Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants

Mizuki Takenaka[1], Anja Zehrmann, Daniil Verbitskiy, Matthias Kugelmann, Barbara Härtel, and Axel Brennicke

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

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RNA editing in plastids and mitochondria of flowering plants changes hundreds of selected cytidines to uridines, mostly in coding regions of mRNAs. Specific sequences around the editing sites are presumably recognized by up to 200 pentatricopeptide repeat (PPR) proteins. The here identified family of multiple organellar RNA editing factor (MORF) proteins provides additional components of the RNA editing machinery in both plant organelles. Two MORF proteins are required for editing in plastids; at least two are essential for editing in mitochondria. The loss of a MORF protein abolishes or lowers editing at multiple sites, many of which are addressed individually by PPR proteins. In plastids, both MORF proteins are required for complete editing at almost all sites, suggesting a heterodimeric complex. In yeast two-hybrid and pull-down assays, MORF proteins can connect to form hetero- and homodimers. Furthermore, MORF proteins interact selectively with PPR proteins, establishing a more complex editosome in plant organelles than previously thought.

In all flowering plants, RNA editing alters more than 400 cytidines to uridines in the mRNAs of mitochondria and converts 30–40 cytidines in plastids (1, 2). In Lycopodiaceae, more than a thousand nucleotide identities in mitochondria and several hundred in plastids are changed (3). This process was recognized about 20 y ago (4–6), but only in recent years have the first determinants involved in the recognition of specific editing sites been identified (7). In target RNAs, the crucial sequence parameters that determine a nucleotide to be edited were identified by transgenic, in vivo, in vitro, and *in organello* assays to be similarly structured in the two organelles (8–10). These *cis* targets, located mostly 5–20 nucleotides 5′ of the target cytidine, are postulated to be recognized by specific *trans*-acting proteins of the 450 members strong pentatricopeptide repeat (PPR) protein family (11–13).

Roughly 30 individual PPR proteins have been assigned to one or several targets by connecting a dysfunctional gene with the loss of RNA editing at specific sites (14, 15). These proteins, which are essential for processing of single or very few RNA editing sites, belong to a subgroup within the PPR family characterized by their patterns of repeats and C-terminal extensions. Some are extended by only an extension (E) domain; others contain an additional conserved region terminating with the name-giving amino acids DYW. This subgroup can supply up to 200 proteins for editing at specific sites, providing an explanation of how the numerous RNA editing sites in flowering plant mitochondria and plastids can be specifically addressed (12, 13). For the enzymatic reaction of converting a cytidine to a uridine, a deaminating activity is required. Because a separate enzyme has not been identified so far, it was proposed that possibly one of the additional C-terminal domains directly contributes the enzymatic activity, in *cis* when present and in *trans* through heterodimer formation (16, 17). We now find that an entirely unexpected class of proteins constitutes an additional, essential component of the plant organellar editosome and is required for processing of almost all editing sites in plastids and of at least many sites in mitochondria.

Results

Mutation of *MORF1* Affects Numerous Editing Sites in Plant Mitochondria. Our forward genetic screen of an ethylmethanesulfonate (EMS)-mutated population of *Arabidopsis thaliana* ecotype Columbia (Col) plants with a multiplexed single-nucleotide extension protocol yielded a number of mutant plants that have lost detectable editing at specific sites (18). The mutations were mapped and the nuclear encoded genes identified several of the site-specific *trans* factors of the PPR family (19). One of the mutants, however, shows reduced RNA editing at more than 40 mitochondrial sites, very different from PPR proteins, which affect only one or several such sites (Fig. 1*A* and *SI Appendix*, Table S1). The effect of the mutation is specific to RNA editing defects; other RNA-processing steps and RNA stability are not affected (*SI Appendix*, Fig. S1). Genomic mapping in a cross of wild-type ecotype Landsberg *erecta* (L*er*) plants and the mutant ecotype Col plant narrowed the locus to a region where no PPR protein is encoded. Sequence analysis revealed an EMS-typical mutation in an unassigned reading frame, At4g20020 (Fig. 1*B*). To confirm this identification, protoplasts from the mutant plant were transfected with the wild-type gene. In these assays, editing at the target sites was increased but not fully restored. In mutant plants stably transformed with the intact Col gene under control of the 35S promoter, RNA editing was fully recovered at all affected sites (Fig. 1*C*). The complementation of the editing defects at the target sites confirms that indeed the right locus has been identified. This gene was named *MORF1* because it encodes a *m*ultiple *o*rganellar *R*NA editing *f*actor. Homozygous mutant plants with a T-DNA insertion in the *MORF1* gene are not viable (*morf1-2*; Fig. 1 *D* and *E*). This finding suggests that the EMS mutant (*morf1-1*) is a "soft" mutation, which only partially disables the function of the encoded MORF1 protein. Therefore, presumably further, essential editing sites are also targeted by the MORF1 protein, and/or those RNA editing sites that are still partially processed in the EMS mutant *morf1-1* are vitally required. The residual level of editing in this EMS mutant is sufficient for the viability of the plant.

MORF3, Another Member of the MORF Family, Is Required for Different RNA Editing Sites in Mitochondria. The *MORF1* gene belongs to a small family of nine genes and one potential pseudogene (Fig. 2*A* and *SI Appendix*, Fig. S2). Of the encoded proteins, four are predicted by Predotar to be targeted to plastids (MORF2, At1g53260, MORF8, and MORF9). In three different proteome analyses, however, fragments of MORF8 were identified in mitochondrial extracts, thus correcting the theoretical prediction. In one of these investigations, the MORF8 protein as well as MORF3 were found

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[1]To whom correspondence should be addressed. E-mail: mizuki.takenaka@uni-ulm.de.

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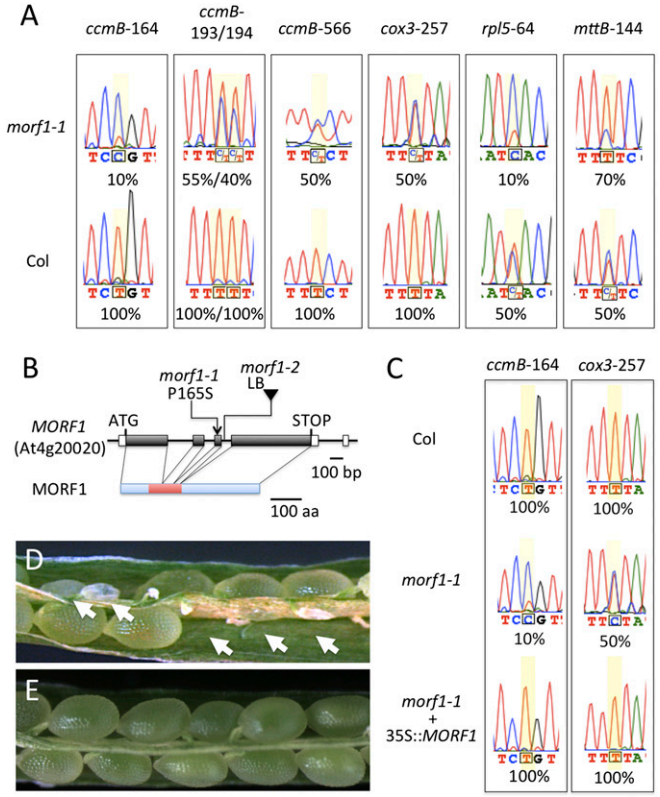
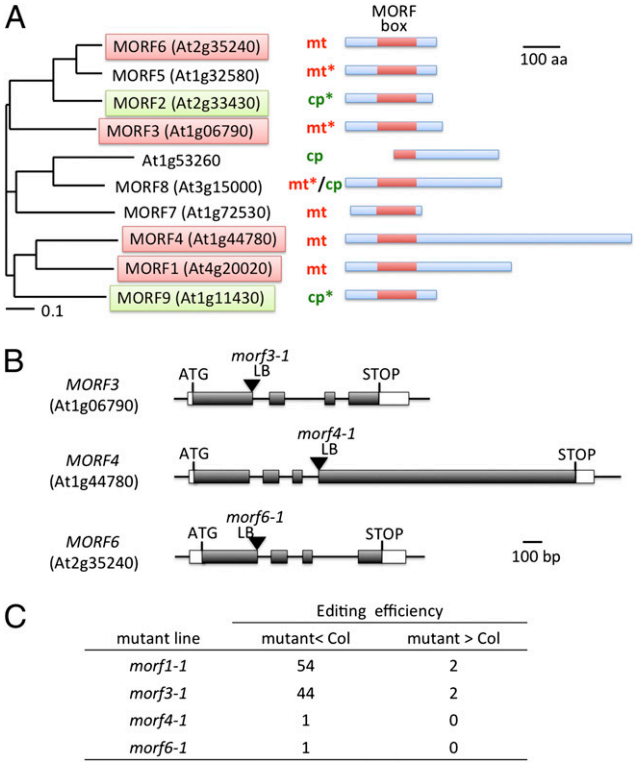


Fig. 1. The MORF1 protein is required for RNA editing at multiple sites. (*A*) Sample sequences of the more than 40 editing sites affected in *morf1-1* EMS mutant plants. The first five sites show editing reduced to different degrees. At the last site, editing increases in the mutant in comparison with wild-type plants of *A. thaliana* ecotype Columbia. (*B*) Structure of the *MORF1* gene and the MORF1 protein. The location of the *morf1-1* single-nucleotide alteration changing a proline to a serine codon and the T-DNA insertion site in *morf1-2* are indicated. LB denotes the location of the left border of the T-DNA. The darker shading in the MORF protein marks the conserved MORF domain. (*C*) Stable transformation of *morf1-1* mutant plants with the wild-type Col gene under control of a 35S promoter complements the editing defects. (*D*) The T-DNA insertion line *morf1-2* is homozygous lethal; homozygous seed growth is aborted (arrows) in pods on a selfed heterozygous plant. (*E*) Wild-type Col plants show the full seed set.



mutant line	Editing efficiency: mutant< Col	mutant > Col
morf1-1	54	2
morf3-1	44	2
morf4-1	1	0
morf6-1	1	0

Fig. 2. The MORF family of proteins contains nine genes and a potential pseudogene in *A. thaliana*. (*A*) The cladogram of similarities between the MORF proteins shows that the plastid editing factors MORF2 and MORF9 are rather distant from each other and more similar to the mitochondrial proteins MORF3 and MORF1, respectively. Predictions (marked mt or cp) and experimental data obtained by GFP-fusion protein localization (only MORF2) or proteomics MS data (marked with an asterisk) for the respective organellar locations are indicated. The MORF8 protein encoded by At3g15000 has been found in mitochondria in three independent assays. Proteins investigated here for their function are boxed. The conserved ~100-amino acids domain is shaded; the other sequences show much less conservation (*SI Appendix*, Fig. S2). The potential pseudogene (At1g53260) contains only the C-terminal part of this conserved region. (*B*) Exon structures of the *MORF3*, *MORF4*, and *MORF6* genes are similar to the *MORF1* locus and contribute similar fragments but differ in their C-terminal extensions. MORF3 is a mitochondrial editing factor involved at more than 40 sites. Locations of the T-DNA insertions in the mutants *morf3-1*, *morf4-1*, and *morf6-1* are shown. LB denotes the location of the left border of the T-DNA. (*C*) Numbers of editing sites affected by T-DNA insertions in the respective *MORF* genes. In the mutants *morf4-1* and *morf6-1*, only one noncoding site each shows somewhat reduced editing.

among mitochondrial proteins with affinity to cobalt ions (20). Genomic locus At1g53260 encodes a protein in which the first half of the otherwise conserved central domain of 100 amino acids (the MORF box) is missing and which is therefore not likely to be functionally competent (*SI Appendix*, Fig. S2). These findings leave MORF2 and MORF9 as functional plastid proteins and assign the seven other MORFs to mitochondria (MORF1 and MORF3–8).

We next investigated whether a second protein predicted for and found in a mitochondrial location, MORF3, is, like MORF1, involved in RNA editing. A T-DNA insertion in the first exon presumably disables the *MORF3* gene in a mutant plant line (Fig. 2*B*). Unlike mutant *morf1-2*, this T-DNA line, *morf3-1*, is viable as a homozygous plant. *morf3-1* plants grow a bit more slowly than wild-type plants but otherwise display no detectably altered morphological phenotype in the greenhouse. The analysis of about 400 mitochondrial editing sites showed that multiple sites are affected by the loss of MORF3. These are almost all different from the sites affected in *morf1-1* (*SI Appendix*, Table S1).

Analogous investigation of RNA editing in homozygous mutant lines of MORF4 and MORF6 showed diminished editing levels at only one site each (Fig. 2 *B* and *C*). These sites are silent; that is, they do not alter the encoded amino acids and are also in wild-type plants variably edited in different tissues of the plant. This finding does not exclude the participation of MORF4 and MORF6 in further editing events. Because MORF4 is similar to MORF1, and MORF6 is very similar to the MORF5 protein (Fig. 2*A*), the related proteins can potentially substitute for each other at some of their targets. Such functional substitutions seem to occur also between a number of mitochondrial PPR proteins at sites where editing in a knockout mutant of a given PPR gene is partially maintained, presumably by another specificity factor (21).

Mutants of Either MORF2 or MORF9 Are Affected at Almost All RNA Editing Sites in Chloroplasts. The protein MORF2 has been experimentally verified to be targeted to the plastid by in vitro import assays (22, 23), and dedicated proteomics analyses detected peptides of MORF2 and MORF9 in plastid proteins (24). Mutation of the *MORF2* gene has been reported to influence mRNA and rRNA accumulation in chloroplasts of *Arabidopsis* with a phenotype similar to an apparent ortholog in *Antirrhinum*

majus (22, 23). These genes have been tentatively assigned *DAG* (*d*ifferentiation *a*nd *g*reening) in *Antirrhinum* and *DAG-like* (*DAL*) in *Arabidopsis* (22, 23, 25).

To investigate whether the severe plastid developmental phenotypes of the *dag* and *dal* and of the *morf2* mutants are caused by deficiencies in plastid RNA editing and to see whether a mutation of *MORF9* also affects growth through RNA editing, we selfed the respective T-DNA insertion lines (Fig. 3*A*) and analyzed the homozygous plants *morf2-1* and *morf9-1* (Fig. 3 *B* and *C–E*). The *morf2-1* mutant has severe problems in chloroplast development similar to the allelic *dag* and *dal* mutants. The *morf2-1* mutant plants stay white, showing no sign of chlorophyll synthesis (Fig. 3*B*). The *morf9-1* mutant also exhibits defects in greening in light, but with features distinct from the *morf2-1* phenotype. When grown on sugar-supplying agar medium, the cotyledons of *morf9-1* are uniformly green, whereas the first true leaves are white. In these, scattered green flecks arise occasionally with advancing age. Subsequent leaves develop a variegated pattern with about 30% green islands, eventually sufficient to sustain autotrophic growth in soil (Fig. 3 *D–F*).

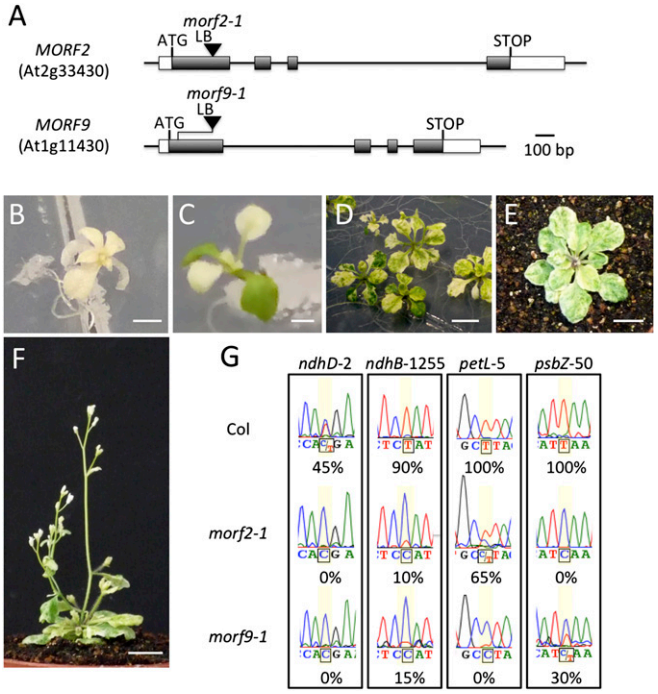


Fig. 3. MORF2 and MORF9 are required for RNA editing in plastid mRNAs. (*A*) Exons of the *MORF2* and *MORF9* genes yield similar-sized proteins, although the intron structures vary. Sites of the T-DNA insertions in the homozygous mutants *morf2-1* and *morf9-1* are shown. (*B*) Phenotype of the *morf2-1* mutant shows a complete lack of chlorophyll biosynthesis in light, and plantlets have to be grown on sugar-containing medium. This mutant is allelic to the *dag* and *dal* mutants described in *Antirrhinum* and *Arabidopsis*, respectively. (Scale bar, 1 mm.) (*C*) In the *morf9-1* mutant, the cotyledons are fully green but the leaves show a variegated appearance with spots of green on otherwise whitish leaves. (Scale bar, 1 mm.) (*D–F*) Sample plants of the *morf9-1* mutants show the variation of the green islands in intensity and distribution between individuals. These plants are able to grow autotrophically on soil. (Scale bars, 1 cm.) (*G*) Several of the affected editing sites are shown that document the differing influence of the *MORF2* and *MORF9* genes. Site *ndhD*-2 canonically requires both intact MORF proteins. Several sites cannot be edited without intact MORF2 (e.g., site *psbZ*-50); others require functional MORF9 proteins (e.g., site *petL*-5). Most of the sites show reduced editing in the absence of either factor, suggesting that the two MORF proteins act in concert at the same sites and that heterodimeric combinations of the two proteins are required for optimal editing.

For the analysis of RNA editing, white leaves were harvested from *morf2-1* and young white first true leaves were collected from *morf9-1*. From these samples, total cellular RNA was purified and the cDNA was investigated for all 34 plastid editing sites documented in *Arabidopsis*. Surprisingly, in both mutants, nearly all plastid editing sites are affected (Fig. 3*G* and *SI Appendix*, Table S2). At several sites editing is completely lost, most editing events are reduced by 10–70%, and some sites are less affected in each mutant. Some of the sites for which MORF2 is essential are different from those that canonically require MORF9, but several sites cannot be edited at all without either MORF. Both MORF2 and MORF9 are thus required for full editing at almost all plastid editing sites. At some sites the reduction in editing is rather small, at the level of experimental variation, that is, less than 10% reduction (*SI Appendix*, Table S2). These findings suggest that MORF2 and MORF9 act together at most editing sites in plastids and that at many sites one can compensate for (or substitute) the respective other factor, except at those sites that remain unedited when one MORF factor is missing. The most parsimonious and straightforward explanation is a direct interaction between the two proteins in a heterodimeric or—at some sites—a homodimeric configuration.

MORF Proteins Can Interact with Each Other. The *Arabidopsis* interactome database predicts MORF9 to interact with MORF6 (26), although MORF9 is plastid-located and MORF6 is a mitochondrial protein. To investigate the possibility of direct interactions between the MORF proteins, we cloned several of these into yeast two-hybrid bait and prey vectors and tested various combinations (Fig. 4*A*). Indeed, most MORF proteins are able to interact with others and also with themselves to form hetero- or homodimers. The physical formation of homodimers was also investigated by pull-down assays with MORF1 as bait (Fig. 5*A*). The bait MORF1 protein was able to retain prey MORF1 molecules, confirming the ability to form homodimers. Among the heterodimers observed in yeast cells, the interaction between the two plastid proteins MORF2 and MORF9 is particularly noteworthy. This observation supports their in vivo potential to act in a heterodimeric connection. Pull-down assays with MORF1 as bait confirmed the general ability of MORF proteins to form heterodimers; the MORF1 protein was able to retain prey MORF2 molecules, although much less effectively than prey MORF1 molecules in the homodimer assays (Fig. 5*A*). This promiscuous interaction between the mitochondrial MORF1 and the plastid MORF2 is also observed in the yeast two-hybrid assay (Fig. 4*A*). Furthermore, in a screen of an *Arabidopsis* cDNA expression library in yeast with mitochondrial MORF1 as bait, several clones of the plastid-located MORF2 were identified. The various combinations of interactions that are observed between plastid MORFs and mitochondrial MORFs suggest a flexible interactive binding that allows different combinations of MORFs in a given organelle. The slightly discriminating interactions of the plastid MORF2 and MORF9 proteins may still result in specific homo- and heterodimers of these two proteins because they seem to be the only MORFs present in this organelle.

In the mitochondrial compartment, the more than 40 sites affected by mutation of MORF1 and the likewise at least 40 sites addressed by MORF3 show almost no overlap; 92 of 95 sites are uniquely targeted (*SI Appendix*, Table S1). This observation suggests that one or more of the other as yet unassigned potential MORF proteins will be required for the remaining fully edited sites and may supply the residual activity for the partially affected sites. Alternatively, MORF1 and MORF3 substitute for each other at the partially affected sites as well as at the unaffected sites; for example, MORF1 potentially supplies the residual editing activities still available in the MORF3 mutant. These substitutions could be governed by the allowed MORF–

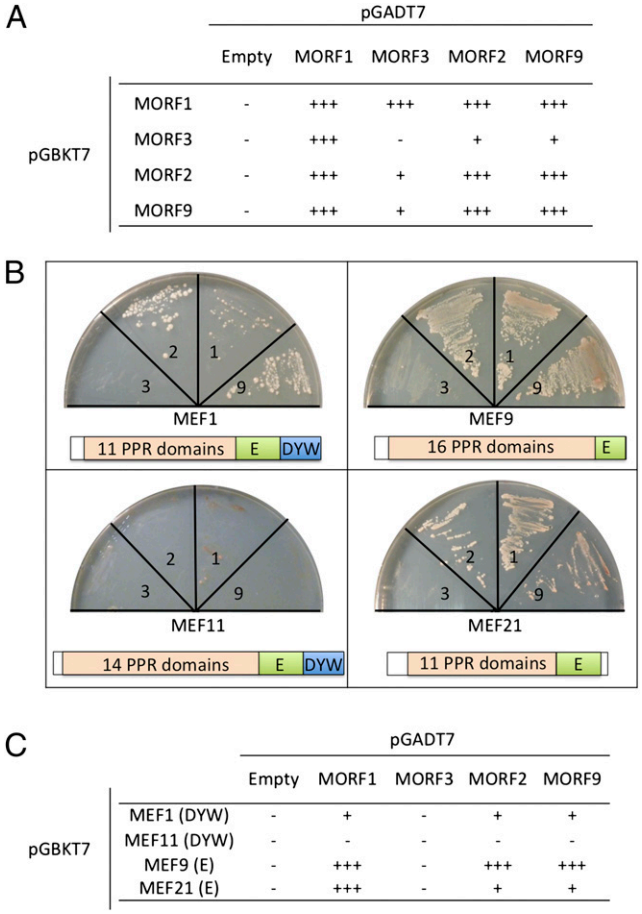


Fig. 4. MORF and MEF proteins can physically interact in yeast two-hybrid assays. (*A*) MORF proteins interact with each other. Reciprocal assays with the MORFs in bait (pGBKT7) or prey (pGADT7) vectors in a yeast two-hybrid analysis reveal that these proteins can interact with themselves in homodimers and with each other in heterodimers. The least specific appears to be the mitochondrial MORF1 protein, which can contact all other MORFs in either direction. Another mitochondrial protein, MORF3, forms strong heterodimers only with the likewise mitochondrial MORF1. The plastid proteins MORF2 and MORF9 interact with each other and with the mitochondrial MORF1, but only weakly with the mitochondrial MORF3. "Empty" is the control for autoactivation. +++ indicates a strong interaction; + represents fewer, slower-growing colonies formed; - indicates no colonies. (*B*) MORF and MEF proteins interact in yeast two-hybrid assays. Respective MEFs are indicated for each plate, and their protein structures are shown. The MORFs tested for binding are numbered in their respective quadrants. The mitochondrial editing protein MORF1, for example, interacts with the mitochondrial editing factors MEF1 (weakly), MEF9, and MEF21, but not with MEF11. These results show that principally MORF and MEF proteins interact rather unspecifically, as, for example, the binding of the plastid proteins MORF2 and MORF9 with the mitochondrial MEF1, MEF9, and MEF21 PPR proteins shows. However, some combinations are preferred, and others do not occur. No-growth quadrants show that there is no autoactivation. (*C*) The interactions between MEF and MORF proteins documented in *B* are interpreted as strong interactions (+++), weak (+), or no (-) binding. The results show that the two DYW-containing proteins, MEF1 and MEF11, interact weakly or not at all with the MORF proteins, whereas the MEF9 and MEF21 proteins, which terminate after the E domain and do not contain a DYW extension, connect more readily and promiscuously with MORF proteins in the yeast two-hybrid assays. The specific target site of MEF21 (*cox3*-257) also requires MORF1, and MEF21 indeed does interact strongly with the MORF1 protein. pGBKT7 is the bait and pGADT7 is the prey vector.

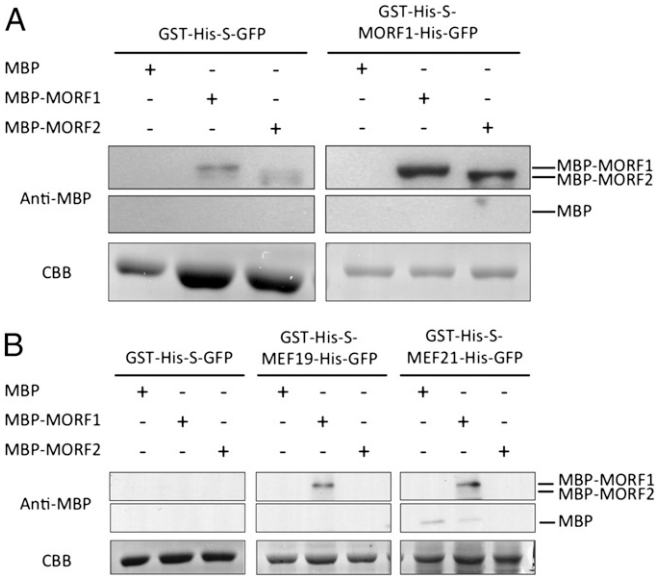


Fig. 5. MORF and MEF proteins interact in pull-down assays. (*A*) In the MORF-MORF pull-down experiment, the GST-His-S-tag-MORF1-His-GFP protein was bound to Ni-NTA agarose beads (*Right*). A parallel bound GST-His-S-tag-GFP protein served as control (*Left*). MORF1 and MORF2 proteins tagged N-terminally with a maltose binding protein (MBP) extension and, as a control, MBP only, were added in separate assays, washed, released, spread on an SDS/PAGE gel, and visualized with an MBP antibody system. (*Right*) The MORF1 protein binds efficiently to the immobilized MORF1 protein. The MORF2 protein binds less effectively: A 20-fold–higher amount of input protein is required to obtain a signal of comparable intensity. (*Left*) Weak interactions of the MBP-MORF1 and MBP-MORF2 proteins to the GST-His-S-tag-GFP control are revealed; to make this background detectable, an approximately fivefold excess of the GST-His-S-tag-GFP control was loaded, as documented by Coomassie stain (CBB; *Bottom*). MBP protein alone is not detectably retained by either the GST-His-S-tag-MORF1-His-GFP protein or the GST-His-S-tag-GFP protein. Agarose beads (400 μL) were loaded with 3.5 nmol of GST-His-S-tag-MORF1-His-GFP protein or 35 nmol of GST-His-S-tag-GFP protein. Input protein was 0.5 nmol of MBP-MORF1, 10 nmol of MBP-MORF2, and 10 nmol of MBP. (*B*) For this MORF-MEF pull-down analysis, the GST-His-S-tag-MEF19-His-GFP protein (*Center*) or the GST-His-S-tag-MEF21-His-GFP protein (*Right*) were immobilized on glutathione agarose beads and probed for interaction with the MBP-fused MORF1 and MORF2 proteins. Retained MORF proteins were detected in the gel blot with an MBP antibody system. Comparison with the control glutathione agarose-bound GST-His-S-tag-GFP (*Left*) shows that the MBP-tagged MORF1 and MORF2 proteins do not bind detectably to the GST-His-S-tag-GFP protein when present in amounts comparable to the MEF19 and MEF21 proteins; the weak signals obtained with excess amounts of the control are shown in *A*. Both MEF19 and MEF21 are able to bind and retain the mitochondrially located MORF1 but not the plastid-targeted MORF2. This result confirms the interaction pattern seen in the yeast two-hybrid assays (Fig. 4), where MEF21 strongly interacts with MORF1 but only weakly with MORF2. The interactions observed between MORF1 and MEF19, and MORF1 and MEF21, agree with the RNA editing site analysis, with MORF1 and MEF19 and MORF1 and MEF21 targeting the same respective RNA editing sites in mitochondria. The agarose beads (400 μL) were loaded with 3.5 nmol of the GST-His-S-tag-MEF19-His-GFP, the GST-His-S-tag-MEF21-His-GFP, or the GST-His-S-tag-GFP protein. Input protein was 0.5 nmol of MBP-MORF1 or MBP-MORF2 and in the control 1 nmol of MBP. In the Coomassie stain (*Bottom*), not all partial MEF proteins that contain the N-terminal GST-His-S tag but not the C-terminal His-GFP tag are documented. The weak signal seen of free MBP retained by immobilized MEF21 in the input lane of MBP-MORF1 is either a much shorter bacterial translation product or a result of protein cleavage before or during the protein preparation from the bacteria.

MORF heterodimer/homodimer combinations of which some may be less efficiently substituted by others and lead to loss of editing when one MORF is mutated. In yeast cells, MORF1 shows little discrimination in connecting to other MORFs, whereas MORF3 only interacts strongly with MORF1 but with none of the other MORF proteins (Fig. 4*A*). MORF3 does not seem to be able to form homodimers.

MORF Proteins Can Interact with Site-Specific RNA Editing PPR Proteins. In plastids as well as in mitochondria, RNA editing is affected by the loss of individual MORF proteins at sites that also require individual PPR proteins for processing. In plastids, for example, editing at site *ndhD*-2 is lost when either MORF2 or MORF9 is disturbed, but also when the PPR protein chlororespiratory reduction 4 (CRR4) is mutated (7). In mitochondria, editing site *ccmB*-566 requires mitochondrial editing factor 19 (MEF19) and MORF1; site *cox3*-257 needs MEF21 as well as MORF1 for processing (14). These coinciding requirements suggest that site-specific PPR proteins (e.g., CRRs and MEFs) are required in conjunction with one or more MORF proteins.

To directly investigate this potential connection between the specific editing factors and the MORF proteins, we tested whether MEF–PPR proteins can interact with MORF proteins in yeast two-hybrid assays (Fig. 4*B*) and in pull-down assays (Fig. 5*B*). Growth of yeast cells on respective selective media shows that most of the MEF proteins indeed interact with MORF proteins. Consistent with their "promiscuous" roles at most editing sites in their organelle, the plastid factors MORF2 and MORF9 interact with several mitochondrial MEFs. Similarly, the MORF1 protein, which is required for more than 40 RNA editing sites in mitochondria, interacts with MEF1, MEF9, and MEF21. This binding is, however, specific and selective, as the DYW–PPR protein MEF11 is not contacted by any of the MORFs investigated. Furthermore, MORF interaction with the second DYW domain-containing MEF protein, MEF1, seems to be rather weak, as suggested by slower establishment and growth of the yeast cells.

The potential of MEF and MORF proteins to interact is supported by the results of a screen of a cDNA library derived from RNA isolated from young *Arabidopsis* seedlings with MEF9 as bait. Several clones of the MORF8 protein were identified in the total plant cDNA library, which strengthens the findings from the yeast two-hybrid assays that MORF and MEF proteins can interact. Both MEF9 and MORF8 are mitochondrially located proteins and may interact in this organelle.

To investigate potential MORF–MEF interactions by another experimental approach, we tested whether MEF21 and MEF19 are able to bind the mitochondrially located MORF1 and the plastid-targeted MORF2 proteins in pull-down assays (Fig. 5*B*). MEF21 can retain MORF1 but not MORF2, as similarly observed in the yeast two-hybrid assays, where MEF21 interacts strongly with MORF1 but only weakly with MORF2 (Fig. 4*B*). In mitochondria, MEF21 and MORF1 are both required for editing at site *cox3*-257 (14). We also probed the potential for physical interaction between the MEF19 PPR protein and the MORF1 protein, which both target the RNA editing site at *ccmB*-566 (Fig. 5*B*). In the pull-down assay, the MEF19 bait protein bound to matrix beads retains the prey MORF1 molecules but not the plastid-targeted MORF2 protein (Fig. 5*B*). The physical interaction between MORF1 and MEF19 can thus connect the site-specific MEF19 PPR protein and the MORF1 protein for RNA editing at their common target site at *ccmB*-566. In summary, these lines of evidence support the potential of MEF and MORF proteins to interact more or less specifically with each other.

Discussion

MORF Proteins Are Unique Components of the Plant Organellar "Editosomes." The involvement of the family of MORF proteins suggests that the previous simple model of RNA editing in higher plant organelles has to be expanded to a more complex editosome model that contains more protein factors than envisaged: A PPR protein recognizes a specific sequence context in the RNA, binds there, and provides the attachment site for one or another of the MORF proteins. Or, vice versa, a MORF protein contacts an RNA molecule (provided MORF proteins bind RNA) and then attracts an MEF protein that then can play out its sequence specificity for binding to its cognate RNA motif. At the initiation step of MEF/MORF contacts to the RNA, additional, less specific ribonucleoproteins (RNPs) may be involved, such as CP31A and CP31B, which have been shown to be required for efficient RNA editing at several sites in plastids (27). These RNPs are partially redundant (27), a feature that seems to be common to MEF and MORF proteins. The interactions between PPR proteins and an RNA sequence appear to be specific yet fluid, because in some instances one PPR protein can be substituted by another: Those editing sites in mitochondria at which residual editing is still seen when a given PPR factor is disabled are not completely dependent on this single PPR protein; the remaining editing must be supported by another (PPR) protein (21).

MORF proteins must also partially overlap in their interactions with E or DYW–PPR proteins, as the numerous partial reductions in RNA editing at specific sites in plastids in the *morf2* and *morf9* mutants show. On the other hand, the complete loss of editing at several sites in the plastid when either MORF2 or MORF9 is disturbed indicates that at these sites both proteins are required and cannot substitute for each other. That both plastid MORFs are canonically required suggests that both should be contained within the editosome, potentially connecting each other directly in a heterodimeric arrangement. The observation that the MORFs interact selectively with each other corroborates this conclusion (Figs. 4*A* and 5*A*).

MORF Proteins Are Present in Plants with Numerous Editing Sites. In evolutionary terms, this type of RNA editing and editosome seems to be a requirement specific to the land plant lineage. PPR proteins and MORF proteins expanded in plants in parallel with an increase of RNA editing sites. MORF-like proteins appear to be absent from other organisms; their presence is correlated with the evolutionary burst of editing site numbers. Genes for MORF proteins are only detected in flowering plants (*SI Appendix*, Fig. S3), and not in the moss *Physcomitrella patens* (17, 28). In this plant, only PPR proteins with C-terminal extended DYW domains are involved in the few editing events. This correlation raises the possibility that MORF proteins may be involved in compensating the loss of the DYW domain in some editing PPRs.

In the editosome, a MORF protein would connect to the RNA-binding PPR protein, either an E or a DYW moiety, and to the cytidine-deaminating or -transaminating activity. The latter may be another PPR protein with a DYW domain that acts as a deaminase, or may be another protein that performs the reaction. Inclusion of a second, different yet specific, PPR protein could have consequences for the decoding of the RNA sequence. Each PPR protein would have to contact just very few nucleotide identities in the *cis* element of the RNA, and only the combination of both PPR proteins would need to have full affinity. In the moss *P. patens*, single PPR proteins may recognize a given RNA nucleotide pattern, because a knockout of one PPR factor always results in full or no editing but never leads to partial loss of editing. In addition, no MORFs are present in the moss.

The involvement of the MORF proteins with many editing events in both organelles of flowering plants shows that the RNA editing processes in plant mitochondria and in plastids are similar and probably coevolved (*SI Appendix*, Fig. S3). In plastids, the process seems to be more stable, with all PPR proteins so far identified being required for fixed sites with no overlapping specificities of the PPR proteins manifested by residual editing at some sites upon the loss of a given PPR factor. Although this is superficially similar to the situation in the organelles of *P. patens*, in flowering plants MORF proteins are required and, for example, in the instance of site *ndhD*-2, two MORFs are needed for any editing to occur (Fig. 3). In mitochondria, a more fluid and flexible editing complex may adapt more rapidly to novel sites and new specificities by small modifications of the activities involved. The connection of the MORF proteins may provide an additional

safety level to avoid deleterious unwanted editing events caused by the often rather loose PPR specificity that is required to address sites with little sequence similarity (14, 15, 29).

Although the actual editing process in plant organelles with "just" C-to-U (but also U-to-C) nucleotide transitions seems biochemically much less complex than the U insertion/deletion editing in trypanosome mitochondria (30–32), likewise more and specialized protein factors such as the MORF proteins are required to make up a functional RNA editing complex.

Materials and Methods

Plant culture, mutant screening, handling of nucleic acids, RNA editing analysis, transfection, and transformation were as described (18, 29). Protein target predictions were analyzed with the Predotar program (urgi.versailles.inra.fr/predotar). Details of yeast two-hybrid and pull-down analyses, full methods, and associated references are described in *SI Appendix, SI Materials and Methods*.

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1. Giegé P, Brennicke A (1999) RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. *Proc Natl Acad Sci USA* 96:15324–15329.
2. Chateigner-Boutin AL, Small I (2010) Plant RNA editing. *RNA Biol* 7:213–219.
3. Grewe F, et al. (2011) A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmannii*. *Nucleic Acids Res* 39:2890–2902.
4. Covello PS, Gray MW (1989) RNA editing in plant mitochondria. *Nature* 341:662–666.
5. Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. *Nature* 341:660–662.
6. Hiesel R, Wissinger B, Schuster W, Brennicke A (1989) RNA editing in plant mitochondria. *Science* 246:1632–1634.
7. Kotera E, Tasaka M, Shikanai T (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* 433:326–330.
8. Bock R, Hermann M, Kössel H (1996) In vivo dissection of *cis*-acting determinants for plastid RNA editing. *EMBO J* 15:5052–5059.
9. Neuwirt J, Takenaka M, van der Merwe JA, Brennicke A (2005) An in vitro RNA editing system from cauliflower mitochondria: Editing site recognition parameters can vary in different plant species. *RNA* 11:1563–1570.
10. Farré J-C, Leon G, Jordana X, Araya A (2001) *cis* recognition elements in plant mitochondrion RNA editing. *Mol Cell Biol* 21:6731–6737.
11. Lurin C, et al. (2004) Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 16:2089–2103.
12. Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: A socket set for organelle gene expression. *Trends Plant Sci* 13:663–670.
13. Fujii S, Small I (2011) The evolution of RNA editing and pentatricopeptide repeat genes. *New Phytol* 191(1):37–47.
14. Takenaka M, Verbitskiy D, Zehrmann A, Brennicke A (2010) Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of *Arabidopsis thaliana*. *J Biol Chem* 285:27122–27129.
15. Hammani K, et al. (2009) A study of new *Arabidopsis* chloroplast RNA editing mutants reveals general features of editing factors and their target sites. *Plant Cell* 21:3686–3699.
16. Salone V, et al. (2007) A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Lett* 581:4132–4138.
17. Rüdinger M, Szövényi P, Rensing SA, Knoop V (2011) Assigning DYW-type PPR proteins to RNA editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*. *Plant J* 67:370–380.
18. Takenaka M, Brennicke A (2009) Multiplex single-base extension typing to identify nuclear genes required for RNA editing in plant organelles. *Nucleic Acids Res* 37:e13.
19. Takenaka M (2010) MEF9, an E-subclass pentatricopeptide repeat protein, is required for an RNA editing event in the *nad7* transcript in mitochondria of *Arabidopsis*. *Plant Physiol* 152:939–947.
20. Heazlewood JL, et al. (2004) Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *Plant Cell* 16:241–256.
21. Zehrmann A, Verbitskiy D, Härtel B, Brennicke A, Takenaka M (2011) PPR proteins network as site-specific RNA editing factors in plant organelles. *RNA Biol* 8(1):67–70.
22. Chatterjee M, et al. (1996) DAG, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*. *EMBO J* 15:4194–4207.
23. Bisanz C, et al. (2003) The *Arabidopsis* nuclear DAL gene encodes a chloroplast protein which is required for the maturation of the plastid ribosomal RNAs and is essential for chloroplast differentiation. *Plant Mol Biol* 51:651–663.
24. Zybailov B, et al. (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS One* 3:e1994.
25. Babiychuk E, Fuangthong M, Van Montagu M, Inzé D, Kushnir S (1997) Efficient gene tagging in *Arabidopsis thaliana* using a gene trap approach. *Proc Natl Acad Sci USA* 94:12722–12727.
26. Cui J, et al. (2008) AtPID: *Arabidopsis thaliana* Protein Interactome Database—An integrative platform for plant systems biology. *Nucleic Acids Res* 36(Database issue):D999–D1008.
27. Tillich M, et al. (2009) Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. *Proc Natl Acad Sci USA* 106:6002–6007.
28. Ohtani S, et al. (2010) Targeted gene disruption identifies three PPR-DYW proteins involved in RNA editing for five editing sites of the moss mitochondrial transcripts. *Plant Cell Physiol* 51:1942–1949.
29. Zehrmann A, Verbitskiy D, van der Merwe JA, Brennicke A, Takenaka M (2009) A DYW domain-containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of *Arabidopsis thaliana*. *Plant Cell* 21:558–567.
30. Grosjean H, Benne R (1998) *Modification and Editing of RNA* (ASM, Washington, DC).
31. Simpson L, Thiemann OH (1995) Sense from nonsense: RNA editing in mitochondria of kinetoplastid protozoa and slime molds. *Cell* 81:837–840.
32. Stuart K (1991) RNA editing in trypanosomatid mitochondria. *Annu Rev Microbiol* 45:327–344.