. By assaying all 523 mitochondrial C targets of editing in *REME1*-silenced leaf tissue in comparison with the wild type, we unexpectedly found that REME1 promotes RNA editing at some C targets while inhibiting the editing at other specific sites. This result was confirmed by the analysis of a T-DNA insertional mutant within the *REME1* coding sequence.

RESULTS

Differential Editing of *nad2*-558 in Columbia and Landsberg *erecta* Is Controlled by a Single Gene

Study of mitochondrial editing polymorphisms between Columbia (Col) and Landsberg *erecta* (L*er*) mitochondrial transcripts led to the first report on the genetic architecture of mitochondrial editing in Arabidopsis (Bentolila et al., 2008). We mapped 12 major quantitative trait loci (QTLs) for 11 of the 13 editing traits analyzed, demonstrating that efficiency of editing of individual mitochondrial C targets is generally governed by a major factor. The largest effect attached to a QTL was detected for editing extent of *nad2*-558. The QTL controlling this trait colocalizes with the marker m246 on chromosome 2; its effect could explain 95% of the phenotypic variation. The segregation of editing extent of *nad2*-558 observed in recombinant inbred lines (RILs; Fig. 1) indicates that the differential editing between Col and L*er* is controlled by a single gene.

Positional Cloning of *REME1*, Which Promotes the Editing of *nad2*-558

We chose the QTL controlling the editing of *nad2*-558 for map-based cloning because of its high heritability and the subsequent simplicity of following its segregation by evaluation of editing extent (Fig. 1). Coarse mapping with 30 RILs delineated a log of the odds 1 interval for this QTL of 9.4 centimorgans between 7.75 and 17.15 centimorgans on chromosome 2 (Bentolila et al., 2008). By definition, the likelihood of finding the QTL in this interval is 10 times higher than finding the QTL outside the interval. The physical coordinates corresponding to this genetic interval were estimated to be 559 and 2,478 kb, thus determining a region of 1,919 kb comprising 503 genes. In order to reduce the size of this interval, we found recombinants in the pool of 70 RILs that had not been used in the original QTL mapping. The additional RILs considerably shortened the interval to 107 kb. We further restricted this interval by selecting 11 recombinants among a population of 691 F2 plants through the use of cleaved-amplified polymorphism markers (Fig. 2).

As a result of this fine mapping, the interval containing the QTL controlling *nad2*-558 editing could be precisely bracketed between the crossover that took place in the individuals 4B8 on the left side and 1A5 and 4A4 on the right side (Fig. 2). Sequencing the PCR products containing the crossovers for these recombinants demonstrated that the interval containing the editing factor could be delineated by the physical coordinates 1182057 and 1185817. At this position lies only one annotated gene, At2g03880, which we have named *REME1*.

One of the fine-mapping recombinants we obtained, 4B8, is recombined inside the *REME1* gene; the first 5′ 498 nucleotides were inherited from L*er*, while the remaining nucleotides from position 666 to the end of the gene were inherited from Col, with crossing over taking place in between positions 498 and 666. This recombinant, which exhibits the Col editing phenotype, reveals which part of the gene is involved in the control of *nad2*-558 editing. Of the 15 nonsynonymous single nucleotide polymorphisms (SNPs) found be-

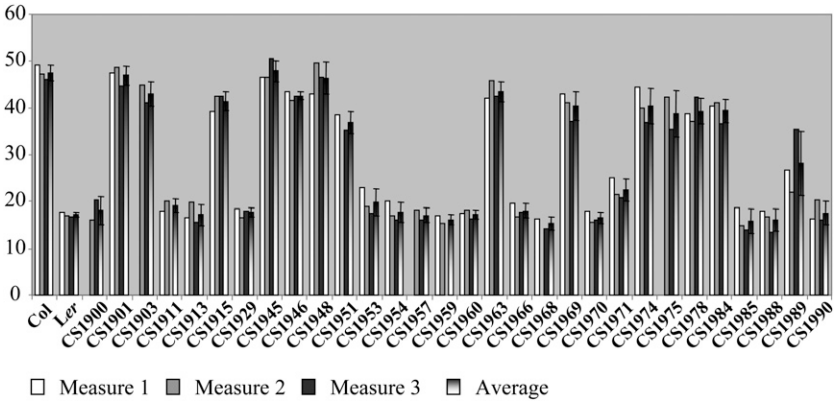

Figure 1. Distribution of *nad2*-558 editing extent in 30 RILs and their progenitors, Col and L*er*. The average of editing values was obtained for each inbred line by assaying three different plants, except for CS1900, CS1903, CS1911, CS1951, CS1957, CS1959, CS1968, and CS1975, where only two measures are available.

tween the Col and the L*er* allele for *REME1*, one or more of the seven SNPs located downstream of the crossover found in the recombinant are implicated in differential editing of the parental alleles. Any one of the individual SNPs or any combination of them could be responsible for the differential editing of the parental alleles.

Confirmation of the Role of REME1 in *nad2*-558 Editing by Virus-Induced Gene Silencing

The positional cloning identified *REME1* unambiguously as the *nad2*-558 editing QTL. As expected, virus-induced gene silencing (VIGS) of *REME1* resulted in a significant decrease of *nad2*-558 editing ($P < 5 \times 10^{-5}$; Fig. 3A). The silencing vector (Robbins et al., 2009) contains a sequence of the gene to be silenced and a GFP sequence, so that we can visually screen for silenced plants after inoculating Arabidopsis plants carrying a GFP transgene. Under UV light, the silenced plants show a characteristic red phenotype resulting from chlorophyll autofluorescence, while unsilenced plants fluoresce green. Poisoned primer extension (PPE) assay on transcripts from uninoculated versus silenced plants shows that silencing of *REME1* results on average in a 29% decrease in *nad2*-558 editing [(49 – 35)/49 = 0.29; Fig 3A]. Because the QTL controlling the editing of *nad2*-558 roughly colocalizes with the one controlling the editing of *orfX*-144 (Bentolila et al., 2008), we tested whether *REME1* was also involved in the editing of *orfX*-144. No significant reduction of *orfX*-144 editing was observed in *REME1*-silenced plants (Fig. 3B; $P = 0.16$), thus indicating that *orfX*-144 editing is under the control of another gene.

REME1 Controls the Editing of Other Mitochondrial Sites

Because editing of *nad2*-558 is silent (editing does not change the encoded amino acid sequence), this site is likely to have no physiological importance. Why should silent editing occur? One possibility is that the trans-factor encoded by *REME1* operates on a non-silent site and also happens to interact with *nad2*-558 because of a fortuitous similarity in recognition cis-sequences between the two sites. This hypothesis was tested by comparing the electrophoretograms obtained from the bulk sequencing of reverse transcription (RT)-PCR products of a silenced plant and a control plant for the entire set of mitochondrial transcripts. In order to have enough material to produce the RT-PCR products for the whole set of mitochondrial genes, the RNA that was assayed for editing extent came from either a mixture of two control plants or a mixture of two silenced plants. Before conducting the bulk sequencing screen, we verified that a mixture of two silenced plants could be distinguished from a mixture of two control plants when sequencing electrophoretograms around *nad2*-558 were compared (Supplemental Fig. S1). As shown in Supplemental Figure S1, there is a noticeable difference between the T peaks of control versus silenced plants. Thus, we can conclude that the bulk sequencing assay can detect a 30% differential in editing extent between RNA samples.

Using RT-PCR and bulk sequencing, we screened the entire 439 C residues known to be edited in suspension cell transcripts of 34 mitochondrial genes for possible differences between the control and the silenced plants (Table I). We also analyzed C targets reported to lie in untranslated regions (UTRs) and in introns (Table I). As we reported previously (Bentolila et al., 2008), tissue specificity of editing results in the absence of editing in rosette leaves for 43 sites previously reported to be edited in suspension cells (Giegé and Brennicke, 1999). Conversely, 56 new sites were found to be edited in rosette leaves that were not reported in suspension cells, leading to the survey of a total of 452 mitochondrial sites in coding sequences (Table I). If a putative difference in editing extent between control and silenced pools was detected in the screen, editing efficiency at that site was then assayed by PPE, which is more time consuming but more reliable and quantitative than bulk sequencing. Because of the labor associated with the PPE assay, only a subset of the differentially edited sites was further checked by PPE. The lower accuracy of bulk sequencing for measuring editing extent is illustrated in Sup-

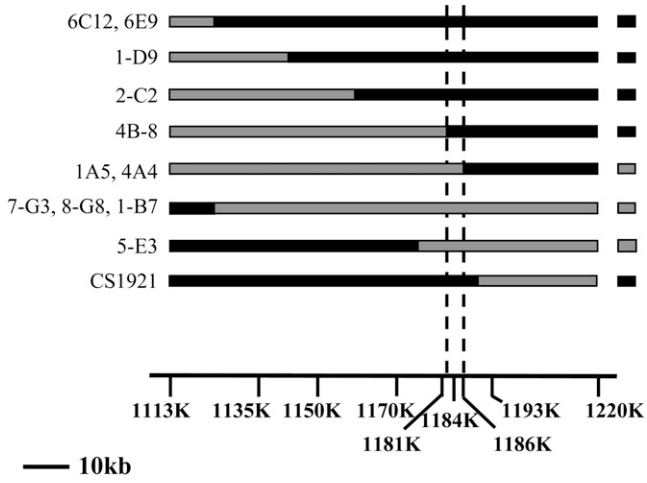

Figure 2. Positional cloning of *REME1*, which controls *nad2*-558 editing. The graphical genotypes of the recombinants between 1113K and 1220K are represented by squares with either a black background (Col) or a gray background (L*er*). The names of the recombinants are given on the left of each graphical genotype, with the *nad2*-558 editing value symbolized by a square at the right of the genotype (black for Col, gray for L*er*). Below the graphical genotypes is drawn a map of the area with markers named after their physical coordinates. The location of the gene At2g03880 (*REME1*) that controls *nad2*-558 editing was bracketed by the recombination points occurring in 4B8 and 1A5 and 4A4.

plemental Figure S1, where the heights of the T peaks for *nad2*-558 for the control and silenced pools show a difference that does not reflect the real contrast in editing extent by PPE (53% versus 31%, respectively). Our PPE measurements were performed on several independent silenced and control plants in order to obtain sufficient data for testing the statistical significance of the differential editing extent (Table I).

Among all the edited sites surveyed, one site showed a noticeable reduction in editing extent between control and silenced pools when comparing their respective electrophoretograms (Fig. 4A; Supplemental Fig. S2). When measured on the individual plants used for *nad2*-558, the C target of editing at position 552 on *orfX*, the tatC homolog, shows a very significant reduction in editing extent in *REME1*-silenced plants ($P < 3 \times 10^{-6}$; Table I; Fig. 4B). The average decrease in editing extent of *orfX*-552 found in *REME1*-silenced tissues, approximately 35%, is similar in range to the decrease observed for *nad2*-558. Like *nad2*-558, editing of *orfX*-552 results in a silent codon change. No nonsilent site was found to exhibit a reduction in editing extent in silenced leaf tissue. While editing of *orf240A*-199, a nonsilent site, appeared to exhibit lower editing on bulk-sequencing electrophoretograms, the reduction in editing extent was not statistically significant when assayed by PPE (Table I).

A surprising result from the bulk-sequencing screen is the finding of 42 sites for which editing appeared to be higher in silenced tissues than in control tissues. Overall, 23 sites that appeared differentially edited in silenced versus uninoculated plants by bulk sequencing were further assayed by PPE; the difference in editing was observed to be not significant for seven of these sites, allowing an estimation of 30% as the rate of false positives in the bulk-sequencing screen (Table I). Using this correction factor, we conclude that *REME1* silencing results in decreased editing in 1.4% of the sites and increased editing in 6.5% of the C targets known to exist in the mitochondrial transcripts. Strikingly, 81% (34 of 42) of the sites showing an increase in editing extent in *REME1*-silenced tissue result in codon changes that alter the encoded amino acid. This proportion closely fits the overall distribution of nonsilent sites in the population of edited sites in the coding sequences of mitochondrial genes (85% = 384 of 452). In contrast, no nonsilent sites were found to exhibit lower editing extent following VIGS of *REME1*.

The magnitude of the increase in editing of some C targets in VIGS tissue is rather pronounced. For example, the edited C at position 1,771 in *matR* exhibits an increase of 22% (13 of 60) in silenced tissues ($P < 8 \times 10^{-5}$; Table I; Fig. 5A). Not all the sites increased in editing are in coding regions; a site located in the 3′ UTR of *rpl5* exhibited a significant increase in editing extent in VIGS plants (Table I).

Increased editing extent in silenced tissues tends to affect several sites within transcripts of the same mitochondrial gene, although not all C targets in the

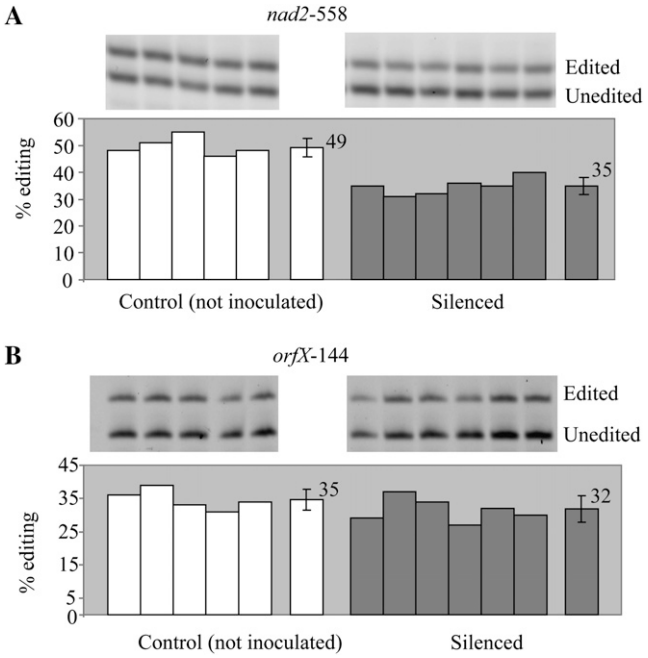

Figure 3. VIGS of *REME1* results in decreased editing of *nad2*-558 (A) but does not affect the editing extent of *orfX*-144 (B). The gel of the PPE products is shown on top of each panel with the quantification of editing below each corresponding lane. On the right of each group is the average with SD. In A, the intensity of the top band, representing editing products, is lower in silenced plants than in the control plants.

Table I. *Number and position of sites showing a differential editing extent in REME1-silenced Arabidopsis rosette leaves*

Sites in boldface are nonsilent sites whose editing results in an amino acid change. NS, Not significant.

Gene	Sites Edited	Control > Silenced		Silenced > Control		Checked by PPE	
		No.	Position[a]	No.	Position[a]	Position	*P*
Coding sequences							
atp1	4	0		0			
atp6-1	1	0		0			
atp9	4	0		0			
ccb203	11			2	**208**, **320**	208	NS
ccb206	37	1	406	1	**80**	80	NS
ccb256	29	1	624	4	**184**, 252, **421**, **673**	184	2×10^{-2}
						421	4×10^{-3}
ccb382	14	0		1	**709**	709	NS
ccb452	19	0		2	**1,246**, **1,280**	1,246	NS
						1,280	5×10^{-2}
cob	10	0		0			
cox2	13	0		0			
cox3	7	0		0			
matR	10	0		7	**374**, **461**, 1,593, 1,731, **1,771**, **1,807**	374	5×10^{-5}
						461	5×10^{-4}
						1,771	8×10^{-5}
						1,807	3×10^{-5}
nad1	22	0		0			
nad2	28	1	558[b]	0		558	4×10^{-6}
nad3	11			1	149		
nad4	38	1	1401	0			
nad4L	10	0		0			
nad5	30	0		0			
nad6	11	0		0			
nad7	31	1	1137	0			
nad9	7	0		0			
orf114	2	0		1	327	327	5×10^{-2}
orf240A	1	1	**199**	0		199	NS
orf25	9	0		0			
orfB	0	0		0			
orfX	34	2	552, 586	1	**97**	552	3×10^{-6}
rpl16	6	0		0			
rpl2	3			1	**212**	212	5×10^{-2}
rpl5	10			6	58, **64**, **92**, **169**, **317**, **329**	92	5×10^{-5}
						329	3×10^{-4}
rps12	8	0		0			
rps14	2			1	99, **194**	194	2×10^{-4}
rps3	12	1	603	4	**64**, **887**, 1,344, **1,534**		
rps4	17	0		10	**77**, **88**, **175**, **235**, **967**, **992**, **1,042**, **1,043**, **1,052**, **1,057**	967	2×10^{-3}
rps7	1	0		0			
Total	452	9		42			
UTRs and introns							
cox3 trailer	4	0		0			
nad5 intron	2	1	803	0		803	NS
nad6 leader	1	0		0			
nad7 leader	1	1	−39	0		−39	NS
nad7 second intron	1	0		0			
rpl5 trailer	1	0		1	+195	+195	2×10^{-2}
rpl16 trailer	2	0		0			
rps7 leader	1	0		0			
Total	13	2		1			

[a]Position of the edited C in the coding sequence is given relative to the start codon. In leader and trailer, the position of the edited C is relative to the start codon and the stop codon, respectively. [b]*nad2*-558 is the site used to map *REME1*.

same transcript exhibit increases to the same extent. For example, PPE demonstrated that *matR* possesses three sites with over a 10% increase in silenced tissues; nevertheless, another C target in this transcript, *matR*-461, exhibited only a 6% increase in silenced tissues (Fig. 5B). Similarly, the *rpl5* site at position 92 exhibits a

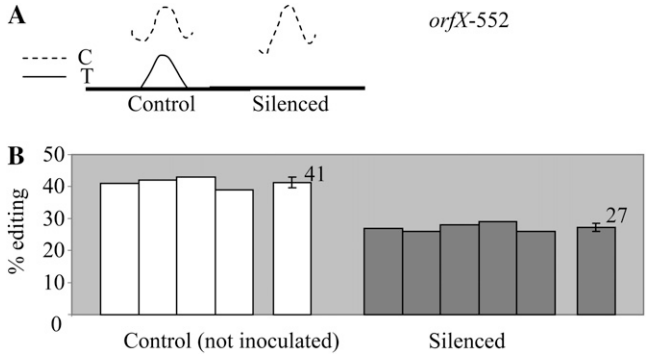

Figure 4. VIGS of *REME1* results in decreased editing of *orfX*-552. A, Comparison of the sequencing electrophoretograms of *orfX* RT-PCR products obtained from control and silenced pools shows a difference in the height of the T peak at position 552. B, PPE assay shows a significant ($P < 3 \times 10^{-6}$) difference in the editing extent of *orfX*-552 between several independent control and silenced plants. On the right of each group is the average value with SD.

15% increase in silenced tissues, while the increase is only 2% at position 329 ($P < 5 \times 10^{-5}$ versus $P < 3 \times 10^{-4}$, respectively; Table I; Supplemental Fig. S3).

Increased Editing Extent in *REME1*-Silenced Tissues Comes from the Virus Inoculation and from the Specific Silencing of *REME1*

We were concerned that the increase in editing extent observed in *REME1*-silenced tissues for certain mitochondrial sites might be an artifact caused by the VIGS technique; it is conceivable that the virus inoculation could stress the plant and as a result impact the editing machinery. The controls in the first set of silencing experiments were not inoculated plants and thus could not control for the possibility of an effect of virus inoculation on the editing extent. Therefore, we embarked on a new set of *REME1*-silencing experiments where controls for virus inoculation were performed by inoculating the plants with a vector harboring the GFP but no part of any plant gene (referred to empty vector in Fig. 6 and Supplemental Fig. S4; for more detail on the construction of the empty vector, see "Materials and Methods"). Because the PPE technique is rather time consuming, we focused our analysis on *matR*-1771, the site that showed the most pronounced increase in editing extent in the previous *REME1*-silencing experiments.

As shown in Figure 6A, the 13% [(77 – 68)/68] increase in editing extent for *matR*-1771 observed in *REME1*-silenced plants relative to noninoculated plants can be partitioned into two components: 6% is attributable to the virus inoculation (empty vector versus noninoculated; Fig. 6A; $P < 2 \times 10^{-2}$), while 7% comes specifically from *REME1* silencing (empty vector versus *REME1* silenced; Fig. 6A; $P < 7 \times 10^{-3}$). The main result from this experiment is the significance of the specific effect of *REME1* silencing on the increase of *matR*-1771 editing extent; another outcome is the significant effect of the virus inoculation on the editing extent. The same observations were made for *nad2*-558, whose editing extent is decreased by *REME1*-VIGS: a part of the decrease can be specifically attributed to *REME1* silencing, while another part comes from the virus inoculation itself (Supplemental Fig. S4A). The difference between *nad2*-558 and *matR*-1771 is the different role played by *REME1*-specific silencing in the overall difference between silenced plants and noninoculated plants (two-thirds for *nad2*-558 versus one-half for *matR*-1771; Fig. 6A; Supplemental Fig. S4A). All these observations were also found to be valid for *orfX*-552 editing extent (data not shown).

The efficiency of silencing in this new experiment seemed to be reduced when compared with the previous experiment, resulting in a variation in editing extent less pronounced when comparing the noninoculated control plants with the *REME1*-silenced plants. For instance, the increase in editing extent for *matR*-1771 is 13%, whereas it was observed to be 22% previously (compare Figs. 6A and 5A). Similarly, the decrease in editing extent for *nad2*-558 is less marked, 18% versus 29% (compare Supplemental Fig. S4A and Fig. 3A). The reason for this drop in silencing efficiency is unclear, but it led us to repeat this experiment in order to see whether results could be affected by the silencing efficiency.

In an independent experiment, the efficiency of silencing was found to be similar to the original *REME1* silencing: the increase in editing of *matR*-1771 averaged 25% [(76 – 61)/61] in *REME1*-silenced plants versus noninoculated plants (Fig. 6B; $P < 2 \times 10^{-5}$),

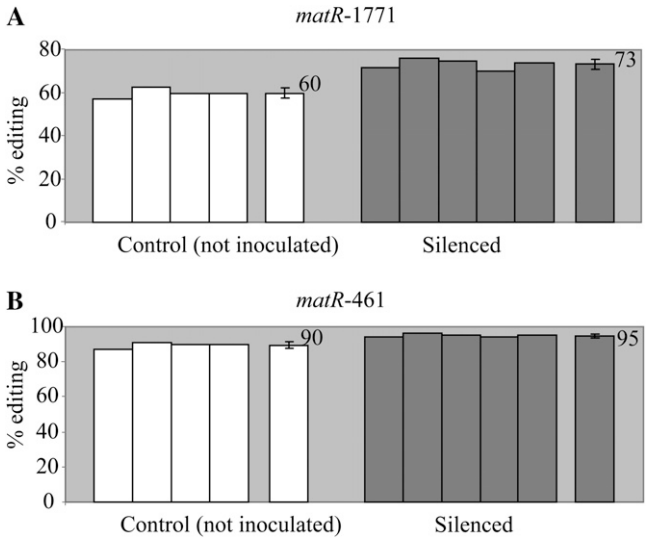

Figure 5. VIGS of *REME1* results in increased editing of sites found in *matR* as detected by PPE assay. A, Editing extent of C at position 1,771 is significantly increased in silenced plants ($P < 8 \times 10^{-5}$). B, The increase in editing is less pronounced for the edited C at position 461 ($P < 5 \times 10^{-4}$). On the right of each group is the average editing extent with SD.

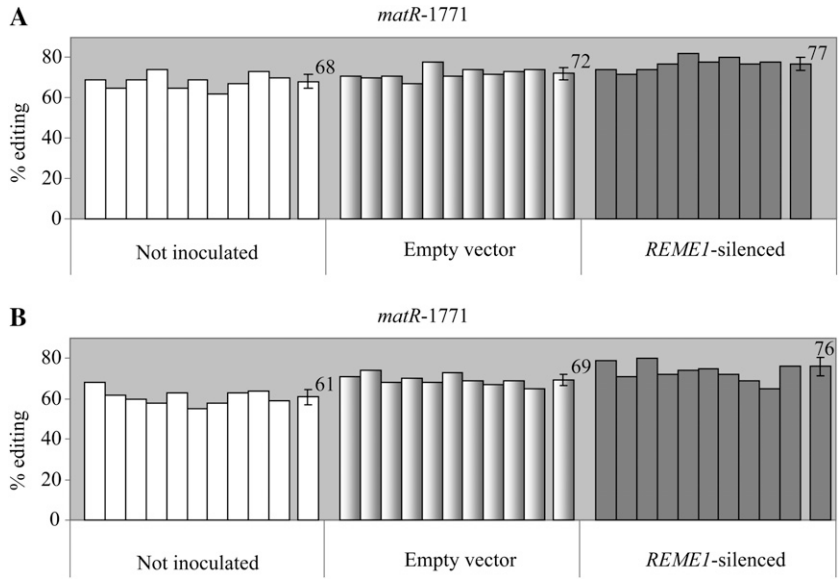

Figure 6. Increase in editing extent of *matR*-1771 in *REME1*-silenced plants comes from the virus inoculation and the specific silencing of *REME1*. Editing extents were assayed by PPE. The effect of virus inoculation can be estimated by comparing the noninoculated plants with the empty vector controls; the specific effect of *REME1* silencing can be estimated by comparing the empty vector control with the *REME1*-silenced plants. A and B show data from two independent experiments. In both experiments, about half the increase of editing extent in *REME1*-silenced plants can be attributed to the specific silencing of *REME1*, the other half being the result of the virus inoculation. On the right of each group is the average editing extent with SD.

whereas the decrease for *nad2*-558 averaged 28% (Supplemental Fig. S4B; $P < 3 \times 10^{-7}$). We can consider the silencing efficiency of this latter experiment to be comparable to the original one because the variation in editing extents for both *matR*-1771 and *nad2*-558 are very close (25% versus 22% and 28% versus 29%, respectively). Again, the overall increase in editing extent of *matR*-1771 in *REME1*-silenced plants can be split into roughly equal parts between the effect of virus inoculation (13%; $P < 2 \times 10^{-5}$; Fig. 6B) and the specific silencing of *REME1* (12%; $P < 3 \times 10^{-2}$; Fig. 6B). The same material was used to assess *nad2*-558 editing extent. Unlike *matR*-1771, there was no effect of virus inoculation on *nad2*-558 editing extent (compare not inoculated with empty vector controls in Supplemental Fig. S4B). In the previous experiment, the effect of virus inoculation on *nad2*-558 editing extent, although half the specific effect of *REME1* silencing, was still significant ($P < 2 \times 10^{-2}$; Supplemental Fig. S4A). The reason for the absence of a virus inoculation effect on *nad2*-558 editing extent in the latter experiment is unclear but was also observed for *orfX*-552 (data not shown).

The main conclusion to be drawn from this new set of *REME1*-silencing experiments where an empty vector control was included is the reproducible and significant effect of *REME1*-specific silencing on the increase of *matR*-1771 editing extent. In addition, a decrease in editing extent of *nad2*-558 and *orfX*-552 was also confirmed in *REME1*-silenced plants.

REME1 Encodes a PPR-DYW Protein

REME1 encodes a PPR-DYW protein that is predicted to be targeted to mitochondria by both Predotar (Small et al., 2004) and TargetP (Emanuelsson et al., 2000) and thus is another example of a member of this subclass of PPR protein implicated in mitochondrial editing (Kim et al., 2009; Zehrmann et al., 2009; Verbitskiy et al., 2010). Although prediction softwares agree on REME1 targeting to mitochondria, and its effect on mitochondrial editing substantiates this prediction, REME1 (At2g03880) was reported to be targeted to chloroplasts (Lurin et al., 2004). Therefore, we examined REME1 subcellular localization in a transient assay where onion (*Allium cepa*) epidermal cells were bombarded with a vector expressing the first 100 amino acids from the REME1 N terminus attached to GFP. A mitochondrial marker was supplied by bombarding the epidermal cells with a vector expressing the cherry protein attached to a mitochondrial transit peptide. Our colocalization results clearly indicate that REME1 is targeted to mitochondria (Supplemental Fig. S5).

Like 80% of the Arabidopsis PPR-containing genes, the gene model for *REME1* contains only one exon, a model that is supported by the isolation of R20567, a full-length cDNA in the RIKEN collection (Seki et al., 2002). The transit peptide length is predicted to be 76 amino acids by TargetP. The modular structure of REME1 (59-P-S-P-32-S-P-33-S-P-L2-S-4-E-E+-DYW) was defined by interrogating the PROSITE database (Fig. 7A) and is in very good agreement with the predicted structure by searching The Institute for Genomic Research (TIGR) protein families. The coordinates of the PPR motifs are shifted by two amino acids in the TIGRFAM output, because the PPR consensus used by TIGRFAM is shifted by two amino acids relative to the PROSITE consensus (Haft et al., 2001; de Castro et al., 2006).

Identification of a L*er* Mutant with a T-DNA Insertion in *REME1*

We looked for an insertional mutation in At2g03880 (*REME1*) in collections of T-DNA mutants. Among several T-DNA insertional mutants present in the SALK

T-DNA express browser (http://signal.salk.edu/cgi-bin/tdnaexpress), only GT_5_23135 presented an insertion within the *REME1* coding sequence. Sequencing of the PCR amplicon obtained from the GT_5_23135 mutant allele confirmed that the insertion was located at nucleotide position 1,025, corresponding to the middle of the fourth P repeat (Fig. 7A). Monitoring *REME1* expression by RT-PCR in GT_5_23135 homozygous mutants demonstrated that the T-DNA insertion prevents the accumulation of transcripts carrying *REME1* sequence downstream of the insertion (Fig. 7B). If translated, the encoded product by the mutant allele will be shortened to a 384-amino acid protein versus 630 amino acids for the wild-type *REME1* allele. The mutant plants show no detectable phenotypic defects and are absolutely indistinguishable from their wild-type siblings when grown in growth room conditions (data not shown).

Assaying editing extent in the GT_5_23135 population showed a very significant reduction in editing extent for both *nad2*-558 and *orfX*-552 in homozygous mutants relative to the wild type ($P < 9 \times 10^{-19}$ and $P < 2 \times 10^{-15}$, respectively; Fig. 8). Two sites that showed the most pronounced increase in editing extent upon *REME1* silencing, *matR*-1771 and *rpl5*-92 (Table I; Figs. 5 and 6), showed moderate but nevertheless significant increases in editing extent in homozygous mutants versus the wild type ($P < 6 \times 10^{-4}$ and $P < 4 \times 10^{-6}$, respectively; Fig. 8).

The accession used to generate GT_5_23135 is L*er*, which is the parent that exhibits low editing of *nad2*-558, Col being the more highly edited parent (Fig. 1). Knocking out the expression of the *REME1* L*er* allele, which has a weaker effect than the Col allele, has a lesser impact on editing extent than knocking down the expression of the Col allele by VIGS. Thus, the difference in editing extent between the mutant and the wild-type plants is smaller than the difference between the silenced plants and the control plants (11% versus 14% and 6% versus 14% for *nad2*-558 and *orfX*-552, respectively; Figs. 3, 4, and 8). Because the only available mutant is in the weaker L*er* allele, we chose the silenced tissue over the mutant tissue in our screen for other mitochondrial sites controlled by REME1. The weaker *REME1* L*er* allele also made the mutant tissue less useful than silenced tissue for testing the inhibitory effect of REME1 on editing. Although the difference in editing extent observed between Col silenced tissues and control plants (empty vector) could reach 7% for *matR*-1771 (Fig. 6B), in GT_5_23135 homozygous mutants, the difference from wild-type plants is only 4% (Fig. 8). This rather moderate but significant increase in GT_5_23135 homozygous mutants can be rationalized based on the comparison of decrease in editing extent for *nad2*-558 and *orfX*-552 between silenced and mutant tissues (14% versus 11% and 14% versus 6%, respectively). The expected difference between the

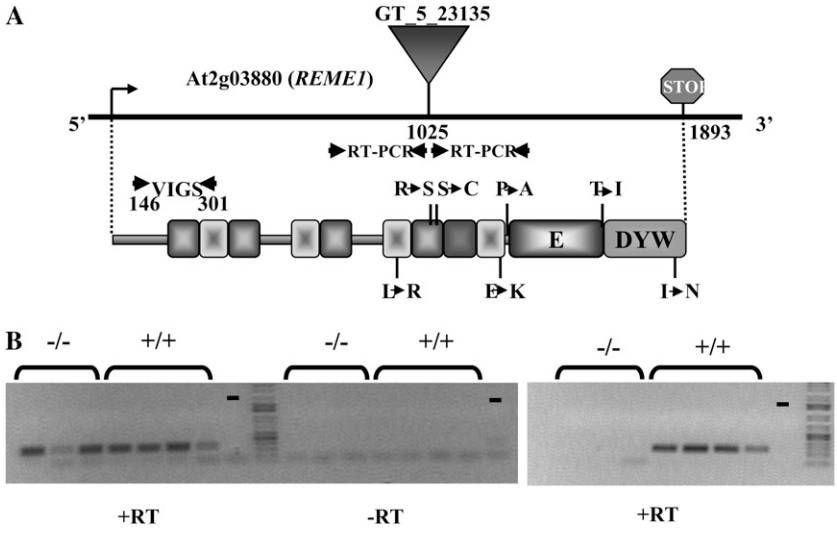

Figure 7. *REME1* encodes a PPR-DYW protein. A, Localization of the T-DNA insertion in the At2g03880 locus and structure of the predicted REME1 protein. The 156-nucleotide region of At2g03880 RNA targeted by VIGS is delimited by facing arrows. Below the gene model is represented the modular structure of the predicted REME1 protein. Each PPR is symbolized by a square with different shading for each form of the PPR motif: P (gray), S (light gray), and L2 (dark gray). The coordinates of each PPR were defined by scanning the PROSITE and the TIGRFAM databases. Amino acid polymorphisms between the products encoded by Col and L*er* alleles are indicated above and below the protein model. Only the amino acid polymorphisms implicated in the differential editing ability of the parental products are shown. The primers used to assess *REME1* expression by RT-PCR in the GT_5_23135 mutant are represented by facing arrows upstream and downstream of the site of insertion. B, T-DNA insertion in GT_5_23135 prevents transcription from occurring downstream of the insertion, as shown by the absence of RT-PCR products in the homozygous mutants (right panel), whereas transcription is not affected upstream of the insertion (left panel).

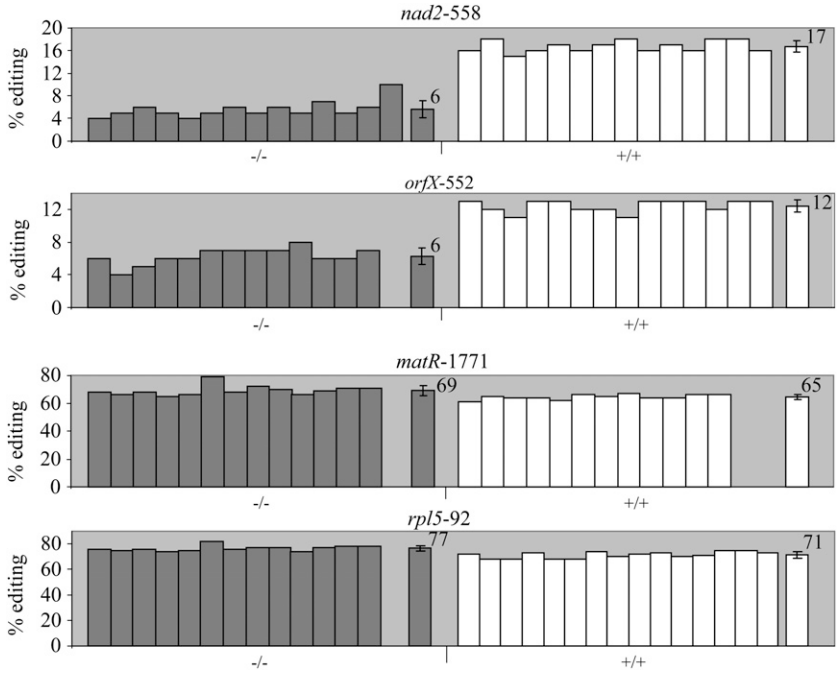

Figure 8. Effect of T-DNA insertion in *REME1* on editing extent in *nad2*-558, *orfX*-552, *matR*-1771, and *rpl5*-92. Editing extents were assayed by PPE. T-DNA insertion in *REME1* coding sequence results in decreased editing of *nad2*-558 and *orfX*-552 in mutant GT_5_23135 (−/−; $P < 9 \times 10^{-19}$ and $P < 2 \times 10^{-15}$, respectively); an opposite effect of the insertion is observed on the editing extent of *matR*-1771 and *rpl5*-92, which are increased in GT_5_23135 (−/−; $P < 6 \times 10^{-4}$ and $P < 4 \times 10^{-6}$, respectively). On the right of each group is the average editing extent with SD.

wild type and homozygous mutants for *matR*-1771 editing extent should range between 5.5% (7 × 11/14) and 3% (7 × 6/14), which is in rather good agreement with the observed 4%.

The *REME1* Col Allele Boosts *nad2*-558 and *orfX*-552 Editing Extent in the L*er* Background

To further prove the function of REME1 in mitochondrial editing, we used a binary vector harboring the *REME1* Col allele (high editor) under the control of a 35S promoter to transform the L*er* accession (low editor). After floral dip transformation, the seeds were harvested and germinated on selective medium. Twenty-three putative transformants were transferred into soil and further assayed for editing extent in *nad2*-558, *orfX*-552, *matR*-1771, and *rpl5*-92. Among the 23 putative T0 L*er* plants, three plants (individuals 5, 10, and 16) did not show a difference in editing extent for either *nad2*-558 or *orfX*-552 with the L*er* parental accession (Fig. 9A). These plants represent either nontransformed material escaping from the selective medium or transgene-silencing events. The remaining 20 T0 plants show an editing extent for both *nad2*-558 and *orfX*-552 that exceeds the one exhibited by L*er*, thus demonstrating that these plants are truly transgenic material and that both sites are controlled by REME1 (Fig. 9A). The very high and positive correlation ($r^2 = 0.95$) between *nad2*-558 and *orfX*-552 editing extent in the T0 population supports the model of control of these two sites by the same mitochondrial editing factor (Fig. 9B).

An interesting outcome of this experiment is the transgressive phenotype exhibited by the majority of the transgenic plants: 15 and 17 of the T0 plants show an editing extent that exceeds the one from Col for *nad2*-558 and *orfX*-552, respectively (Fig. 9A). Among those transgressive plants, individuals 1, 13, and 17 present the most extreme phenotype, with an *orfX*-552 editing extent twice the value of Col (Fig. 9A). The outperforming of the Col accession by the majority of the transgenic plants in relation to *nad2*-558 and *orfX*-552 editing extent could result from the 35S promoter used to drive the expression of the Col transgene. The 35S promoter sometimes drives high expression of the transgene, depending on the site of the transgene insertion in the genome. The variation in *nad2*-558 and *orfX*-552 editing extent observed in the T0 plants might thus result from variation in the level of expression of the transgene. We tested this hypothesis by assaying the level of *REME1* expression by semiquantitative RT-PCR in some of the transgenic plants showing differential *nad2*-558 editing extent (Fig. 10). There is a very clear relationship between *REME1* expression level and *nad2*-558 editing extent. The transgenic material exhibiting high *nad2*-558 editing extent exhibits a high level of *REME1* expression; conversely, transgenic plants with a low *nad2*-558 editing extent also exhibit a low level of *REME1* expression. Transgenic plants with an intermediate level of *nad2*-558 editing extent exhibit an intermediate level of *REME1* expression (Fig. 10). A control experiment in which a reference gene expression was assayed in a similar way on the same material (Supplemental Fig. S6) validates the variation in *REME1* expression level in transgenic material.

We also assayed *matR*-1771 and *rpl5*-92 editing extent in the T0 population to test whether a decrease could be induced by the expression of the Col allele in the less-edited L*er* background. A decrease in *matR*-1771 and *rpl5*-92 editing extent would be expected if

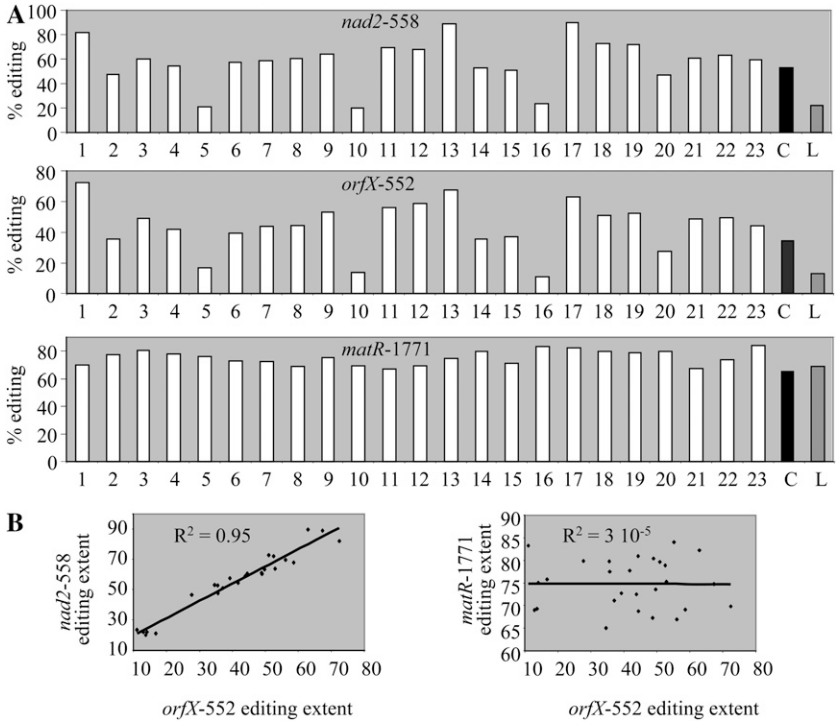

Figure 9. Overexpressing the REME1 Col allele in the L*er* background has an effect on editing extent of *nad2*-558 and *orfX*-552 but not *matR*-1771. A, Editing extent was assayed by PPE on 23 T0 transgenic plants and the two parental accessions Col (C) and L*er* (L). B, A very strong correlation between editing extent of *nad2*-558 and *orfX*-552 in T0 plants supports the control of both sites by REME1; the absence of correlation between editing extent of *orfX*-552 and *matR*-1771 does not support the inhibitory effect of REME1 on *matR*-1771 editing.

REME1 has an inhibitory effect on these editing sites, as was suggested by our previous VIGS and mutant experiments. As apparent in Figure 9A, none of the T0 plants shows a *matR*-1771 editing extent less than what is observed in L*er*. The same observation was made for *rpl5*-92 editing extent (data not shown). The absence of correlation between *orfX*-552 and *matR*-1771 editing extent casts doubt on a direct inhibitory function of REME1 on mitochondrial editing at that site (Fig. 9B). A negative correlation between *orfX*-552 and *matR*-1771 editing extent would be expected if the expression of REME1 results in inhibition of *matR*-1771.

REME1 Is Not Involved in *nad2* trans-Splicing

REME1 controls the editing of a C target in *nad2*, which encodes subunit 2 of the mitochondrial NADH ubiquinone oxidoreductase complex. Maturation of the *nad2* transcript requires four RNA splicing events, one of which involves trans-splicing of independently transcribed RNAs and concerns the second intron (Fig. 11A). Given that the edited C at position 558 is only 20 nucleotides upstream of the trans-splicing site, we investigated whether editing of this C might affect trans-splicing efficiency. Two sets of plants with marked differential levels of *nad2*-558 editing extent were assayed for a possible difference in the amount of transcripts with or without the second intron spliced. Semiquantitative RT-PCR with appropriate primers was performed using RNAs from +/+ and −/− siblings from the GT_5_23135 population and two transgenic T0 plants, individuals 1 and 16, that exhibit a very different *nad2*-558 editing extent (Fig. 9A). The amount of cDNA used as a template for the quantitative PCR was adjusted to be similar between +/+ and −/− GT_5_23135 siblings (2.75 ng) and between T0 individuals 1 and 16 (3.5 ng). If a link existed between *nad2*-558 editing extent and trans-splicing of the second intron, then the amount of second intron-spliced cDNAs should be higher for +/+ versus −/− and for individuals 1 versus 16. Conversely, the amount of second intron-adjoining cDNAs should be lower for +/+ versus −/− and for individuals 1 versus 16. A more abundant template cDNA would in turn result in detection of the amplified product earlier during the cycles of amplification. No significant difference in the amount of intron-spliced and intron-adjoining cDNAs for the second intron was found between the low-edited and high-edited plants tested, indicating that *REME1* does not indirectly control trans-splicing through its action on *nad2*-558 editing (Fig. 11B).

The similarity in the amount of starting template for the RT-PCR was validated by comparing the intron-spliced and intron-adjoining amplified products for the third intron of *nad2*. No detectable difference in the abundance of cDNAs with or without the third intron was observed between +/+ and −/− GT_5_23135 or between transgenic individuals 1 and 16 (Fig. 11B). Nevertheless, the slightly more abundant cDNA material for individuals 1 and 16 relative to +/+ and −/− (3.5 versus 2.75 ng, respectively) was enough to result in more abundant third intron-amplified products (e.g. intron-spliced product is absent at 19 cycles for −/− and +/+ but readily detectable for individuals 1 and 16; Fig. 11B). However, we cannot rule out an effect of *REME1* on trans-splicing that is too small to be

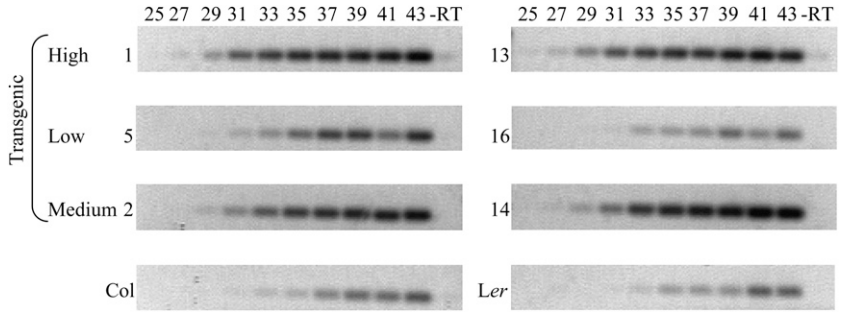

Figure 10. Editing of *nad2*-558 is positively correlated with the level of *REME1* expression in transgenic plants but not in the parental accessions. RT-PCR products of *REME1* transcript were amplified and electrophoresed on 1% agarose gels after completion of the number of cycles indicated above each lane. –RT indicates a negative control in which the reverse transcriptase was omitted and was performed at 43 cycles. On the left of each gel is indicated the origin of the RNA used for the RT-PCR: 1, 13, 5, 16, 2, and 14 are T0 L*er* plants expressing the *REME1* Col allele and showing high, low, and medium levels of *nad2*-558 editing extent, as indicated. The same amount of template RNA (50 ng) was used for each RT reaction. In transgenic material, the correlation between the abundance of the *REME1* RT-PCR product and the level of *nad2*-558 editing is readily observable at 29 and 31 cycles as follows: 1, 13 > 2, 14 > 5, 16. In contrast, no obvious difference in the abundance of *REME1* RT-PCR is apparent between Col and L*er* (similar intensity of the bands at 31 and 33 cycles).

detected by semiquantitative RT-PCR. In mock experiments, we were able to detect, with good reproducibility, a 3- to 4-fold reduction of the trans-spliced product, a range comparable to the difference in *nad2*-558 editing extent between −/− and +/+ GT_5_23135 siblings and high-edited or low-edited transgenic individuals (Supplemental Fig. S7).

DISCUSSION

Mapping of Editing QTLs Can Lead to the Identification of Mitochondrial Site-Specific Editing Factors

Our identification of a mitochondrial RNA editing QTL in 2005 demonstrated the promise of natural variation for the identification of editing factors (Bentolila

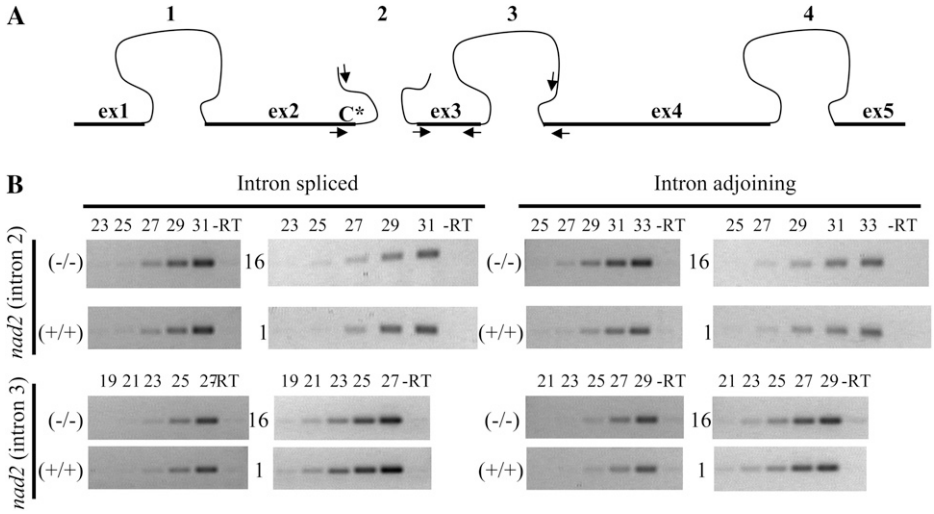

Figure 11. Editing of *nad2*-558 and trans-splicing of the second intron are not coupled. A, Schematic representation of *nad2* pre-mRNA and primers used in the semiquantitative RT-PCR. Exons are represented by thick bars and are drawn to scale. The maturation of *nad2* involves three cis-splicing events (1, 3, and 4) and one trans-splicing event (2). Primers used in the semiquantitative RT-PCR are represented by facing arrows. Targeted C for editing at position 558 is represented by C*. B, Semiquantitative RT-PCR of different portions of *nad2* transcript. Intron-spliced and intron-adjoining RT-PCR products for the second and third introns were amplified and electrophoresed on 2% agarose gels after a completed number of cycles indicated above each lane. –RT indicates a negative control in which the reverse transcriptase was omitted and was performed for each combination of primers at the maximum number of cycles (e.g. 31 for intron 2 spliced). On the left of each gel is indicated the origin of the RNA used for the RT-PCR: (−/−) and (+/+) are siblings from the GT_5_ 23135 population with or without the T-DNA insertion in *REME1*, and 16 and 1 are T0 L*er* plants expressing the *REME1* Col allele and showing low and high *nad2*-558 editing extent, respectively.

et al., 2005). Subsequently, our group (Bentolila et al., 2008) and another group (Zehrmann et al., 2008) identified a number of variations in the extent of RNA editing in plant mitochondria between ecotypes of Arabidopsis. Our identification of an editing QTL by precise mapping to a single gene, *REME1*, illustrates the power of this approach.

Because a number of PPR proteins have now been found to be chloroplast or mitochondrial editing factors (Kotera et al., 2005; Zehrmann et al., 2009), an alternative approach to identifying such factors is analysis of mutants in genes predicted to encode organelle-targeted PPR motif-containing proteins. Analyses of homozygous T-DNA PPR gene insertional mutants have identified a number of chloroplast editing factors (Hammani et al., 2009; Okuda et al., 2009; Robbins et al., 2009), as only 34 C targets need to be analyzed in putative mutants to determine if editing extent is affected. Reverse genetics is a far more cumbersome approach for the identification of editing factors in plant mitochondria, where there are over 500 C targets. PPR proteins are involved in other aspects of organelle gene regulation (Schmitz-Linneweber and Small, 2008; Stern et al., 2010); thus, laborious measurements of editing at hundreds of sites in a mutant could result in the unsatisfying conclusion that a particular PPR protein is not involved in editing. Recently, a reverse genetics approach identified five E-class PPR proteins involved in Arabidopsis mitochondrial RNA editing (Takenaka et al., 2010). However, the assay used in that study covers only 269 annotated editing sites, roughly just over half the total of the annotated mitochondrial sites. This incomplete coverage could explain why the mitochondrial editing factors identified were reported to control only one site. Furthermore, homozygous mutations in some PPR editing factors are likely to be embryo lethal. In contrast, by starting with a QTL that affects editing efficiency between Arabidopsis accessions, whatever gene is identified as encoding the QTL will definitely be involved in RNA editing, and even factors that are essential for embryo viability can be identified.

This study showed VIGS to be an excellent substitute for T-DNA insertional mutants whenever the background used to generate such mutants has a weak allele. VIGS should also become the method of choice to assay a number of candidate genes for mitochondrial editing, because it is much faster and less labor intensive than growing mutant populations and characterizing them. Furthermore, some putative T-DNA insertional mutants either lack the insertion or have a misannotated location.

REME1 Is a Site-Specific PPR-DYW Editing Factor

With the identification of REME1 as a PPR-DYW protein, the number of PPR proteins reported to be involved in organelle editing has now increased to 28. Among the 15 PPRs known to affect chloroplast editing, four belong to the E subclass (Kotera et al., 2005; Okuda et al., 2007; Chateigner-Boutin et al., 2008; Hammani et al., 2009) and 11 belong to the DYW subclass (Zhou et al., 2008; Cai et al., 2009; Hammani et al., 2009; Okuda et al., 2009; Robbins et al., 2009; Yu et al., 2009). All the mitochondrial editing factors identified so far but one also belong to the PLS subfamily: six mitochondrial editing factors are members of the DYW-PPR subclass (Kim et al., 2009; Zehrmann et al., 2009; Takenaka, et al., 2010; Tasaki et al., 2010; Verbitskiy et al., 2010; this study), while six members of the E subclass were shown to be necessary for mitochondrial editing (Takenaka, 2010; Takenaka et al., 2010). A member of the P subfamily denoted PPR596 was reported to control mitochondrial editing efficiency at partially edited sites (Doniwa et al., 2010).

Extensive fine mapping allowed us to identify a plant exhibiting a recombination event within the *REME1* gene. This recombinant delineates the area responsible for the differential editing abilities of the product encoded by the two parental accessions, Col (high editor) and L*er* (low editor). Seven nonsynonymous SNPs between Col and L*er* lie in this area (Fig. 7) and thus are implicated either individually or in any possible combination in the differential editing ability of the accessions. However, the complementation analysis showed that the level of expression of *REME1* also has an impact on the editing extent of the target sites. Three transgenic plants showed a level of editing extent almost twice the one found in Col, the accession providing the transgene. This observation is not surprising given the common low level of PPR proteins (Lurin et al., 2004) and the titration of editing factors when overexpressing a target site (Chateigner-Boutin and Hanson, 2002). Thus, the differential editing extent of *nad2*-558 and *orfX*-552 between Col and L*er* could also come from a different level of *REME1* expression in the two accessions. However, a differential *REME1* expression level could not be detected between Col and L*er* by our semiquantitative RT-PCR methodology (Fig. 10). This result does not rule out the involvement of *REME1* expression in differential *nad2*-558 editing extent between the parental accessions, as a difference too small to be detected between Col and L*er* might still have an impact on their *nad2*-558 editing ability.

Among the 27 C residues edited in *nad2* transcripts, *REME1* silencing only affects one site, *nad2*-558. Similarly, *orfX*-552 and *orfX*-586 are the only C residues among 34 editing sites found on the *orfX* transcript whose editing extent appears to be reduced in *REME1*-silenced plants (*orfX*-586 was identified by bulk sequencing but not tested further by PPE; Table I). Like MEF1 (Zehrmann et al., 2009), MEF11 (Verbitskiy et al., 2010), OGR1 (Kim et al., 2009), and SLO1 (Sung et al., 2010), REME1 affects editing at multiple mitochondrial sites, thus reconciling the high number of mitochondrial targeted C residues in angiosperms (e.g. more than 500 in Arabidopsis) with the more limited number of PPRs belonging to the DYW or the E subclasses and predicted to be targeted to mitochon-

dria. How a single PPR protein can recognize multiple C targets of editing is unknown but could be related to some sequence similarity in the cis-elements commonly found 5′ to C targets of editing. High-level transgenic expression of some individual chloroplast transcripts results in reduced editing of multiple C targets, a phenomenon thought to be due to saturation of a limiting amount of a trans-factor responsible for specifying editing at more than one site (Chateigner-Boutin and Hanson, 2002). The chloroplast sites that are coinhibited following transcript overexpression exhibit limited sequence similarity, sometimes requiring introduction of gaps into the alignment (Chateigner-Boutin and Hanson, 2002). Similarly, the number of conserved nucleotides is limited to seven in the 15 nucleotides upstream of the edited C in *nad2*-558 and *orfX*-552 (Fig. 12). However, despite the limited nucleotide conservation, when purine or pyrimidine conservation was considered in the 15-nucleotide sequence upstream of the targeted C in chloroplasts, the targeting of multiple editing sites by single factors could be rationalized (Hammani et al., 2009). On the other hand, some mitochondrial editing sites sharing the same editing factor show a high sequence similarity in the 15 nucleotides upstream of the edited C; for instance 60% of the nucleotides from −1 to −15 relative to the edited C are identical between *nad4*-449 and *nad9*-328, both of which are under the control of SLO1 (Sung et al., 2010). The occurrence of high sequence similarity in the cis-element upstream of the edited C was also reported for *cox3*-422 and *nad4*-124, the targets sites of MEF11, and allowed the identification of a third target site by an in silico search for similar upstream sequences (Verbitskiy et al., 2010). No sequence similarity was found in the sequences surrounding the edited C residues whose editing extent is negatively regulated by REME1.

Multiple Factors May Control Editing Efficiency of Mitochondrial C Targets

Several lines of evidence indicate that single site-specificity factors do not completely control editing of individual mitochondrial C targets. Earlier, we reported that differential editing extent in Col and L*er* at some sites was under the control of at least two QTLs located at different positions on the genetic map (Bentolila et al., 2008). Zehrmann et al. (2009) reported a total lack of editing in *mef1* mutants for only two of the three mitochondrial sites under the control of MEF1. In *mef1* mutants, the editing extent of *nad2*-1160 is reduced to 20%. Perhaps another factor is able to provide the function needed for low-level editing of *nad2*-1160. In addition, the level of editing extent in the study by Zehrmann et al. (2009) was assayed by bulk sequencing, which is rather inaccurate, particularly in evaluating low editing extent. For instance, no edited peak for *orfX*-552 is detectable in the electrophoretogram of the *REME1*-silenced pool RT-PCR product (Supplemental Fig. S2), even though the editing extent was measured to be 26% by PPE. Similarly, editing extent of *nad4*-C433 in the rice *ogr1* mutant, a null mutant, is only reduced to 17%, but in that study, a reliable measurement was achieved by sequencing cDNA clones (Kim et al., 2009). In the *REME1* insertional mutant we studied, the location of the insertion results in a predicted truncated product of 384 amino acids that lacks the E and DYW domains and three of the nine PPRs. Even if this truncated protein accumulates, it is very unlikely to be functional; the E motif has been shown to be essential for several of the chloroplast RNA editing factors (Okuda et al., 2007, 2009). Thus, the residual editing activity in *REME1* homozygous mutant plants for both *nad2*-558 and *orfX*-552 may be explained by the existence of another factor active at these sites (Fig. 8).

Figure 12. Alignment of sequences around editable C residues and putative cis-elements in the sites controlled by REME1. Thirty nucleotides upstream and 10 nucleotides downstream of the target C are shown. The target C is shown in larger font, and the edited C residues are underlined. Letters in boldface represent conserved nucleotides, and blocks of conserved nucleotides are shaded.

REME1 Is a Positive Regulator and Might Be a Negative Regulator of Mitochondrial RNA Editing

All the approaches used in this study, positional cloning, VIGS, T-DNA insertional mutant, and complementation analysis, prove that REME1 is a positive regulator of mitochondrial editing for both *nad2*-558 and *orfX*-552. Unexpectedly, we observed that silencing of *REME1* results in increased editing at multiple C targets in Arabidopsis mitochondria. Thirteen targets were verified by the precise PPE assay as significantly increased in editing efficiency in mitochondrial coding regions in plants subjected to VIGS of *REME1*. However, repetition of the VIGS experiment incorporating an empty vector control showed that about half of the increase in editing extent for *matR*-1771 was due to the virus inoculation, while the remaining half was due to specific *REME1* silencing. This significant increase in editing extent upon *REME1*-specific silencing was

further supported by an increase observed in T-DNA insertional mutants. While these two lines of evidence support an inhibitory role for REME1 in mitochondrial editing, the complementation analysis did not. Even transgenic plants outperforming the Col accession (high editor) with regard to *nad2*-558 and *orfX*-552 editing extent did not show any editing extent reduction in the expected sites, *matR*-1771 and *rpl5*-92. The observed absence of any decrease in *matR*-1771 and *rpl5*-92 editing extent in plants overexpressing *REME1* would be expected if more than one editing factor positively controls these sites. This hypothesis is consistent with the fact that the *REME1* knockout mutant still exhibits residual editing extent for *nad2*-558 and *orfX*-552, implying that another editing factor can operate on these sites. According to this theory of multiple factors, only one of the editing factors that increases *matR*-1771 and *rpl5*-92 editing extent is subjected to the REME1 inhibiting effect; thus, a reduction in REME1 by VIGS would release only the factor susceptible to the REME1 inhibitor effect, hence increasing editing extent of *matR*-1771 and *rpl5*-92 in *REME1*-silenced tissues. On the other hand, overexpressing *REME1* would have no further inhibitory effect on editing, because the first factor susceptible to REME1 is already inhibited in the normal physiological state and the second, REME1-independent factor would still be free to function; hence, there is no change in *matR*-1771 and *rpl5*-92 editing extent.

Site-specific inhibition of editing by protein factors has previously been reported only once in plant organelles. PPR591, the only plant editing factor that belongs to the P subfamily of PPR-containing proteins, is a negative regulator of several mitochondrial sites that lie mostly on the *rps3* transcript (Doniwa et al., 2010). There is also precedence for editing inhibition in the apolipoprotein B (*apoB*) RNA editing system in mammals. Two RNA-binding proteins, GRY-RBP and CUGBP2, have been shown to inhibit *apoB* mRNA editing in vitro (Anant et al., 2001; Blanc et al., 2001). In addition, antisense inhibition of expression of GRY-RBP or CUGBP2 in McA cells led to a 2- to 3-fold increase in endogenous *apoB* RNA editing, suggesting that both these factors may participate in the *apoB* editing complex as negative regulators in vivo. In contrast to GRY-RBP and CUGBP2, REME1 is able to promote editing at some sites while inhibiting editing at other sites. Future work may identify additional organelle editing factors with dual inhibitory and stimulatory activities. In fact, such activity could have been overlooked in some of the studies reported so far. For example, editing was assayed only by bulk sequencing and only for 120 mitochondrial C targets in *mef1* mutants (Zehrmann et al., 2009), so that C targets with enhanced editing may not have been detected.

The existence of PPR proteins that inhibit editing efficiency may explain a previously unexpected result we obtained when studying the editing QTL that affects *ccb206*-406 in Col and L*er* (Bentolila et al., 2005). We observed that the less edited phenotype was dominant: the F1 exhibited the same editing extent at *ccb206*-406 as the parent with the lower editing efficiency. We suggested that editing QTLs might encode inhibitory factors, which has now been demonstrated by *REME1* silencing by VIGS. The mechanistic process by which REME1 is able to inhibit editing requires further investigation. REME1 might exert its inhibitory effect through binding to and sequestering of editing factors, as has been shown for GRY-RBP (Blanc et al., 2001). Alternatively, REME1 might directly compete with editing factors for binding to the cis-elements upstream of the C targets. We favor the first model, as the absence of sequence similarity in the surrounding sequences around the edited C residues suggests that REME1 does not exert its inhibitory effect by a direct interaction with the target RNAs.

Our finding raises the question of what functional roles in gene expression may be played by factors that inhibit plant mitochondrial RNA editing. Is the inhibitory effect on editing of particular C residues merely an accidental by-product of a complex system that edits over 500 C residues? Or does inhibition of editing of particular transcripts sometimes serve to increase the expression of mitochondrial protein variants? Although incompletely edited transcripts are known to be translated in plant mitochondria, no useful protein variation has ever been detected as a result (Lu and Hanson, 1994; Lu et al., 1996; Phreaner et al., 1996; Williams et al., 1998). Proteins derived from incompletely edited transcripts are usually thought to be degraded or nonfunctional. However, the presence of variants derived from incompletely edited transcripts has been tested rigorously for only a limited number of plant mitochondrial proteins. Further studies will be needed to discover whether reduced editing at particular C residues may play a role in the generation of protein diversity in plant mitochondria.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

The population of Arabidopsis (*Arabidopsis thaliana*) RILs used to map editing QTLs and their parental accessions, Col and L*er*, have been described in a previous work (Bentolila et al., 2008). One of the RILs, CS1985, which is L*er*/L*er* at the *REME1* locus, was crossed with Col to generate the fine-mapping population. The F1 hybrid was allowed to self-pollinate to produce the F2 progeny. The F2 recombinants between 1113K and 1220K were grown in 16 h of light/8 h of dark under full-spectrum fluorescent lights in a growth room at 26°C. These growing conditions were also used for the Col line expressing GFP, kindly donated by Dominique Robertson (North Carolina State University), the F3 progeny from F2 recombinants, and the T-DNA insertional mutants. Line GT_5_23135 was obtained from the John Innes Center SM lines collection (Tissier et al., 1999).

Screening of Fine-Mapping Recombinants

Genomic DNA extraction of the 691 F2 plants followed the same protocols as described by Bentolila et al. (2008) but was scaled for the use of the Genogrinder 2000 apparatus (Spex CertiPrep) that allowed high-throughput DNA extraction in microtiter plate format. Screening for recombinants was done with the cleaved-amplified polymorphism markers 1027K and 1276K (Supplemental Table S1). PCR was performed with the Taq PCR Master Mix

Kit (Qiagen). After amplification, the PCR products were digested by the appropriate restriction enzyme (Supplemental Table S1), and the digestion products were separated on a 1% agarose gel. All the markers used to genotype the recombinants are listed in Supplemental Table S1. For the sequencing markers, the PCR products after purification by using Exosap (USB) underwent bulk sequencing at the Cornell University Life Sciences Core Laboratory Center. Evaluation of the editing extent of the 11 recombinants for *nad2*-558 was done on three F3 progeny selected to be homozygous recombinant.

Identification of T-DNA Insertional Mutants

The T-DNA population was segregating for the presence of the T-DNA. Plants grown in soil were genotyped with the following primers: the wild-type allele was amplified with GT_5_23135-F1 (5′-AGGAGGGGTTGG-AGTCTGAT-3′) and GT_5_23135-R1 (5′-GAACCCTACAAGCACCAAGC-3′). The mutant allele was amplified with GT_5_23135-F1 and Ds5-1 (5′-ACGG-TCGGGAAACTAGCTCTAC-3′) or with GT_5_23135-R1 and Ds3-1 (5′-ACC-CGACCGGATCGTATCGGT-3′). Both mutant amplicons were sequenced in order to precisely determine the location of the T-DNA insertion in *REME1*. Genotyping the T-DNA populations was performed by amplifying the mutant and wild-type alleles with BioMix Red (Bioline). The PCR products were then visualized on a 1% agarose gel.

VIGS

The protocol and materials used for VIGS are detailed by Robbins et al. (2009). The silencing fragment for *REME1* was amplified by PCR using the following gene sequence tag primers designed by the CATMA database (Crowe et al., 2003): REME1-F1 (5′-TGGATTCATTGCAAAGTCATGG-3′) and REME-R1 (5′-CATTAACCAGAAACATCATTGGTCG-3′). The PCR product was cloned into the pCR8-GW-TOPO vector (Invitrogen), its sequence was verified, and then it was transferred to the silencing vector by using Gateway technology (Invitrogen). The empty vector control was built by first cutting the pCR8-GW-TOPO vector with *Eco*RI, which releases the amplicon, and then self-ligating the vector with a T4 DNA ligase (Fermentas). The minimal and only vector component of the self-ligated pCR8-GW-TOPO vector was transferred via Gateway technology to the silencing vector, which after validating its sequence was designated the empty vector in silencing experiments.

Screening of Mitochondrial Transcripts for Editing Sites Affected by *REME1* Silencing

The screening of the mitochondrial transcripts in *REME1*-silenced and control plants relied on the production of cDNA for the entire set of mitochondrial genes. The methods and primers used for RT-PCR have been described previously (Bentolila et al., 2008); however, we had to design new primers for some of the genes in order to cover the entirety of the published sites (Supplemental Table S2). New primers were also developed to amplify UTRs and introns (Supplemental Table S2). Bulk sequencing of the RT-PCR products with the same primers used for the amplification of the cDNAs was performed at the Cornell University Life Sciences Core Laboratory Center. Whenever the amplicon was too long to obtain the full sequence with the primers used to amplify it, internal primers were designed (Supplemental Table S2).

Measurement of Editing Values

All the techniques and primers used to measure editing values have been reported in a previous work (Bentolila et al., 2008). The only difference concerns the use of PPE oligonucleotides with a fluorescent tag, 5′-hexachlorofluorescein, purchased from Integrated DNA Technologies to measure the editing extent of *nad2*-558, *orfX*-144, and *rpl2*-212. 5′-Hexachlorofluorescein-tagged extension products were detected by a Typhoon 9400 imager (GE Healthcare). Editing extent at other sites was detected by radiolabeled oligonucleotides as described previously (Bentolila et al., 2008). PPE primers are listed in Supplemental Table S3.

Intracellular Localization of REME1

The first 300 nucleotides of *REME1* starting from the AUG codon were amplified and cloned into the pCR8-GW-TOPO vector (Invitrogen). After validating the sequence, the amplicon was transferred to the pEarleygate103 vector (35S-GW-GFP; Earley et al., 2006) by Gateway technology. Plasmid DNA was transiently introduced into onion (*Allium cepa*) epidermal cells by using a helium-driven particle accelerator (PDS-1000; Bio-Rad) according to the manufacturer's instructions. After bombardment, the onion peels were kept for 36 h on Murashige and Skoog plates in the dark. The localization of the expressed proteins was visualized with a laser scanning confocal microscope (Leica SP2). The mitochondrial marker was developed by Nelson et al. (2007).

Plant Transformation

The coding sequence of the *REME1* allele from Col, including the stop codon, was amplified and cloned into a Gateway-converted pBI121 binary vector (Wade Heller, personal communication). This construct was transformed into the L*er* accession via *Agrobacterium tumefaciens* through the standard floral dip protocol.

Semiquantitative RT-PCR Conditions

RNA extracted by a combination of TRIzol and the PureLink RNA minikit (Invitrogen) was cleared from contaminating trace amounts of DNA by using the Turbo DNA-free kit (Ambion). Quantification of RNA was performed with a nanodrop spectrophotometer (Nanodrop Technologies). cDNA was produced with SuperScript III reverse transcriptase (Invitrogen) and gene-specific primers (Supplemental Table S4). Semiquantitative PCR was performed using the primers listed in Supplemental Table S4.

Statistical Analysis

Comparison of the ratio of cis-spliced and trans-spliced cDNAs and editing extent averages between *REME1*-silenced and control plants was performed by Student's *t* test (two-tail *t* test with equal variances; Microsoft Excel).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Bulk sequencing assay can detect differential editing extent in *REME1*-silenced tissues.

Supplemental Figure S2. An example of differential editing between pools of control and silenced plants assayed by bulk sequencing.

Supplemental Figure S3. VIGS of *REME1* results in increased editing of sites found in *rpl5*.

Supplemental Figure S4. Editing extent of *nad2*-558 is decreased in *REME1*-silenced plants.

Supplemental Figure S5. REME1 is targeted to mitochondria.

Supplemental Figure S6. Semiquantitative RT-PCR of a reference gene (At4g26410) shows no difference in expression level between transgenic lines and parental accessions.

Supplemental Figure S7. Semiquantitative RT-PCR is able to detect a 3- to 4-fold reduction in *nad2* second intron-spliced or intron-adjoining product.

Supplemental Table S1. Markers used in REME1 fine mapping.

Supplemental Table S2. New primers used in mitochondrial gene screening.

Supplemental Table S3. New PPE primers used in this report.

Supplemental Table S4. Primers used for semiquantitative RT-PCR.

ACKNOWLEDGMENTS

Some of this work comprised an undergraduate biology honors thesis submitted by W.K. We are grateful to Wade Heller for excellent technical advice on VIGS and to John Robbins for the identification of a *REME1*

insertional mutant. We acknowledge valuable technical help from Huijun Yang for use of the biolistic particle delivery system and from Amir Sattarzadeh for confocal microscopy. We thank Catherine Colas des Francs-Small for providing us with the primer sequences used in the semiquantitative RT-PCR.

Received September 12, 2010; accepted October 19, 2010; published October 25, 2010.

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