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The RanBP2 zinc finger domains of chloroplast RNA editing factor OZ1 are required for protein–protein interactions and conversion of C to U

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SUMMARY

In the chloroplast, organelle zinc finger 1 (OZ1) is a RanBP2-type zinc finger (Znf) protein required for many RNA editing events, a process by which specific cytosines are enzymatically converted to uracils as a correction mechanism for missense mutations in the organelle genomes. RNA editing is carried out by a large multi-protein complex called the 'editosome' that contains members of the pentatricopeptide repeat (PPR) protein family, the RNA editing factor interacting protein (also known as MORF) family and the organelle RNA-recognition motif (ORRM) family, in addition to OZ1. OZ1 is an 82-kDa protein with distinct domains, including a pair of Znf domains and a unique C-terminal region. To elucidate the functions of these domains, we have generated truncations of OZ1 for use in protein–protein interaction assays that identified the C-terminal region of OZ1, as well as the Znf domains as the primary interactors with PPR proteins, which are factors required for site-specificity and enzymatic editing. Expression of these OZ1 truncations *in vivo* showed that the Znf domains were required to restore chloroplast RNA editing in *oz1* knockout plants. Mutation of key structural residues in the Znf domains showed that they are necessary for editing and required for interaction with ORRM1, a general editing factor with an RNA-binding domain. These functional characterizations of the Znfs and novel C-terminal domain contribute to our understanding of the model for the chloroplast plant editosome.

Keywords: RNA editing, zinc finger, plant editosome, chloroplast.

INTRODUCTION

RNA editing in plants compensates for missense mutations in organellar genes by converting select cytidines in mRNA to uridines (Small et al., 2020; Smith et al., 1997; Stern et al., 2010). To date, in *Arabidopsis thaliana*, 43 sites in the chloroplast and over 600 sites in the mitochondria have been identified as targets for editing (Bentolila et al., 2013; Ruwe et al., 2013). These editing events are important for proper plant growth. Many cases of mutant Arabidopsis plants that lack editing at key sites are characterized by defects linked to incomplete development of the chloroplasts or mitochondria, such as lack of pigmentation, slow growth in the absence of an external sugar supply or embryo lethality (Hackett et al., 2017; Shi et al., 2016, 2017; Wang et al., 2021).

The 'editosome' complex is composed of nuclear-encoded proteins for which the diversity and subfunctions are not fully characterized. Evidence from genetic assays and protein interaction studies revealed a core set of four protein families: the numerous pentatricopeptide repeat (PPR) proteins (Barkan and Small, 2014), the RNA editing factor interacting proteins (RIPs), also known as MORFs (Sun et al., 2016; Takenaka et al., 2012), the organelle RNA-recognition motif (ORRM) protein family (Hackett et al., 2017) and the organelle zinc finger (OZ) family (Gipson et al., 2020; Sun et al., 2015). The particular protein members of the families within an editosome differ from one edited site to the next. The identification of these editing factors and their interactions with each other and the RNA substrate have led to a model for the editosome, although the individual contributions of these factors to the editing process is still being uncovered.

Plant RNA editing factors exhibit a variety of functionally distinct domains, such as the RNA-binding, editing-essential RRM domain versus the protein-binding Gly-rich regions of ORRM proteins (Shi et al., 2015, 2016, 2017) and the RNA-binding PPR tract, protein-binding E-domains and nucleotide deaminase DYW domain of editing PPR proteins (Bayer-Császár et al., 2017; Boussardon et al., 2012, 2014; Hayes and Santibanez, 2020; Okuda et al., 2006;

Wagoner et al., 2015). OZ1 also contains a number of distinct domains: a group of N-terminal motifs shared by all four members of the OZ family, two zinc finger (Znf) domains and a C-terminal region specific to OZ1 that contains three spaced motifs with strong conservation across many species (Sun et al., 2015). Many Znf proteins perform various roles in plant physiology, with some of these roles being as yet unidentified (Gipson et al., 2020). The particular subfamily of Znf in the OZ family is the RanBP2 family, named for the nuclear pore factor that binds to the Ran nuclear localization factor (Singh et al., 1999). Previous analysis (Sun et al., 2015) showed that the RanBP2 Znfs in OZ1 are similar to those of ZRANB2, a human splicing factor known to bind to single-stranded RNA via its Znf domains (Loughlin et al., 2009).

Because OZ1 is an 82-kDa protein with several predicted domains, we hypothesized that OZ1 would have specialized domain functions as other editing factors do. Based on the three-part domain structure, we prepared truncated forms of OZ1 for protein–protein interaction testing in yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays with known binding partners from the other editosome protein families. These experiments have identified the C-terminal domain of OZ1 and the Znf domains as the primary interactors with PPR proteins, whereas the Znf domains alone are necessary for interaction with ORRM1. Using mutational analysis of the Znf domains of the editing factor OZ1, we have demonstrated the importance of the domains to the contribution of OZ1 to RNA editing. The ability of OZ1 truncations consisting of either the Znf domains alone or Znfs with a C-terminal domain to rescue RNA editing defects in the knockout mutants further establishes that the Znfs of OZ1 are necessary for its role in editing at target sites.

RESULTS

Domain boundary analysis of OZ1 predicts several topologically independent regions

oz1 knockout mutants exhibit reduced seed viability and early chlorosis (Figure 1a), although they eventually develop functional chloroplasts that allow them to grow on soil autotrophically (Sun et al., 2015).

To determine possible secondary structure and delineate domains in preparation for cloning truncation constructs, we performed domain boundary analysis on the OZ1 protein sequence using a suite of online tools for structure prediction (Cooper and Marsden, 2017). Our analysis determined cut-off points for three putative functional regions: the N-terminal domain, shared between all four OZ family proteins and spanning OZ1 residues 34–273, the pair of RanBP2 Znfs spanning residues 274–337, and the C-terminal stretch, unique to OZ1 and spanning residues 358–stop (Figure 1b).

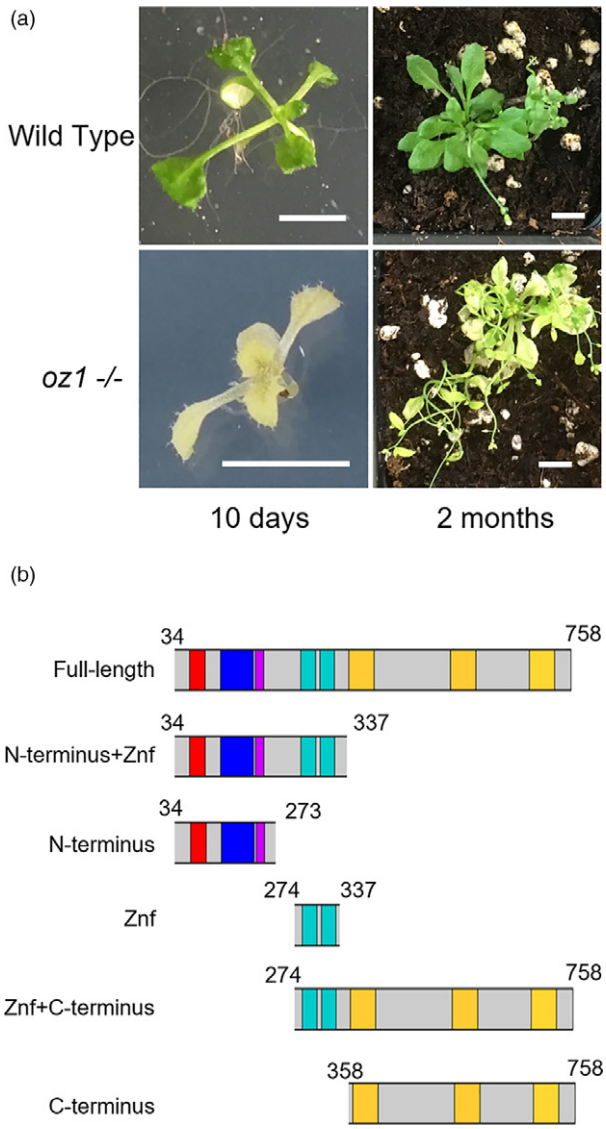

Figure 1. OZ1 is necessary for normal plant development and consists of multiple domains. (a) Wild-type Col and *oz1* homozygous mutant Arabidopsis plants at 10 days (left two images; scale bars = 5 mm) were grown in Magenta boxes and, at 2 months (right two images; scale bars = 1 cm), they were transferred to soil. (b) Map of OZ1 domains as identified in Sun et al. (2015) and truncations used in the present study. Numbers above cartoons label the first and last amino acid residues of each construct. All constructs were cloned with the first 33 amino acids removed (the estimated size of the transit peptide) and then fused with the first 65 amino acids of RecA as a replacement chloroplast transit peptide at the N-terminus. Red = 19 residue-long motif; blue = 60 residue-long motif; purple = 16 residue-long motif; teal = RanBP2 ZnFs; yellow = 47 residue-long motif.

The original analysis of the OZ family protein sequences using MEME (Sun et al., 2015) showed the presence of four unique sequence motifs. The N-terminal region of every OZ protein (Figure 1b) contains a 19 residue-long motif (red), a 60 residue-long motif (blue) and a 16 residue-long motif (purple), all with variation in the length of linkers

between them. Based on the domain boundary analysis of OZ1, the 19 residue-long motif is predicted to contain an alpha helix; the 60 residue-long motif contains three alpha helices along with a short, disordered region; and the 16 residue-long motif contains another short alpha helix. The fourth motif (Figure 1b) identified is unique to the long C-terminal region of OZ1, which contains three of these 47 residue-long motifs. Domain boundary analysis was inconsistent with secondary structure prediction of the three C-terminal motifs, predicting two alpha helices in the most C-terminal motif but largely disordered regions in the first two such motifs.

The C-terminal domain of OZ1 mediates protein-protein interactions with other editing factors except for ORRM1

Previous work identified OZ1 as a protein binding partner with the chloroplast RNA editing factor ORRM1 along with chloroplast editing PPR proteins CRR28 and OTP82 (Sun et al., 2015). To evaluate which domain or domains of OZ1 participate in these interactions, we prepared truncated constructs of OZ1 based on our domain boundary analysis (Figure 1b) for expression in a GAL4-based Y2H assay. Along with CRR28 and OTP82, we included RARE1 and QED1 (named in the older literature as 'OTP81') in the assays because these PPR proteins share target editing sites with those of OZ1 (Hammani et al., 2009; Robbins et al., 2009; Sun et al., 2015; Wagoner et al., 2015). Finally, we assayed for interaction with DYW2, an unusual member of the PPR family in terms of having a very short tract of just five PPR domains upstream of the E and DYW domain (Andrés-Colás et al., 2017). DYW2 associates with so-called E-type PPR proteins, which lack a DYW domain of their own and provide editing catalysis *in trans* to those editosomes (Brehme et al., 2020; Malbert et al., 2020). Testing these constructs for interaction with other editing factors showed that the C-terminal domain construct alone was able to establish the interactions that occur between mature-length OZ1 and RARE1 (a weaker interaction than with the other PPR proteins), OTP82, CRR28 and DYW2; however, the OZ1_C-terminus did not interact with QED1 or ORRM1 (Figure 2 and Table 1).

These interactions with PPR proteins also occurred with both the N-terminal domain plus the zinc finger domains (N-terminus+Znf) and the zinc finger domains alone (Znf), albeit with a weaker strength (Figure 2). We also observed an interaction between ORRM1 and both the N-terminus+Znf truncation of OZ1 as well as the Znf alone. This interaction was the only one that was not sustained by the C-terminal domain of OZ1. The N-terminal domain of OZ1 did not show interaction with any of the editing factors tested (Figure 2). Furthermore, it appears that this N-terminal domain has a repressive effect on the binding ability of the Znf domain, as seen with RARE1 and QED1. The Znf alone interacts with all the editing factors tested, including QED1, whereas the N-terminus+Znf does not show an interaction with QED1. Moreover, the Znf alone showed interaction with DYW2 and ORRM1 at the lowest concentration tested, which is not observed in the N-terminus+Znf truncation of OZ1.

We confirmed these Y2H results through BiFC in *Nicotiana benthamiana* leaves (Figure 3 and Table 1). In several cases, BiFC detected interactions that had not been observed in the Y2H assays, such as the interaction of QED1 and the OZ1 C-terminus, and most notably the interaction of the N-terminal domain of OZ1 with RARE1, OTP82, CRR28 and DYW2 (Figure 3g–i,k and Table 1). In conclusion, when compiling the results from both the Y2H and the BiFC assays, the Znf domain alone recapitulates all

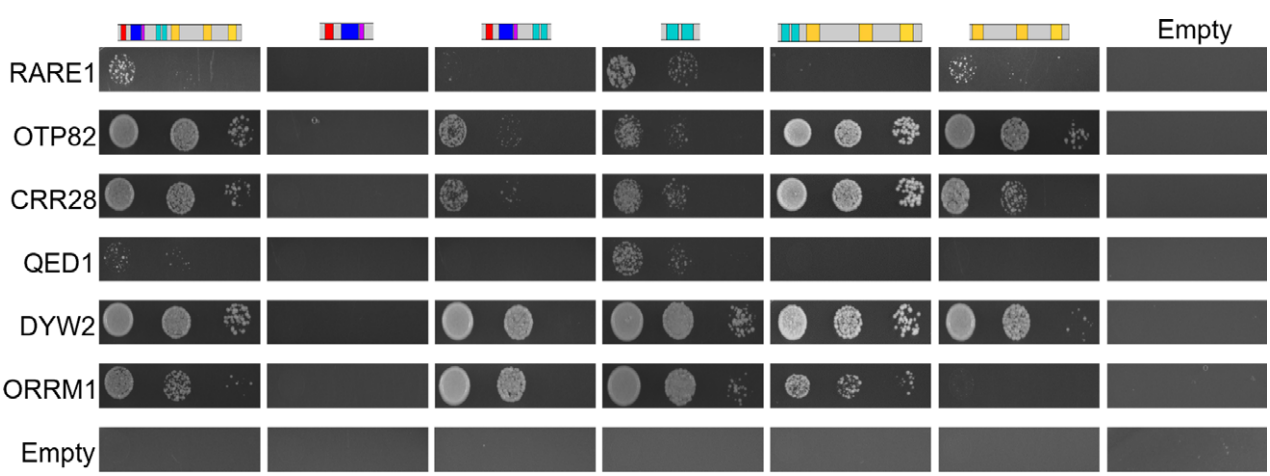

Figure 2. A Y2H assay shows that the Znfs of OZ1 bind to other editing factors, and the C-terminal domain of OZ1 mediates the interaction with most other editing factors except ORRM1. Serial dilutions of yeast (from left to right: 10^7 cells ml^{-1}, 10^6 cells ml^{-1} and 10^5 cells ml^{-1}) are shown, demonstrating interactions between mature-length OZ1 or OZ1 truncations (OZ1_FL, OZ1_N-terminus, OZ1_N-terminus+Znf, OZ1_Znf, OZ1_Znf+C-terminus and OZ1_C-terminus) and PPR proteins (RARE1, CRR28, OTP82, QED1), DYW2 or ORRM1. Empty = yeast strains expressing empty pGADT7 or pGBKT7 vectors as a negative control.

Table 1 Summary of yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) interaction assay results between truncations of OZ1 and known RNA-editing factor binding partners

	RARE1		OTP82		CRR28		QED1		DYW2		ORRM1	
OZ1_full-length	Yes†	Yes†	Yes	Yes	Yes	Yes	Yes†	Yes	Yes	Yes	Yes	Yes
OZ1_N-terminus	No	Yes	No	Yes	No	Yes	No	No	No	Yes	No	No
OZ1_N-terminus+Znf	No	No	Yes†	Yes	Yes†	Yes	No	Yes†	Yes	Yes	Yes	Yes
OZ1_Znf	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes†	Yes	Yes
OZ1_Znf+C-terminus	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
OZ1_C-terminus	Yes†	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No
	Y2H	BiFC	Y2H	BiFC	Y2H	BiFC	Y2H	BiFC	Y2H	BiFC	Y2H	BiFC

†Weaker than average interactions, based on growth density in Y2H or fluorescent puncta abundance in BiFC.

the interactions observed for the full length OZ1. This observation is particularly relevant for ORRM1, for which the presence of the Znf is both sufficient and necessary for the interaction to occur (Table 1).

The Znf domains of OZ1 are essential for chloroplast RNA editing

After observing in the Y2H and BiFC assays that the Znf domains of OZ1 are responsible for all of the protein–protein interactions, we produced stable transformants of *oz1* homozygous plants expressing OZ1 truncations under control of the 35S promoter and targeted to the chloroplast with the RecA transit peptide (the first 65 amino acids of RecA). Seedlings and mature plants expressing the full-length OZ1 construct resembled wild-type Arabidopsis, but plants expressing truncation constructs exhibited varying degrees of chlorosis and delayed growth (Figure 4a). The plants expressing the N-terminal part of OZ1 or the N-terminus+Znf showed severe chlorosis similar to the *oz1* mutant plant. On the other hand, the transgenic plants expressing constructs with the Znf alone, Znf+C-terminus or the C-terminus alone exhibited some greening (Figure 4a). We aimed to determine whether the restoration of greening could be coupled with the ability of the constructs to restore plastid editing extent. After chloroplast RNA extraction and reverse transcription quantitative real-time PCR (RT-qPCR) experiments, we assayed several chloroplast sites for their editing extent in the various OZ1 truncation transgenic plants. The *ndhB*-C1255 editing site exhibited severe reduction of its editing extent in the *oz1* mutant compared to wild-type, and expression of full-length OZ1 in the *oz1* mutant rescued editing at that site (Figure 4b). Expression of the OZ1 N-terminus, N-terminus+Znf and C-terminus did not rescue *ndhB*-C1255 editing, although the Znf+C-terminus and Znf did rescue editing, albeit not to the extent of the full-length construct in the case of the OZ1_Znf construct (Figure 4b). Other sites repeated this pattern, where the Znf and the Znf+C-terminus truncation are able to rescue editing (Figure S1). The ability of these constructs to rescue editing is also apparently concentration-dependent because biological replicates that have lower expression levels of either the Znf or Znf+C-terminus construct have correspondingly lower editing extents (Figures 4b and 5a). However, most other chloroplast editing sites, such as *ndhB*-C467 and *rpoA*-C200, remain at the *oz1* knockout editing level in the OZ1_Znf truncation-expressing transgenic plants but are rescued in the Znf+C-terminus transgenic plants (Figure 4b; Figure S1). More importantly, the inability of certain truncation constructs to restore editing extent in the *oz1* mutant, such as the N-terminus or N-terminus+Znf, cannot be attributed to a defective level of expression; both constructs are expressed at a higher or similar level

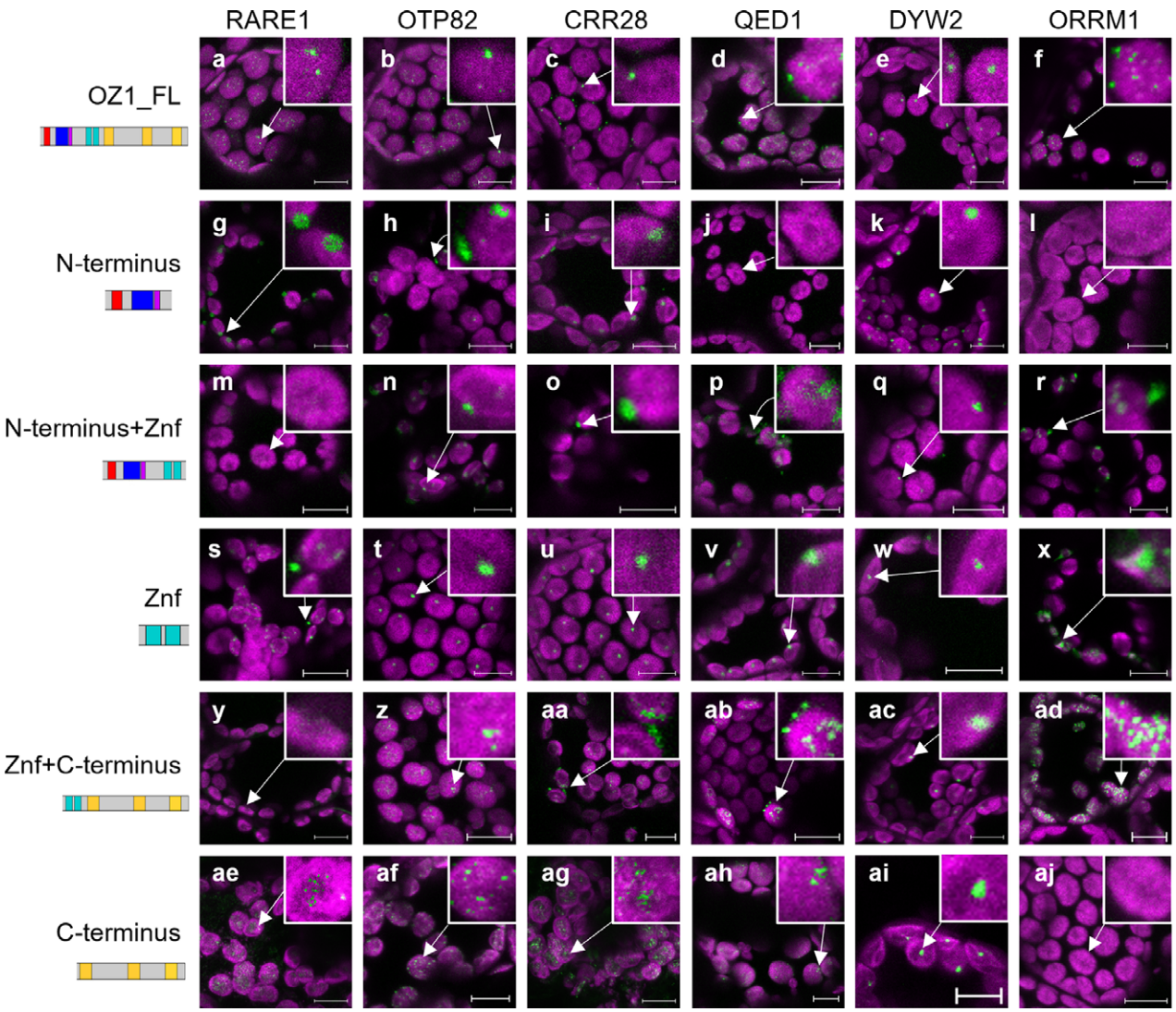

Figure 3. BiFC of OZ1 truncations with other editing factors shows that all domains interact with PPR proteins while ORRM1 only interacts with truncations containing the Znf domains. (a–aj) Confocal images of interactions between OZ1 truncations and RARE1, OTP82, CRR28, QED1, DYW2 and ORRM1. Arrows indicate regions magnified to approximately 10× in the inset. Magenta = chlorophyll autofluorescence; green = YFP. YFP puncta that appear to be adjacent to (but not within) the chloroplast is a result of chlorophyll autofluorescence only marking the grana in the core of the chloroplast and not the entire stroma. Scale bars = 10 μm.

relative to the OZ1_Znf constructs in all the transgenic plants tested (Figure 5a).

To confirm that the Znf domains of OZ1 are essential domains for RNA editing, we mutated the four conserved cysteines in each Znf domain because those cysteines are predicted to be essential for their structural folding (Figure 6a) (Gipson et al., 2020). We transformed *oz1* plants with full-length OZ1 carrying the Znf cysteine-to-alanine mutations and observed more robust growth and greening in the Znf-1 plants (where the four C have been changed to A in the first Znf domain) versus the Znf-2 (where the four C have been changed to A in the second Znf domain) or Znf-1&2 mutants (where all the C have been changed to A in both Znf domains) (Figure 6b).

As performed with the plants carrying truncation constructs, we aimed to determine whether the morphological aspect of the transgenic plants could be explained by the level of plastid editing caused by the different constructs. RNA editing analysis revealed no editing rescue in *oz1* homozygous background plants expressing OZ1 constructs with the cysteine-to-alanine mutations in both Znfs or just Znf2 (Figure 6b; Figure S2), regardless of how highly the construct was expressed (Figure 5b). This demonstrates that the Znf domains are essential for the editing function of OZ1. For example, the transgenic plant 64-3 expressing the Znf-2 construct had the highest level of expression of the transgene among all plants tested, but it still failed to show any restoration of editing (Figure 5b; Figure S2).

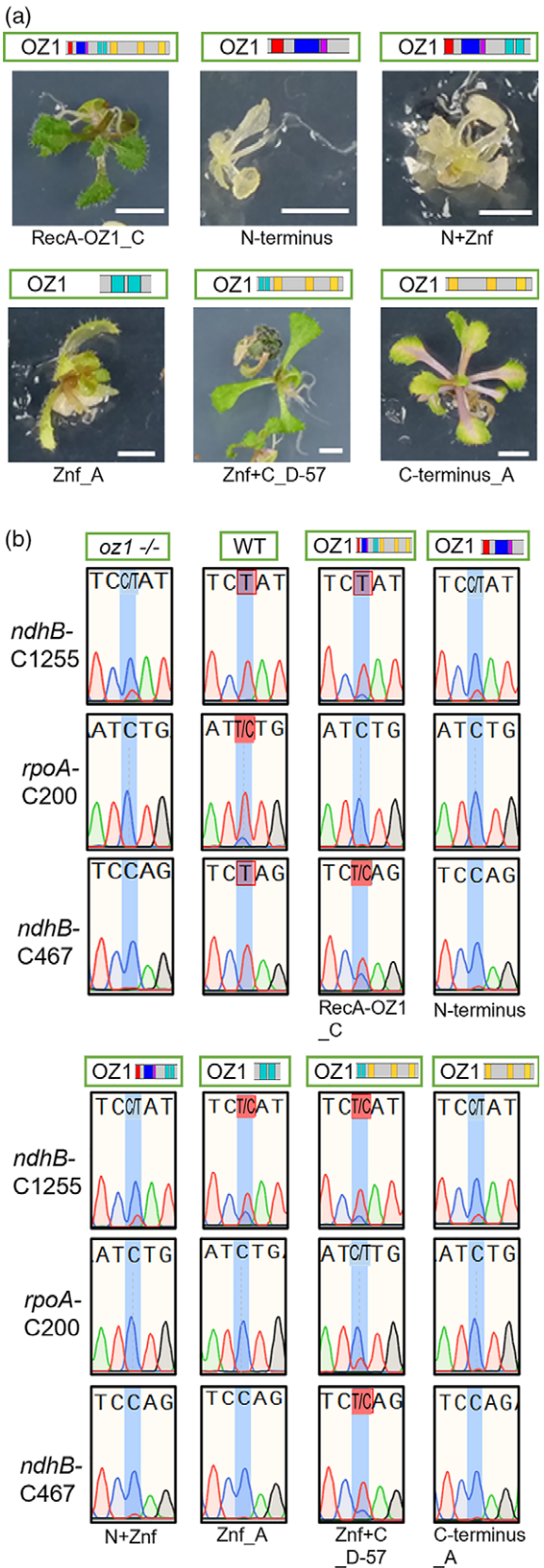

Figure 4. The OZ1 Znf domains alone can partially rescue certain editing sites. (a) One-month old *oz1* homozygous seedlings expressing OZ1 truncation constructs. Scale bars = 5 mm. (b) Sanger sequencing traces of editing sites *ndhB*-C1255, *rpoA*-C200 and *ndhB*-C467 in *oz1* mutants, WT-sequence OZ1 in *oz1* mutants and *oz1* mutants expressing OZ1 truncation constructs. Chosen traces are representative of ≥ 3 different plants analyzed per construct. Text labels under seedlings images and Sanger traces indicate which transformant line is depicted, corresponding to the line names in Figure 5.

Furthermore, editing is largely restored by the OZ1_Znf-1 mutant construct, but some sites, such as *ndhG*-C50, are not significantly rescued by Znf-1 (Figure 6b; Figure S2). As observed in plants expressing OZ1 truncations capable of rescue, plants with greater expression of Znf-1 have a greater extent of editing rescue (Figure 5b; Figure S2). Altogether, these results indicate that Znf2 alone is the editing-essential domain.

Znf mutation does not affect PPR-OZ1 interactions but does perturb ORRM1-OZ1 binding

Aiming to understand why the second Znf domain is needed for the editing function of OZ1, we hypothesized that the domain is important for interaction with other editing factors. We therefore evaluated the possibility that the putative structural disruption caused by mutating zinc-coordinating cysteines into alanines could be interfering with the ability of OZ1 to bind with other editing factors and incorporate into the editosome. Y2H with PPR proteins and the Znf-1, Znf-2, or Znf-1&2 mutant of OZ1 showed that mutation of the Znfs does not interrupt binding between OZ1 and PPR proteins (Figure 7a). However, the interaction with ORRM1 was abolished when Znf2 was mutated but not when Znf1 was mutated.

The requirement for Znf2 was also observed when interactions were tested by BiFC (Figure 7b), where PPR proteins interacted with OZ1 regardless of OZ1 mutation, whereas ORRM1 did not interact with OZ1 protein that has the cysteine-to-alanine mutations in Znf2.

DISCUSSION

In the present study, we delineated functional domains in the plastid RNA editing factor OZ1. Three modular domains, the N-terminus, a central Znf pair and a C-terminus, were tested individually or in combination for their ability to bind to other editing factors. In addition, these domains were assayed for their ability to restore a wild-type morphology and/or editing extent in an *oz1* mutant background. The goal was to determine whether there was a connection between these different biological processes.

In every instance where the full-length OZ1 protein interacted with a PPR protein, we found that the C-terminal

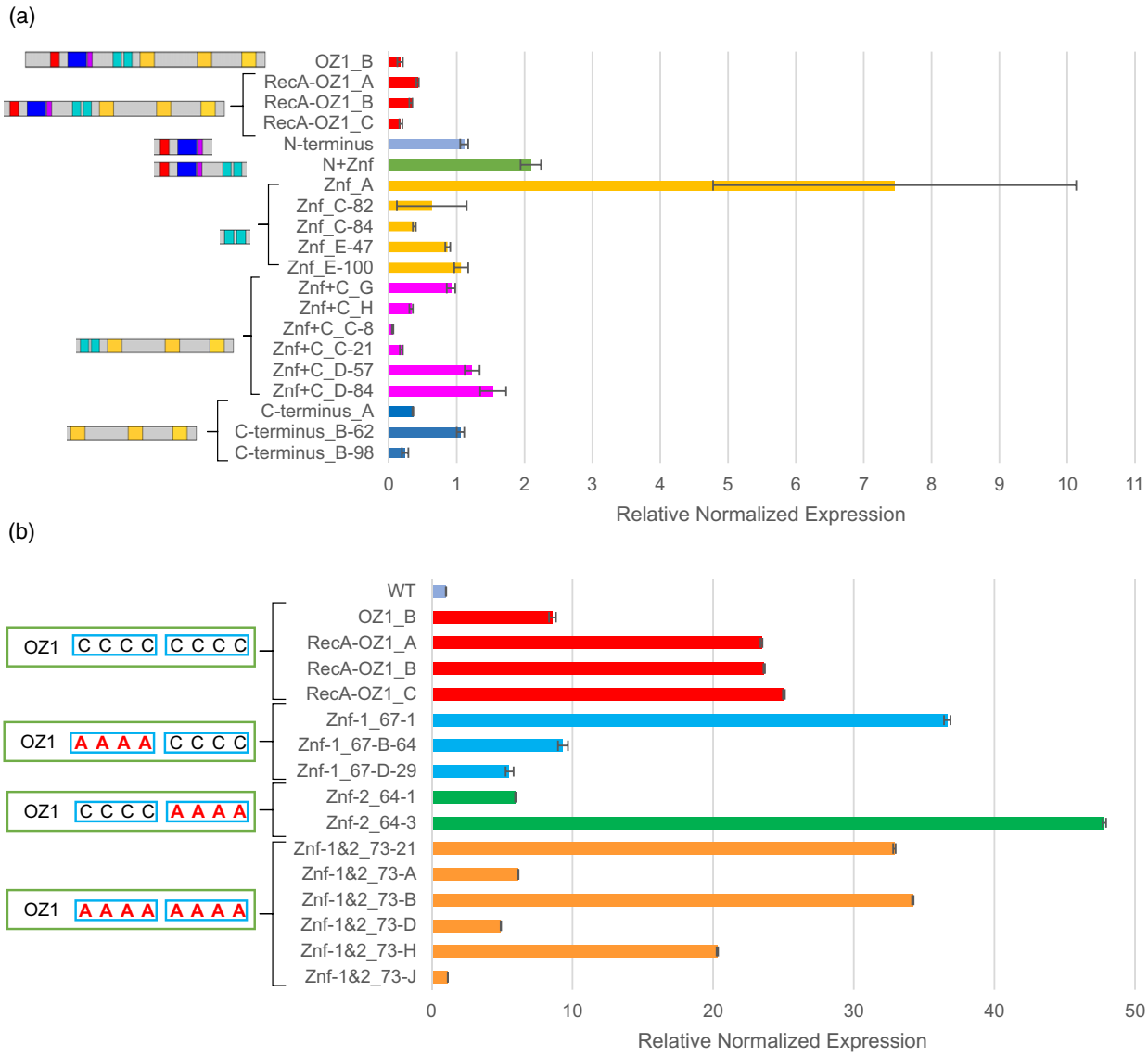

Figure 5. RT-qPCR of plants expressing OZ1 constructs. (a) RT-qPCR of OZ1 truncation expression constructs. All plants are *oz1* homozygous background. 'OZ1' construct refers to the full-length OZ1 sequence, whereas 'RecA-OZ1' refers to constructs of the RecA chloroplast transit peptide fused to the N-terminus of the OZ1 sequence minus the first 100 nucleotides. Primers used: pBI121_qPCR_F and pBI121_qPCR_R for OZ1 constructs, SAND_qPCR_F and SAND_qPCR_R for the SAND reference gene. Error bars indicate the SEM, corrected for expression differences relative to reference gene. (b) RT-qPCR quantification of OZ1 Znf mutants, obtained as above except using OZ1q_1464-1681_F and OZ1q_1464-1681_R for OZ1 constructs and native OZ1 in the wild-type sample.

portion of the protein recapitulated protein–protein binding (Table 1), leading to the conclusion that the C-terminal domain of OZ1 is a main contributor to those interactions. The motifs in the C-terminal region of OZ1, shown as yellow in Figure 1b and in the sequence displayed in Sun et al. (2015), are unique to OZ1. Future structural elucidation of this editing factor will likely deepen the understanding of the incorporation of OZ1 into the editosome.

Surprisingly, the OZ1_Znf truncation was also able to interact not only with PPR proteins (Figures 2 and 3 and Table 1), but also with ORRM1, a major component of the plastid editosome as defined by the complete loss of editing for 12 sites in the *orrm1* mutant (Sun et al., 2013). We found that both the C-terminal domain and the Znf domains of OZ1 contribute to protein–protein interactions, although the physical nature of interactions mediated by the C-terminal domain is likely distinct from those mediated by the Znfs. There is precedent for RanBP2 Znfs acting as protein-binding domains; indeed, the namesake protein of the family is Ran-binding protein 2, which resides in the nuclear pore complex of human cells and binds a nuclear export factor through its Znf domains (Singh et al., 1999). The rigidly structured Znf domains of OZ1 may contribute to ORRM1 interactions, although the ability of OZ1 to

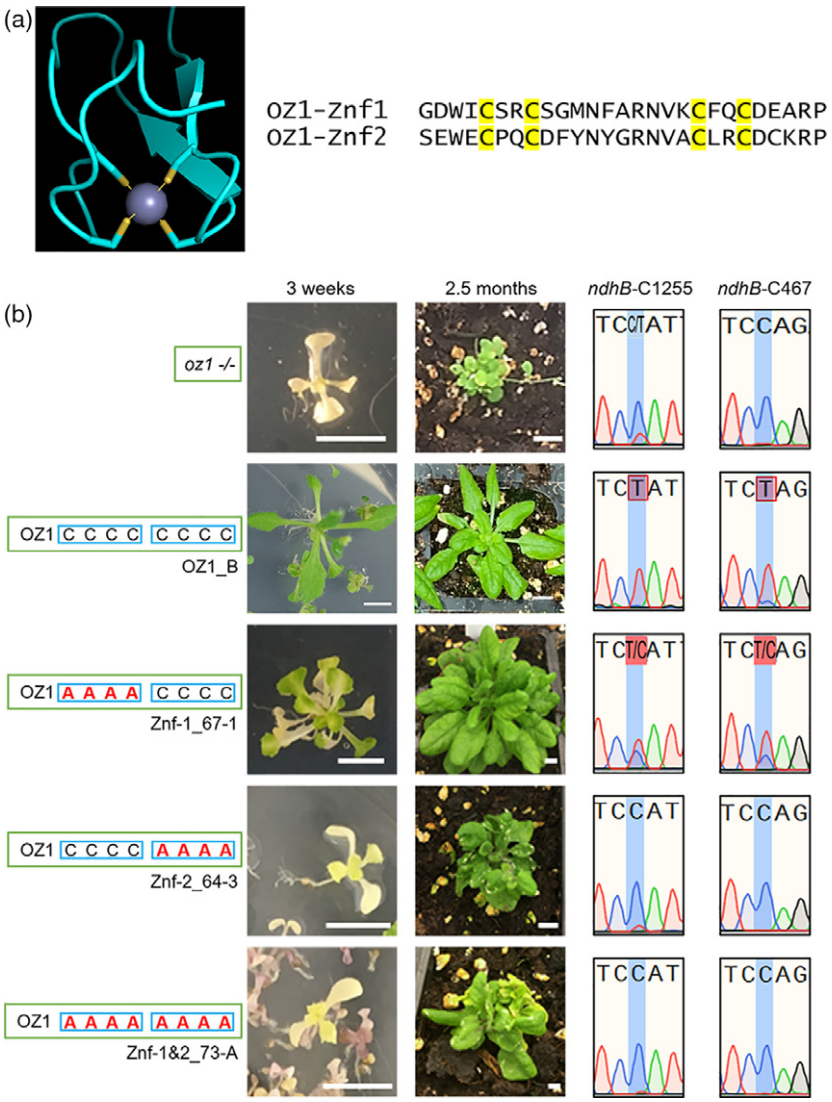

Figure 6. The ZnF domains of OZ1 are essential for chloroplast RNA editing. (a) Predicted structure of the second OZ1 ZnF domain based on the crystal structure of ZRANB2 (3g9y). Gray sphere = coordinated Zn^{2+} ion; yellow = zinc-coordinating cysteine residues; teal = rest of Znf structure, showing predicted beta sheet secondary structure. To the right, alignment of the sequences of the first and seconds Znfs of OZ1. Yellow highlights indicate the putative zinc-coordinating cysteine residues. (b) Znf mutant seedlings and representative editing traces. *oz1* homozygous seedlings and mature plants expressing OZ1 Znf mutant constructs. Text labels under construct diagrams indicate which transformant line is depicted, corresponding to the line names in Figure 5. Scale bars = 5 mm. Sanger sequencing traces show the *ndhB*-C1255 and *ndhB*-C467 editing sites in *oz1* mutants, *oz1* mutants expressing WT-sequence OZ1 and *oz1* mutants expressing cysteine-to-alanine ZnF mutant constructs.

accumulate in a sufficiently high concentration and associate with putative editosomes in spite of its low native expression level (Hruz et al., 2008) could be explained by the presence of low-complexity domains in the C-terminal region, which were predicted by domain boundary analysis. Much recent research has characterized the ubiquity and necessity of so-called phase-separated droplets, comprising membraneless organelles created by the loose interactions between proteins with low-complexity domains, as well as interactions between such proteins and RNAs (Cuevas-Velazquez and Dinneny, 2018). If OZ1 is associated with editosome-containing phase-separated droplets, then sensitivity to component concentration could explain the variation seen in the fluorescent puncta morphology in our BiFC experiments (Figures 3 and 7b–m).

The BiFC assay revealed more interactions than the Y2H assay, an observation that was already encountered during our study of the mitochondrial splicing factor OZ2 (Bentolila et al., 2021). As proposed in our previous work, the Y2H assay, which is a heterologous system, might not allow the proper folding of some of the proteins tested, preventing their interactions. In addition, the removal of the transit peptide from the factors assayed in the Y2H assay is based on prediction software and, as such, might be inaccurate with respect to removing too much or too little of the protein, impairing its proper folding. Finally, these interactions may be assisted in *N. benthamiana* by cofactors that do not exist in yeast.

Complementation experiments demonstrated that the presence of the Znf domains is necessary but not sufficient to restore editing in the *oz1* mutant background. The N-terminus+Znf construct was unable to complement the editing defect in the mutant, whereas the Znf alone and the Znf+C-terminus was able to significantly increase the plastid editing extent (Figure 4b). The failure of the N-

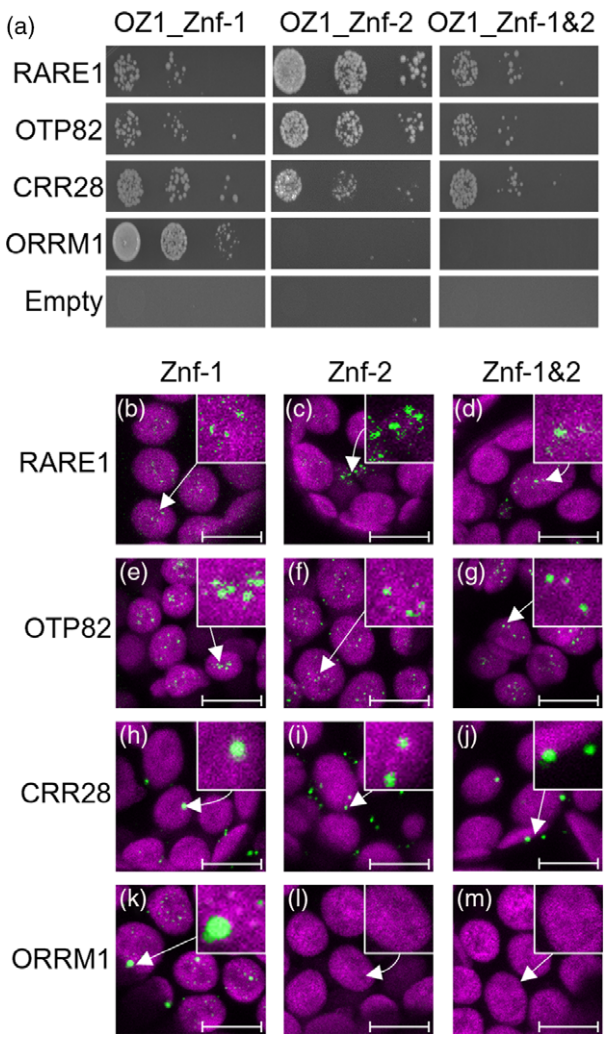

Figure 7. Mutation of the OZ1 Znf domains perturbs interaction with ORRM1 but not PPR proteins. (a) Y2H assays between OZ1 Znf mutants and editing factors RARE1, OTP82, CRR28 (PPR proteins) or ORRM1. Empty = yeast strains expressing empty pGADT7 or pGBKT7 vectors as a negative control. (b–m) BiFC confocal images of interactions between OZ1 Znf mutants and RARE1, OTP82, CRR28 and ORRM1. Magenta = chlorophyll autofluorescence; green = YFP. Arrows indicate regions magnified to approximately 10× in the inset. YFP puncta that appear to be adjacent to (but not within) the chloroplast is a result of chlorophyll autofluorescence only marking the grana in the core of the chloroplast and not the entire stroma. Scale bars = 10 μm.

terminus+Znf construct to restore editing is somewhat puzzling but might be explained by the repressive effect of the N-terminus on the binding of Znf that was observed in the Y2H assay (Figure 2). For the constructs exhibiting any ability to rescue editing (e.g. OZ1_Znf-1 and OZ1_Znf+C-terminus), the expression level of the construct in a given plant as measured by RT-qPCR (Figure 5) correlates with the extent of editing rescue in that plant (Figures S1 and Figure S2). Furthermore, relatively low expression levels of the Znf+C-terminus construct are able to rescue editing to a much greater extent than even the high expression of Znf only. This may indicate that the presence of the C-terminus assists with the association of the truncation into the editosome more effectively than the Znf alone can do. This consideration is also relevant to the lack of restoration observed with the N-terminus+Znf construct.

In the light of the interaction and complementation experiments, it is tempting to link the ability of the Znf domain to bind to ORRM1, a major component of the plastid editosome, and the capacity of OZ1 to fulfill its editing function. This model is supported by the mutation analysis that we performed. The inability of ORRM1 to interact with OZ1_Znf-2 and OZ1_Znf-1&2 may impair the incorporation of the mutated OZ1 into a functional editosome, which could explain the lack of editing rescue in plants expressing either of these constructs. Furthermore, through the mutant analysis, we were able to implicate the second Znf domain as being essential for both the binding of OZ1 to ORRM1 and for the editing function of OZ1. We confirmed that the loss of editing function in OZ1_Znf-2 and OZ1_Znf-1&2 could not be attributed to the misfolding and degradation of those proteins because YFP labeling demonstrated that OZ1_Znf-1&2 still accumulates in the chloroplast (Figure S3). Many of the editing sites rescued by Znf-1 but not Znf-2 or Znf-1&2 are abolished in the *orrm1* knockout mutant, such as *ndhB*-C467 and *ndhD*-C878 (Figure S2a,f) (Sun et al., 2015), supporting a link between the binding of OZ1 to ORRM1 via Znf2 and the editing function of OZ1. However, among the sites that are moderately affected in the *orrm1* mutant (Sun et al., 2015), there are sites such as *ndhB*-C1255 (Figure S2d) that are rescued by Znf-1 and then sites that are very weakly rescued by Znf-1 (*rpoA*-C200 and *clpP*-C559). For the handful of sites where OZ1_Znf-1 rescue does not correspond with a total editing loss in *orrm1*, a mechanism independent of ORRM1 likely allows editing to occur, possibly through contact of OZ1 with the RNA target.

The structure of the RanBP2 Znf domain was solved with the first domain of the ZRANB2, a human splicing factor (Plambeck et al., 2003). NMR spectra in both the presence and absence of zinc demonstrated that this domain was a genuine zinc-binding domain. Previously, the Znf domains of OZ2 were observed to be able to bind to two zinc atoms using performing mass spectrometry analysis under native and denaturing conditions (Bentolila et al., 2021). We did not perform this analysis for OZ1 in the present study; however, given the high similarity between the Znf domains of OZ1 and OZ2 (Sun et al., 2015) and the severe impact of mutating the OZ1 cysteines predicted to coordinate the zinc atom, it is likely that the Znf domains of OZ1 also bind zinc.

Previous work aligning the RNA sequences surrounding editing sites focused on PPR binding (Barkan et al., 2012; Ruwe et al., 2019; Wagoner et al., 2015), which involves sequences ranging from 15–30 nucleotides upstream of the

editing site (Barkan and Small, 2014). The two RanBP2-type Znf domains found in ZRANB2 are able to bind single-stranded RNA with high affinity and specificity (Loughlin et al., 2009), raising the possibility that the Znf domains found in OZ1 might also bind to an RNA target. However, if OZ1 interacts with RNA, it would only be with very short sequences, no more than three to six nucleotides long, based on the length of known RanBP2 Znf binding sites (Loughlin et al., 2009). The possibility of OZ1 binding to RNA through its Znf domains adds another level of complexity that was not explored in the present study, and future studies may further refine how OZ1 is integrated in the editosome.

We have demonstrated that OZ1 interacts with PPR proteins through the C-terminal domain, which is likely to be necessary for efficient incorporation into the editosome, whereas interaction with ORRM1 through its second Znf is required for RNA editing at particular sites targeted by OZ1. A recent study by our group revealed OZ2, the closest relative of OZ1 in the Arabidopsis proteome, to be a mitochondrial splicing factor (Bentolila et al., 2021). Because the C-terminal region of OZ1, which mediates PPR interaction, is unique to OZ1, it is possible that the other two OZ proteins may function in organelle RNA metabolism other than editing.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis Col-3 plants were used for wild-type controls. *oz1* T-DNA insertional mutants (SAIL_358_H03) were purchased from the Arabidopsis Biological Resource Center (https://abrc.osu.edu). Because of the weak germination and chlorotic phenotype of the homozygous mutant, seeds were sown on 0.5× MS with Gamborg's Vitamins (Caisson Labs, Smithfield, UT, USA) + 1% sucrose agar plates and grown at 25°C with a day length of 16 h. After 5–8 weeks, seedlings with greening leaves were transferred to LM-111 soil and grown under short-day (10 h) conditions for RNA analysis or long-day (16 h) conditions for seed harvesting and floral dipping.

Domain boundary analysis of OZ1

When designing truncation constructs of OZ1 to test the function of different domains, we performed domain boundary analysis (Cooper and Marsden, 2017) to identify the best cut-off points for each construct that would still preserve predicted domain folding. In short, PSIPRED, FoldIndex (Prilusky et al., 2005) and GlobPlot 2 (Linding et al., 2003) were used to analyze the OZ1 amino acid sequence for secondary structure prediction and disordered region identification, providing a basis for determining construct boundaries.

Y2H assay

The mature coding sequences (lacking the first 33 amino acids, the predicted length of the plastid transit peptide) (Sun et al., 2015) of OZ1, OZ1 truncations (containing the last eight residues of the RecA chloroplast transit peptide at the N-terminus as an artifact of cloning), OZ1 Znf mutants, and editing factors RARE1, OTP82, CRR28, QED1, DYW2 and ORRM1 were amplified from Col-3 Arabidopsis cDNA using Phusion polymerase (Thermo Scientific, Waltham, MA, USA) and the Y2H primer pairs in Table S1. 3′-A overhangs were added with Taq DNA Polymerase (Qiagen, Germantown, MD, USA) by incubating at 37°C for 10 min. After purification, the amplicons were TA cloned into pCR8/GW/TOPO (Invitrogen, Waltham, MA, USA) to use in Gateway cloning reactions with the Y2H destination vectors pGADT7GW and pGBKT7GW (Lu et al., 2010). Empty pGADT7GW and pGBKT7GW vectors were used as negative controls in the Y2H assays. Yeast mating strains PJ69-4a and PJ69-4α were individually transformed with pGADT7GW and pGBKT7GW plasmids, respectively. Single transformants were mated to produce diploid double-transformant yeast on YPAD agar plates. Diploid yeast were grown in -Leu -Trp media overnight (Takara Bio USA, Mountain View, CA, USA), and then 10 μL of each culture was spotted onto -Leu -Trp -His -Ade media plates (Takara Bio USA) after being diluted with water until OD_{600} of 0.5, 0.05 or 0.005 was reached. Survival/growth plates were imaged after 3 days of incubation at 30°C.

BiFC

The full-length coding sequences (including N-terminal plastid transit peptides) of OZ1, OZ1 Znf mutants and editing factors RARE1, OTP82, CRR28, QED1, DYW2 and ORRM1 were amplified using the BiFC primer pairs in Table S1. For experiments with OZ1 truncations, all OZ1 constructs were cloned without the first 33 amino acids (the predicted transit peptide), and in its place was a RecA chloroplast targeting sequence corresponding to the first 65 residues of that plastid-localized protein (Kohler et al., 1997; Lin et al., 2014). PCR products were first cloned into pCR8/GW/TOPO and then pEXSG-nYFP and pEXSG-cYFP BiFC destination vectors via Gateway cloning reactions, as described above.

Agroinfiltration of *N. benthamiana* with pEXSG plasmid-carrying *Agrobacteria* was performed as reported in Sparkes et al. (2006). For this, *Agrobacterium tumefaciens* GV3101::pMP90RK were transformed with pEXSG plasmids via electroporation using 1 μg of plasmid. Electroporation was conducted with the parameters: capacitance 25 μF, voltage 2.0 kV, resistance 200 Ω, pulse length approximately 5 ms. Agrobacteria were selected on LB agar plates containing kanamycin (50 μg ml^{-1}) and carbenicillin (25 μg ml^{-1}). Cultures (5 ml) of individual transformed Agrobacteria were incubated for 2 days at 28°C and resuspended in a solution of 50 mM 2-(*N*-morpholine)-ethanesulphonic acid (pH 5.6), 2 mM Na_3PO_4, 1.4 mM glucose and 100 mM acetosyringone. Infiltration samples were made by mixing 0.3 OD_{600} of each pEXSG plasmid-carrying bacteria and 0.3 OD of P19-carrying bacteria to a final OD of 0.9. Leaves of 4–6-week-old *N. benthamiana* plants grown under long-day conditions were infiltrated with the *Agrobacteria* solution. Two to 3 days post-infiltration, 2-mm squares were cut from the infiltrated leaf area and imaged using an Axio Observer LSM 710 microscope and a C-Apochromat 40×/1.20 W Korr M27 objective (Zeiss, Oberkochen, Germany). Chlorophyll was excited at 514 nm and the emission filter range was set to 617–735 nm; YFP was excited at 514 nm, with emission at 519–602 nm.

Plant transformation

OZ1 truncation constructs with the RecA targeting sequence (as described above) were cloned into pBI121. OZ1 Znf point mutants with the native transit peptide were synthesized with the Q5 Site-Directed Mutagenesis kit (NEB, Ipswich, MA, USA), TA cloned into

pCR8 and then cloned into a Gateway-compatible pBI121 vector as described above. *Agrobacterium tumefaciens* GV3101 were transformed with pBI121 expression plasmids as above, selecting with kanamycin (50 μg ml^{-1}) and gentamicin (25 μg ml^{-1}).

oz1 heterozygous plants underwent floral dipping using standard protocols (Zhang et al., 2006). After 24 h of recovery in the dark, floral-dipped plants were returned to long-day conditions and grown for 4–6 weeks before drying and harvesting seeds. Seeds were sterilized and stratified before plating for selection on 0.5× MS with Gamborg's Vitamins + 1% sucrose agar plates containing kanamycin (50 μg ml^{-1}), BASTA (glufosinate ammonium) and cefotaxime (250 μg ml^{-1}). Surviving seedlings were transferred to soil 5–6 weeks after sowing.

RNA analysis

Leaves were taken from 1–5-month-old Arabidopsis, and RNA was extracted using Trizol and the PureLink RNA Mini Kit (ThermoFisher, Waltham, MA, USA). RNA was treated with TURBO DNAse (Invitrogen) and quantified with a Qubit II Fluorometer (ThermoFisher). cDNA was amplified using Superscript III (Invitrogen) and pooled chloroplast transcript primers (Table S1). Gene-specific amplicons were then amplified with the corresponding primer pairs (Table S1) and sequenced.

RT-qPCR was used to evaluate the relative expression levels of OZ1 constructs in transformed *oz1* plants. Total RNA was extracted and treated as above, and cDNA was amplified as above with random hexamer primers. The qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the primers in Table S1 in a CFX Connect reaction module (Bio-Rad). ΔCq was calculated from three technical replicates per sample by comparing Cq values of the OZ1 expression construct target with those of the *SAND* (At3g28390) reference gene.

ACCESSION NUMBERS

The data are available under accession numbers: OZ1 (At5g17790), RARE1 (At5g13270), OTP82 (At1g08070), CRR28 (At1g59720), QED1 (At2g29760), DYW2 (At2g15690), ORRM1 (At3g20930) and SAND (At3g28390).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ABG, MRH and SB conceptualized the project. ABG performed the experiments. ABG, SB and MRH analyzed the data. ABG drafted the original manuscript. MRH and SB performed revisions and editing to produce the final manuscript.

DATA AVAILABILITY STATEMENT

All data referenced in this study is provided either in the main text or in the supporting information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sanger sequencing traces of RNA editing sites from OZ1 truncation construct-expressing plants and homozygous *oz1*.

Figure S2. Sanger sequencing traces of RNA editing sites from OZ1 zinc finger mutant construct-expressing plants, homozygous *oz1* and wild-type Arabidopsis.

Figure S3. A-to-C mutation of the putative zinc-binding residues of the OZ1 zinc fingers does not prevent accumulation of OZ1 in the chloroplasts.

Table S1. Primers used in the present study.

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