

A synthetic RNA editing factor edits its target site in chloroplasts and bacteria

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Letter

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Abstract

Targeted cytidine to uridine RNA editing is a widespread phenomenon throughout the land plant lineage. Members of the pentatricopeptide repeat (PPR) protein family act as the specificity factors in this process. These proteins consist of helix-turn-helix domains, each of which recognises a single RNA nucleotide following a well-elucidated code. A cytidine deaminase-like domain (present at the C-terminus of some PPR editing factors or provided in trans via protein-protein interactions) is the catalytic domain in the process. The huge expansion of the PPR superfamily in land plants provides the sequence variation required for design of novel consensus-based RNA-binding proteins. We used this approach to construct a synthetic RNA editing factor designed to target one of the two sites in the *Arabidopsis* chloroplast transcriptome naturally recognised by the RNA editing factor CHLOROPLAST BIOGENESIS 19 (CLB19). We show that this designed editing factor specifically recognises the target sequence in *in vitro* binding assays and can partially complement a *clb19* mutant. The designed factor is specific for the target *rpoA* site and does not recognise or edit the other site recognised by CLB19 in the *clpP1* transcript. We show that the designed editing factor can function equally specifically in the bacterium *E. coli*, and shows some activity even in the absence of the editing cofactors that are often required for natural editing factor activity in plants. This study serves as a successful pilot into the design and application of programmable RNA editing factors based on plant PPR proteins.

Introduction

RNA editing is a term denoting a type of post-transcriptional modification of a transcribed RNA such that it differs from the sequence predicted from the genomic DNA¹. This process occurs in diverse forms across all kingdoms of life, including the deamination of adenosines to inosines (which are read as guanosines in the mRNA) by ADAR enzymes in animals², the deamination of cytidines to uridines by APOBEC enzymes in animals³ and by PPR-DYW proteins in plants⁴, and the unknown mechanism of uridine modification to cytidine in an as yet unelucidated pathway present in some groups of plants (hornworts, lycophytes and some ferns)^{5,6}. Recently, several tools have been developed to perform single nucleotide editing of mRNA⁷. These tools utilise the ADAR deaminase domain as the base modification tool, with specificity encoded in a programmable guide RNA^{8,9}. The reliance on RNA-guided enzymes, whilst facilitating design, has some drawbacks (for example, it makes it hard to target organelles) and these artificial protein fusions have poor spatial accuracy around the desired target site due to flexibility in the linker region^{8,9}. These limitations justify examining the possibility of redesigning natural protein-guided editing systems such as those found in plants.

RNA editing in plants is carried out by PPR proteins¹⁰, particularly the PLS-class PPR proteins which generally contain triplets of three different types of PPR motifs in a (P1-L1-S1)*n*-P2-L2-S2 arrangement¹¹. These PPR arrays are followed by PPR-like E1 and E2 helix-turn-helix motifs, extending the characteristic repeating structure and a ~136 aa domain (named the DYW domain) with a conserved C-terminal D, Y, W tripeptide^{11,12}. This DYW domain contains a cytidine deaminase-like active site and is necessary and

sufficient for editing to occur^{13–18}. PLS-class PPR protein-mediated editing has been characterised in several model plants notably *Arabidopsis*, the moss *Physcomitrella patens*, rice and maize⁴, but is far more prevalent in some other little-studied plants including hornworts, lycophytes and ferns^{5,6,19–23}.

Correlation of PPR protein sequences with their target sites has led to a proposed ‘code’ for identifying interactions of PPR motifs with an RNA base^{24–27}. These specificity-determining interactions involve hydrogen bonding between the amino acids at two positions in each motif and the aligned RNA base²⁸. With minor variations this code can be used to describe and predict interactions of any of the P, L or S motifs with RNA²⁶. This code has been employed to make many successful predictions about which editing factors target which sites in mitochondria and chloroplasts^{25,26,29,30}. It has also been used in attempts to modify target recognition by editing factors. CLB19 is an *A. thaliana* chloroplast editing factor required for editing of the *rpoA-78691* and *clpP1-69942* sites³¹ that acts as the targeting factor in a complex with the DYW donor protein DYW2 and another editing cofactor, NUWA^{32,33}. We previously showed that the target preference of CLB19 could be modified in vitro and in vivo³⁴ although we were unable to make it entirely specific for one or other of the two target sites.

The modular nature of PPR proteins, their large diversity, and the predictability of their interactions with RNA has prompted the design of synthetic PPR proteins based on consensus motifs, as first performed by³⁵. Such synthetic proteins have proved to be useful tools for understanding the structure of PPR proteins and demonstrating their potential as programmable RNA binding proteins^{28,36–40}. Recently, the first crystal structure of a designer PLS protein⁴¹ based on the consensus from genomic PLS sequences has revealed a similar structure to natural and synthetic P-class PPR proteins. This work suggests that the L-motifs within this synthetic protein are slightly misaligned with the P and S motifs, but this misalignment is rescued by the formation of a complex with the cofactor MORF9 to allow for effective RNA recognition⁴¹. MORF9 is a member of a 10 protein family in *Arabidopsis* that have been identified as important RNA editing cofactors in seed plants^{42,43}, but until this crystal structure was published, it had been unclear how these proteins influence the process. MORF proteins are only one of several classes of cofactor theorised to form the ‘editosome’ in angiosperm organelles^{44,45}. The apparent complexity of the editing process in angiosperms contrasts with its apparent simplicity in other plants where MORF proteins are absent and PPR editing factors are able to edit RNA on their own, as demonstrated by the reconstitution of editing in bacteria¹⁸ and in vitro¹⁷ with editing factors from *Physcomitrella*. These recent results are promising developments on the road to utilising PPR RNA editing factors as programmable biotechnological tools, but to go further we need to be able to design new editing factors, which is facilitated by the use of synthetic proteins based on consensus motifs. Here we demonstrate that such a synthetic editing factor of this type can be designed and expressed in plants and in bacteria, that it edits the chosen target RNA with high specificity, and that it is at least partially independent of plant-specific cofactors.

Results

Design and expression of a synthetic PPR RNA editing factor

We designed a novel editing factor named dsn3PLS-DYW with the motif arrangement (P1-L1-S1)₃-P2-L2-S2-E1-E2-DYW (Fig. 1). This design was based on the most representative amino acids at each position in each motif based on 9730 PPR protein sequences from 38 different species, largely seed plant species, but also including *Physcomitrella patens*, *Selaginella moellendorffii*, and *Picea abies* (from data collated by ¹¹). The P1-L1-S1 triplets were designed to be position-specific, which means that the first P1 motif is slightly different to the second or the third P1 motif, and likewise for the L1 and S1 motifs

(**Supplementary Figure S1**). The dsn3PLS-DYW protein was designed to start with a four amino acid cap composed of a starting methionine, followed by a glycine, asparagine and serine prior to the first P1 motif, based on work by ⁴⁶, who used the same cap in front of TPR motifs to stabilize α-helices.

Compared to the only previously published design of a synthetic PLS-class PPR protein ⁴¹(PLS)3-PPR, ⁴¹, dsn3PLS-DYW has additional domains at the C-terminus, but lacks the extensive cap and solvating helix present in (PLS)3-PPR that are derived from the N- and C-termini of *ZmPPR10* ^{28,47} (**Supplementary Figure S1**). A key difference is also the residues at the 5th and last positions of each PPR motif, which in the case of dsn3PLS-DYW, were programmed to target the *rpoA*-78691 site recognised by CLB19 (Fig. 1).

The dsn3PLS-DYW protein was expressed as an N-terminal thioredoxin-hexahistidine-TEV protease site fusion in BL21 (DE3) *E. coli* and purified by nickel affinity purification. The N-terminal tag was cleaved off with TEV protease and the dsn3PLS-DYW further purified by reverse nickel affinity purification to about 85% purity (**Supplementary Figure S2**). In parallel, a truncated version lacking the DYW domain (named dsn3PLS) and a short form of MORF9 (amino acids 75–196) were purified similarly from *E. coli* Rosetta 2 (DE3) (**Supplementary Figure S2**). RNA Electrophoretic Mobility Shift Assays (REMSAs) were performed on dsn3PLS and dsn3PLS-DYW with and without MORF9 protein, using Cy5-labelled oligonucleotide probes representing the *rpoA*-78691 and *c/pP1*-69942 binding sites of CLB19 (Fig. 2). In the absence of MORF9, dsn3PLS and to a lesser extent, dsn3PLS-DYW show slight binding of the *rpoA* target at the highest concentrations tested. The addition of MORF9 greatly enhances RNA binding, as previously observed for (PLS)3-PPR (⁴¹, with optimal binding requiring at least 2 MORF molecules to each PPR molecule. The dsn3PLS/MORF9/RNA complex is clearly super-shifted with respect to the dsn3PLS/RNA complex. The formation of a dsn3PLS-DYW/MORF9/RNA complex was confirmed by analytical size-exclusion chromatography (**Supplementary Figure S3**). No binding was observed to the *c/pP1* target with or without MORF protein present, and MORF9 on its own displayed no binding to either target even at the highest concentrations.

RNA editing in planta

In order to test the RNA editing capacity of the designer editing factor, dsn3PLS-DYW was introduced into *Arabidopsis thaliana* in a *clb19-3* mutant background. The construct used for plant transformation included a 60 amino acid rubisco small subunit (RbcS) transit peptide from pea (*Pisum sativum*) and a 4 × c-Myc tag at the N-terminus of the protein, prior to the dsn3PLS-DYW sequence, in order to facilitate chloroplast localisation and detection of the protein. The *Arabidopsis* CLB19 promoter and 5'

untranslated region, consisting of the 1 kb of DNA sequence from upstream of the start codon, was used to drive expression of the transgene. As a negative control an almost identical construct was prepared, with the only difference being an inactivating mutation (E70A) in the DYW domain, which removes the conserved glutamate implicated as the catalytic residue in the deamination mechanism^{14,15}. As a positive control, the native *CLB19* sequence (codons 34–500) was cloned into an equivalent RbcS-cMyc construct. Plants homozygous for the *cbl19-3* mutation are slow growing and light-sensitive, but were successfully transformed via *Agrobacterium*-mediated floral dipping. After hygromycin selection and growth, plants from the dsn3PLS-DYW lines displayed a greener phenotype than the *cbl19-3* mutant line or the dsn3PLS-DYW (E70A) line, but the phenotype was less healthy than those complemented with *CLB19* (Fig. 3a).

As a sensitive test of physiological state, RNA-seq data was obtained from leaves of plants grown under relatively low light and analysed to quantify expression of chloroplast genes (Fig. 3b). In plants expressing CLB19 or dsn3PLS-DYW, genes expressing components of the photosynthetic apparatus were generally expressed at a higher level than in *cbl19* samples whereas genes encoding components of the gene expression machinery showed the opposite pattern. Plants expressing dsn3PLS-DYW (E70A) showed an intermediate gene expression phenotype. Copious editing of both the *rpoA-78691* and *clpP1-69942* editing sites was observed in three biological replicates of the control line expressing CLB19 (Fig. 3c). Partial editing of *rpoA-78691* and no editing of *clpP1-69942* was observed in three biological replicates of the plant line expressing dsn3PLS-DYW. Very little editing of *rpoA-78691* was observed in the line expressing inactive dsn3PLS-DYW (E70A). Thus dsn3PLS-DYW is capable of editing *rpoA-78691* but not *clpP1-69942*.

Off-target editing

The overexpression of the editing factors coupled with the extreme sensitivity of high-coverage RNA-seq allowed us to confidently detect off-target editing. The clearest example of this is a site in the second intron of *ycf3* (position 43350), which is a reported low frequency site (12%) in wild type Col-0⁴⁸ but which is edited at relatively high levels (~ 20%) in plants over-expressing CLB19 (and not significantly edited at all in *cbl19* plants) (Fig. 4). The putative binding site for CLB19 upstream of this editing site closely matches expectations (Fig. 4b). This prompted us to search for other possible rare editing events that may represent ‘off-target’ events. All possible editing sites were screened for significant differences in editing between the overexpressing lines and negative controls (*cbl19* was used as a negative control for lines expressing dsn3PLS-DYW (E70A), and these in turn were used as negative controls for lines expressing CLB19 or dsn3PLS-DYW). Only one potential site, the intended target *rpoA-78691*, was found in lines expressing dsn3PLS-DYW (E70A), with 24 events out of 2654 reads (0.89%). As the E70A mutation should completely prevent the deaminase activity of this protein, this residual activity is, at first sight, surprising. However, CLB19 completely lacks this part of the DYW domain and still effectively induces editing at this site by recruiting other cofactors, so it is possible that the dsn3PLS-DYW (E70A) can do the same, albeit with much reduced efficacy. Besides the *ycf3-43350* intron site already mentioned, twelve other sites were identified as potential off-target events induced by CLB19 (Fig. 4a)

and eleven as potential off-target events induced by dsn3PLS-DYW, using an arbitrary threshold of 2 for the log(odds ratio). We believe that two of these sites are unlikely to be targeted by dsn3PLS-DYW or CLB19. The site 49209 is a known low-frequency editing event in the inter-cistronic region of *ndhK-ndhJ* transcripts, reportedly edited at ~ 6% in wild-type *Arabidopsis*⁴⁸. Although we found this site to be edited at substantially higher levels in plants expressing CLB19 or dsn3PLS-DYW (~ 14%), it is also significantly edited in *cbl19* (~ 2%) and dsn3PLS-DYW (E70A) plants (~ 2%), making unlikely that it is a direct target of the introduced editing factors. The site 1502 is barely edited at all (0.03%) and at approximately equal levels in dsn3PLS-DYW, CLB19 and *cbl19* plants. Excluding these two probable ‘false positives’, the other potential off-target sites show similarity to the *rpoA-78691* site (Fig. 4b).

RNA editing in bacteria

Based on the work by Oldenkott *et al*¹⁸, the bacterial expression vector containing the dsn3PLS-DYW gene was modified to insert 39 bp regions covering either the *A. thaliana rpoA-78691* or *clpP1-69942* editing sites into the 3' untranslated region of the dsn3PLS-DYW transcript. Expression of this modified dsn3PLS-DYW transcript including a downstream editing site was induced together with MORF2 co-expressed from pETM11, or as a negative control, unmodified pETM11 (which expresses human max dimerization protein 1). The presence of either pETM11-based plasmid greatly reduces dsn3PLS-DYW expression, presumably through sequestration of T7 RNA polymerase as the effect is primarily transcriptional. RNA editing was assessed by sequencing of cDNA derived from total RNA of 1 mL aliquots of bacterial culture after 18 h growth post induction at 16 °C (Fig. 5). Under these conditions, editing of the *rpoA-C200* site was observed in the presence of MORF2 or MORF9, but only at reduced levels in the absence of MORF protein. This editing is abolished by the DYW mutation E70A, regardless of MORF co-expression. No off-target events were detected using the same significance thresholds as used in the analysis of chloroplast RNA.

In order to identify if dsn3PLS-DYW was capable of uridine to cytidine ‘reverse’ editing, the *rpoA-78691* base was mutated at the DNA level in the plasmid to a thymidine (*T200*), which would produce a uridine at this position in the transcribed RNA. No conversion of this site to a cytidine was observed in the sequenced cDNA for dsn3PLS-DYW co-expressed with MORF. No editing of the *clpP1-69942* site was seen under any conditions tested (Fig. 5c).

Discussion

The novel editing factor dsn3PLS-DYW was designed to bind specifically to the region upstream of the *A. thaliana* chloroplast *rpoA-78691* editing site recognised by the natural editing factor CLB19. The REMSA results (Fig. 2) and the observed RNA editing in plants and bacteria expressing dsn3PLS-DYW (Figs. 3 and 5) show that this aim was achieved successfully. CLB19 and dsn3PLS-DYW differ quite considerably (only 45% sequence identity in the motifs aligned to the same nucleotides) and notably most of the residues known to be implicated in sequence recognition are different between these two proteins. CLB19 recognises one other major site in *Arabidopsis* chloroplasts, the *clpP1-69942* originally reported in³¹. This site is not detectably bound by dsn3PLS-DYW in vitro (Fig. 2) and not edited in plants or bacteria

expressing dsn3PLS-DYW (Figs. 3 and 5). Hence by rational design we have succeeded in engineering an RNA editing factor that is more specific than its natural counterpart, whereas our previous attempts to achieve the same goal by modification of the natural protein achieved a far less dramatic shift in specificity³⁴. The fact that complementation of only the *rpoA* editing defect was sufficient to almost fully restore normal growth and chloroplast gene expression, at least under relatively low light conditions (Fig. 3), indicates that it is the loss of this editing event that primarily contributes to the strong phenotype of *clb19* mutants. Under higher light conditions growth defects become apparent even in plants expressing dsn3PLS-DYW (in a *clb19* background) (Fig. 3); we cannot be certain whether this is due to the lower degree of editing of the *rpoA-78691* site than in wild-type plants or the complete lack of editing of the *clpP1-69942* site.

Interestingly, *clb19* plants expressing the inactivated dsn3PLS-DYW(E70A) construct were phenotypically distinguishable from untransformed *clb19* (Fig. 3), suggesting that they were partially complemented, and this was confirmed by the RNA-seq data indicating a low level (0.89%) of editing of the *rpoA-78691* site (Figs. 3 and 4). As this construct was not active in *E. coli* (Fig. 5), we presume this low level editing is due to a weak association between the dsn3PLS-DYW(E70A) protein and one of the DYW ‘donor’ proteins known to form complexes with other editing factors, such as DYW2 which forms a complex with CLB19^{32,33}. From an evolutionary point of view, it is noteworthy that less than 1% editing at a single site is sufficient to give rise to phenotypic differences that could provide a selective advantage. This might hint at how new editing events may arise and become selected for.

The high coverage RNA-seq that detected editing of *rpoA-78691* in the dsn3PLS-DYW(E70A) samples also detected ‘off-target’ editing at numerous sites in plants expressing dsn3PLS-DYW and CLB19 (Fig. 4). Only one of these sites has been reported as an editing site previously⁴⁸, to our knowledge – the *ycf3-43350* site in intron 2 of the *ycf3* transcript. We do not think that this event has any functional significance as we did not detect any significant difference in *ycf3* intron 2 splicing (**Supplementary Figure S4**) between plants expressing CLB19 (where the *ycf3-43350* is edited) or dsn3PLS-DYW (where the *ycf3-43350* is not edited). This site is specifically edited by CLB19, and according to our binding predictions (Fig. 4), is an even better match to CLB19 than either *rpoA-78691* or *clpP1-69942*, and yet is edited to a much lower extent. This may be due to a shorter half-life of the intron RNA, or because of the A at position -1 relative to the editing site; purines at this position are known to have an inhibitory effect on editing^{18,50,51} and are rare in our collection of off-target events. Other off-target events were detected at much lower levels, below 2% (Fig. 4). Perhaps surprisingly, no off-target events were detected in bacteria, despite the much greater sequence complexity of the transcriptome and therefore the higher probability of close matches to the target site occurring by chance. We suspect that there may be two explanations for this; firstly the read coverage of the chloroplast transcripts is generally much higher than for *E. coli* transcripts, allowing the detection of lower rates of editing, and secondly, whereas the *rpoA* target is a low abundance transcript in chloroplasts, the target transcript in the *E. coli* experiments is extremely highly expressed and may sequester a large fraction of the editing factor.

We believe that the putative off-target events in chloroplasts are true editing events catalysed by the introduced editing factors because of the statistically significant difference in the transformed lines with respect to the controls, and because they are generally consistent with expectations based on predicted binding by dsn3PLS-DYW and CLB19. The putative off-target sites for CLB19 are generally consistent with the *in vitro* analysis of the contribution of individual CLB19 motifs to target binding, notably the major contribution of the 2nd P1 motif (5/35 combination TD, recognising G)³⁴. Promisingly for applications of synthetic editing factors, editing at off-target sites of dsn3PLS-DYW did not exceed 1.5% at any of the 9 sites we detected. Although eight of these events lead to non-synonymous changes in coding sequences (**Supplementary Table S1**), such low amounts of editing are unlikely to be significant through any loss-of-function effect on the encoded protein. These off-target events are informative for the design of future synthetic editing factors as they provide information on the specificity of recognition (or the lack of it) of individual motifs. For example, the S2 motif aligns with an A in all 21 sites recognised by either dsn3PLS-DYW or CLB19 (Fig. 4), suggesting a hitherto unrecognised importance of this motif in determining site specificity. Other motifs proved to be less specific than expected; for example, the 5/35 combination NS, thought from previous data^{26,27} to be relatively specific for C over U, did not prove to be when considering dsn3PLS-DYW off-target events, where in 12/18 cases NS motifs aligned with a U in the target (Fig. 4). This type of data will be helpful for optimising future designs.

Binding of dsn3PLS-DYW to its target sequence *in vitro* and editing *in vivo* was strongly (but not completely) dependent on the presence of MORF proteins (Figs. 2 and 5). MORF2 and MORF9 were equally able to promote editing by dsn3PLS-DYW (Fig. 5), and the evidence suggests that the PPR-MORF-RNA complex that is formed contains multiple copies of the MORF protein (Fig. 2 and **Figure S3**). These results are entirely consistent with those obtained with a different synthetic protein based on consensus P-L-S motifs⁴¹. Hopefully this confirmation helps remove some of the confusion concerning the role of MORF/RIP proteins in RNA editing.

The currently favoured biotechnological tools for RNA editing, the REPAIR and RESCUE systems comprised of a base editor coupled to a deactivated Cas13, achieve a high degree of specificity via the short guide RNA complementary to the target RNA, but once bound, show significant promiscuity, resulting in undesirable off-target editing of any deaminable bases within a window of at least four nucleotides on either side of the editing site^{8,9}. In this study, we have demonstrated the potential of a new type of designer editing factor that can edit with high specificity. Natural PPR editing factors are extremely precise – editing almost always occurs at the 4th nucleotide 3' of the nucleotide aligned with the S2 motif^{25–27}. This is also the case for dsn3PLS-DYW, as all the off-target events observed are consistent with this positioning of the editing factor relative to the edited C; indeed, no editing was detected at adjacent C residues where this would have been possible (sites 49646 and 69992). Thus it is reasonable to imagine that the specificity of ‘designer’ editing factors based on PPR-DYW scaffolds could ultimately exceed that of Cas13-ADAR fusions. On the other hand, designing the specificity of the PPR array remains complex due to the uncertain contributions of MORF cofactors and the C-terminal S2-E1-E2 motifs. Given that the *Physcomitrella* editing factors PPR56 and PPR65 can edit without MORF proteins

present^{17,18}, it should be possible to design a synthetic editing factor that does not require cofactors for optimal specificity and editing activity. Of particular interest in this context are the monotypic S motif arrays found in putative editing factors in lycophytes¹¹, likely to be MORF-independent⁶.

In conclusion, this work demonstrates the successful use of a synthetic PPR protein as an RNA editing factor, and lays the foundation for detailed structural and mechanistic studies into the mechanism of RNA editing. Designer PPR proteins represent an attractive multipurpose scaffold for targeted RNA binding, particularly as programmable RNA editing factors.

Methods

Plant materials

All plant genetics experiments performed in this study were undertaken on the *Arabidopsis thaliana* *cbl19-3* T-DNA insertion line (SALK_123752)³¹. Seeds were germinated on 0.5 x MS plates (0.5% sucrose) under dim light ($40 \mu\text{E m}^{-2} \text{s}^{-1}$) in long day cycles (16 hours light, 8 hours dark at 25 °C with 45% humidity) for 3 weeks. When the leaves started greening, seedlings were transferred to $120 \mu\text{E m}^{-2} \text{s}^{-1}$ light. In order to perform transformation by flower dipping, pale green seedlings were transferred to soil until they were able to produce flowers.

Bacterial strains

Escherichia coli strains used in this study were DH5a (Thermo Fisher Scientific, Waltham, MA, USA; <https://www.thermofisher.com/>) for cloning, BL21 (DE3) (Novagen, Merck KGaA, Darmstadt, Germany, <https://www.merckmillipore.com/>) for standard protein expression, and Rosetta 2 (DE3) (Novagen) for *E. coli* RNA editing experiments.

Plasmids

Bacterial expression vectors pETM11 and pETM20⁵² were a gift from Dr. Gunter Stier (EMBL, Heidelberg, Germany; <https://www.embl.de>). The plant expression vector used was pCAMBIA1390 (CAMBIA, Canberra, Australia; <https://www.cambia.org>).

Oligonucleotides & primers

Primers and unlabelled oligonucleotides (**Supplementary Tables S2 and S3**) were obtained from Integrated DNA Technologies (IDT; Singapore; <https://www.idtdna.com>) or Sigma-Aldrich (now part of Merck KGaA; Darmstadt, Germany), while labelled oligonucleotides were obtained from Integrated DNA Technologies (IDT, USA).

Design and synthesis of designer editing factor sequences

Domain sequences of putative DYW-subgroup editing factors from 38 land plant genomes containing three P1-L1-S1 motifs were obtained as previously described¹¹. Motifs of canonical length for their type were selected. Consensus sequences of the aligned motifs were constructed with the EMBOSS cons tool⁵³ with plurality 0. The dsn3PLS-DYW protein was designed to start with a four amino acid Met, Gly, Asp,

Ser cap, followed by three repeating modules of position specific P₁-L₁-S₁ triplets, a P₂-L₂-S₂-E₁-E₂ module, and terminating in a consensus DYW domain (**Supplementary Figure S1**). Amino acids residues at the fifth and last positions of the P1, L1, S1, and P2 motifs were selected to bind to the RNA sequence upstream of the *A. thaliana* chloroplast *rpoA-78691* editing site, based on the 2 amino acid PPR code for those motifs^{24–27}, while the residues at the fifth and last positions of the L2, S2, E1 and E2 motifs were chosen to be identical to those of *A. thaliana* CLB19 (Chateigner-Boutin *et al.*, 2008). A synthetic gene encoding the dsn3PLS-DYW sequence was synthesised as a single gene block (GenScript, New Jersey, USA; <https://www.genscript.com/>).

Cloning, bacterial expression and purification

The dsn3PLS-DYW gene was cloned into the *Ncol* and *Xhol* sites of the bacterial expression vector pETM20⁵² by Gibson assembly. Sequences encoding MORF2 (residues 73–193; C82S) and MORF9 (residues 75–196; C85S, C187S) were cloned into the *Ncol* and *Xhol* sites of the bacterial expression vector pETM11⁵² by standard subcloning. The dsn3PLS-DYW expression vector was transformed into *E. coli* strain BL21 (DE3), while the MORF2 and MORF9 expression vectors were transformed into *E. coli* strain Rosetta 2 (DE3). Strains were grown in LB medium at 37 °C, 200 rpm to an absorbance of 0.6–0.8 at 600 nm, induced with addition of 0.1 mM IPTG, with addition of 20 µM ZnSO₄ to the dsn3PLS-DYW expressing cultures, and maintained at 16 °C, 200 rpm for 16–18 h. Bacterial pellets were resuspended in 40 mL of binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT) supplemented with 125 units of Benzonase Nuclease (Sigma). Cells were lysed on ice using an Emulsiflex C5 high-pressure homogeniser (Avestin) to a maximum operational pressure of 16000 psi until the lysate was clarified. The soluble fraction was separated from the insoluble fraction by centrifugation at 24000 g for 45 minutes at 4 °C and passed through a 0.22 µm syringe filter (Merck Millipore) before purification. Filtered bacterial lysate in binding buffer containing recombinantly-expressed proteins was loaded onto a 5 mL His-Trap HP column (GE Healthcare) and the protein was eluted using a gradient of imidazole (20–500 mM) on an NGC Quest Plus (Bio Rad Laboratories) FPLC instrument (**Supplementary Figure S2**). Fractions of interest were pooled and supplemented with 10 mM DTT and recombinant His-tagged TEV protease. The sample was transferred to a dialysis sac and dialysed against 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM DTT at 4 °C for 16 hours. The digest was then applied to the 5 mL HisTrap HP column (GE Healthcare) to remove the tagged protease and residual uncleaved protein (**Supplementary Figure S2**). Purified MORF2 and MORF9 protein was then concentrated using a Vivaspin 3 kDa MWCO concentrator (GE Healthcare), while purified dsn3PLS-DYW protein was concentrated using a Vivaspin 10 kDa MWCO concentrator (GE Healthcare). Concentrated protein was divided into 200 µL aliquots, frozen in liquid N₂ and stored at -80 °C until further use.

RNA electromobility shift assays

Aliquots of concentrated protein were thawed on ice, diluted in dialysis buffer and the protein concentrations were verified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). REMSAs were performed as described previously³⁴.

Editing complementation in *A. thaliana*

The genes encoding dsn3PLS-DYW, dsn3PLS-DYW (E70A) or CLB19 (34–500) were cloned into pCAMBIA1390, along with the sequences for the native CLB19 promoter (-1000 to -1), the N-terminal chloroplast targeting peptide from pea RbcS, and a 4 × c-Myc epitope tag 5' to the editing factor gene. Expression plasmids were transformed into 2-month-old *clb19-3* plants by floral dipping using *Agrobacterium tumefaciens* strain GV3101 Rif^R Gent^R. Total RNA was isolated with RNazol RT reagent (Sigma) from 3.5-week-old seedlings (T3) grown on half-MS plates under dim light conditions (40 μmol m⁻² s⁻²).

Plant RNA Sequencing

Two (for *clb19-3* and *clb19-3 + dsn3PLS-DYW(E70A)* lines) or three (*clb19-3 + CLB19* and *clb19-3 + dsn3PLS-DYW* lines) independent libraries were made from 250 ng of total RNA treated with Turbo DNase (Ambion, Thermo Fisher) using an Illumina TruSeq Stranded Total RNA with Ribo Zero (Plant) library preparation kit. The sequencing run (150 nt, paired ends) was performed on an Illumina HiSeq4000 sequencer by Novogene (Beijing, China; <https://en.novogene.com>). The read data is available from NCBI (BioProject ID PRJNA680434). Reads were first de-duplicated using *clumpify* (using parameters *dedup optical dist = 40*) from the *bbmap* package (<https://sourceforge.net/projects/bbmap/>), then trimmed of adapters with *bbduk* (parameters: *ktrim = r k = 23 mink = 11 hdist = 1 tpe tbo ftm = 5*) and mapped to the Col-0 plastid genome (accession AP000423) with *bbmap* (parameters: *ambiguous = random mappedonly = t*). To ensure that reads mapping across the arbitrary linear ends of the reference genome were not lost, the reference was extended by 1 kb prior to mapping. For analysis of levels of gene expression, mapped read pairs overlapping annotated CDS features were counted using a script based on biojulia (<https://biojulia.net>) available at <https://github.com/ian-small/pyrimid>. Counts were normalised using a pseudo-reference approach taken from DESeq2⁵⁴. For analysing editing, strand-specific nucleotide counts were obtained using the same in-house package (<https://github.com/ian-small/pyrimid>). Nucleotide count data was analysed statistically with a Fisher exact test as implemented in the Python *scipy.stats* package and the p-values were corrected for multiple testing using *statsmodels.stats.multitest.multipletests* with the Simes-Hochberg procedure. Odds ratios were calculated after adding a pseudocount of 0.5 to all observations to avoid division by zero.

Bacterial RNA editing experiments

E. coli RNA editing systems were set up based on the methods described by Oldenkott *et al.*¹⁸. Sequences 33 bp upstream to 5 bp downstream of the *rpoA-78691* or *clpP1-69942* editing sites were introduced into the 3'UTR of dsn3PLS-DYW in the pETM20 expression plasmid by PCR. The plasmids were assembled using NEBuilder HiFi DNA Assembly Master Mix (NEB) according to the manufacturer's instructions. For editing assays, pETM20:dsn3PLS-DYW plasmids containing the editing sites were transformed into Rosetta 2 (DE3) cells, or co-transformed with the empty pETM11 plasmid, pETM11-MORF9 (75–196; C85S, C187S) or pETM11-MORF2 (73–193; C82S). Single colonies were used to inoculate 5 mL LB starter cultures with the appropriate antibiotics and grown at 37 °C, 200 rpm for 16 hours. 250 μL of the starter culture were then used to inoculate 50 mL LB expression cultures with the same antibiotics. The cultures were grown at 37 °C, 200 rpm to an OD₆₀₀ of 0.5–0.6. Cultures were then

cooled on ice for 10 minutes prior to supplementation with 0.4 mM ZnSO₄, and induction with 0.4 mM IPTG. Cultures were incubated at 16 °C, 200 rpm for 18 h before harvesting. Total RNA was extracted from 1 mL of *E. coli* bacterial pellet using the Direct-Zol RNA extraction kit (Zymo Research; Irvine, CAL, USA) according to the manufacturer's instructions. Reverse transcription was performed on 2 µg of DNase-treated total RNA extract using Superscript™ III Reverse Transcriptase (Invitrogen) through random hexamer primers, according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed with Q5 DNA Polymerase (NEB) according to the manufacturer's instructions, using the SynthSEQ.FOR and T7short primers. Sanger Sequencing of the RT-PCR products using the SynthSeq3.FOR primer was performed by Macrogen Inc. (Seoul, South Korea). Editing efficiency was determined from the raw sequencing chromatograms using the EditR webserver available at <http://baseeditr.com/>. For RNA-seq analyses, 500 ng of DNase-treated RNA was rRNA-depleted⁵⁵ and then used for preparing sequencing libraries with the Illumina TruSeq Stranded total RNA library preparation kit as recommended by the manufacturer. The libraries were sequenced on an Illumina HiSeq4000 sequencer by Novogene. 29–34 million 150 nt paired-end reads were obtained for each sample. The read data is available from NCBI (BioProject ID PRJNA680433). Analysis of the data was carried out as described above for the chloroplast RNA data, except that the reads were mapped to the *E. coli* BL21 genome (accession CP010816) and the relevant pETM11 and pETM20 constructs.

Declarations

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Figures

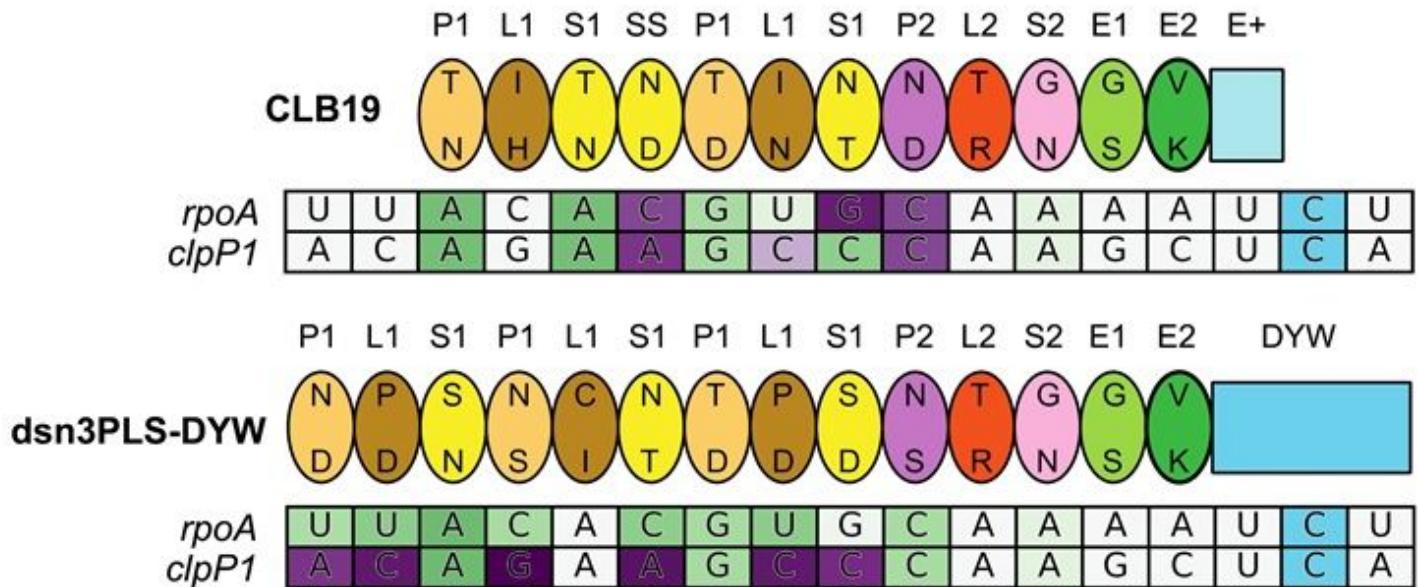


Figure 1

Schematic representation of CLB19 and dsnPLS-DYW bound to their target sites. The proteins are represented by ovals for each PPR motif including the 5th and last specificity-determining amino acids. The target sites are coloured according to the predicted favourability of the alignment at each position, from favoured (green) to neutral (white) to disfavoured (purple). These values are taken from 26. The C at the editing site is shaded in blue.

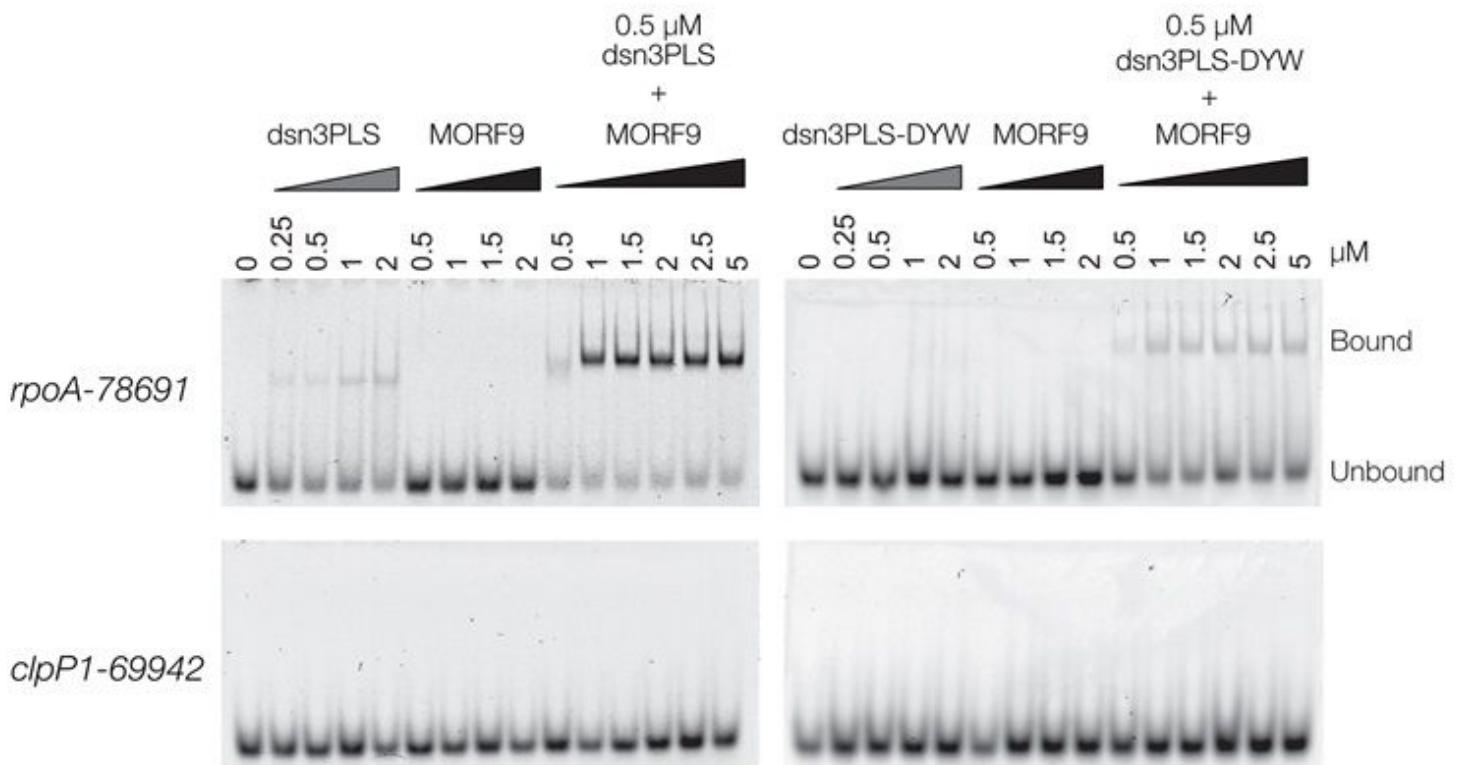


Figure 2

MORF-enhanced sequence-specific binding of Dsn3PLS proteins to *rpoA-78691* target RNA. Binding assays were performed with dsn3PLS (left panels) or dsn3PLS-DYW (right panels) in comparison to, or together with, MORF9 in the presence of 1 nM *rpoA-78691* target RNA (top panels) or *clpP1-69942* (bottom panels).

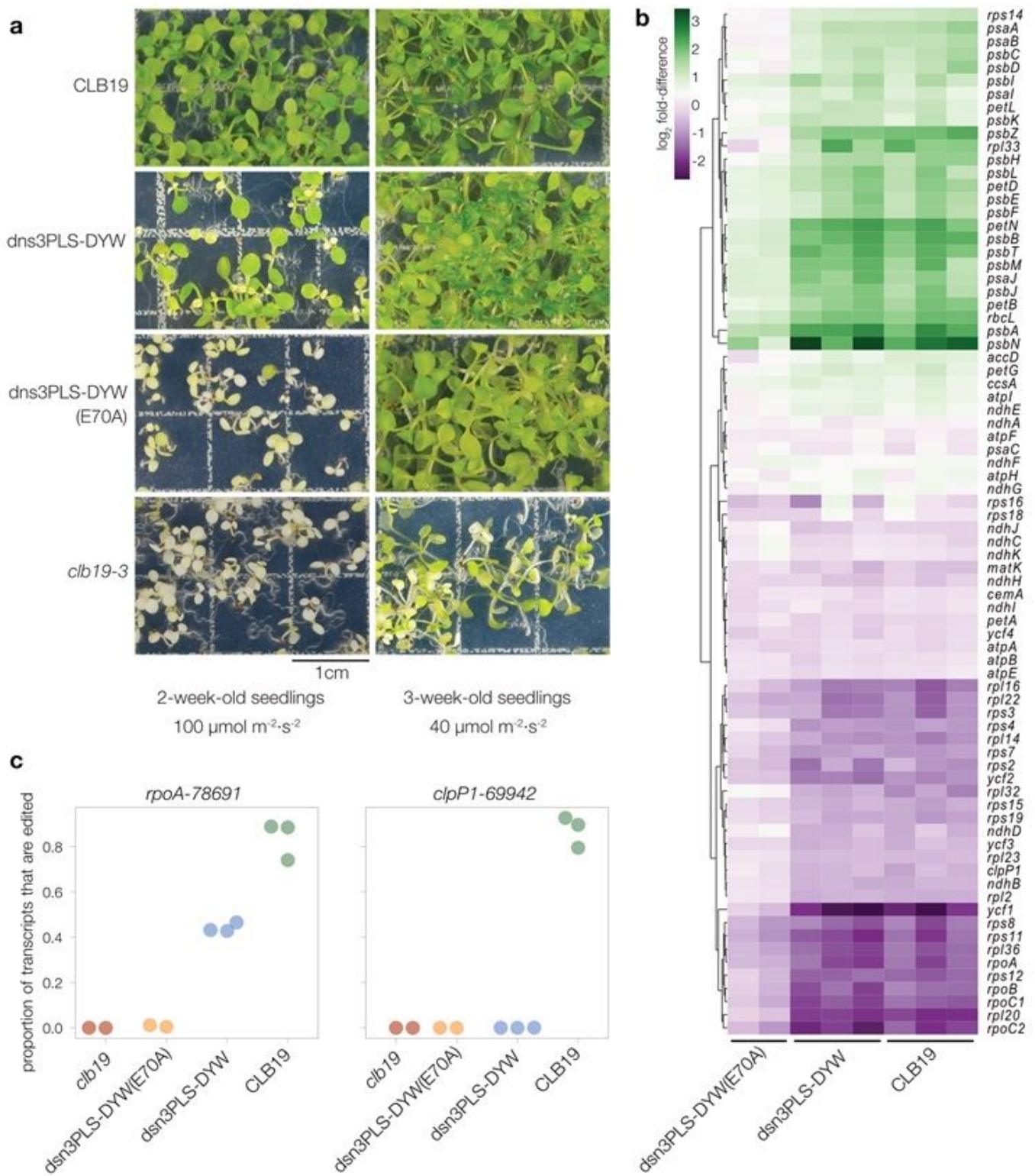


Figure 3

Visible and molecular phenotypes of *clb19* transformants. a: Seedlings were grown under a 16 h photoperiod with a light intensity of either 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$ (left panel) or 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-2}$ (right panel). The construct used to transform the *clb19-3* mutant line is indicated on the left. The scale bar is 1 cm. b: Chloroplast gene expression shown as log₂ fold-difference compared to the transcript levels in *clb19*. The

RNA was extracted from plants grown under low light (equivalent to the right panel in a). c: RNA editing at the *rpoA*-78691 and *clpP1*-69942 sites in the four genotypes.

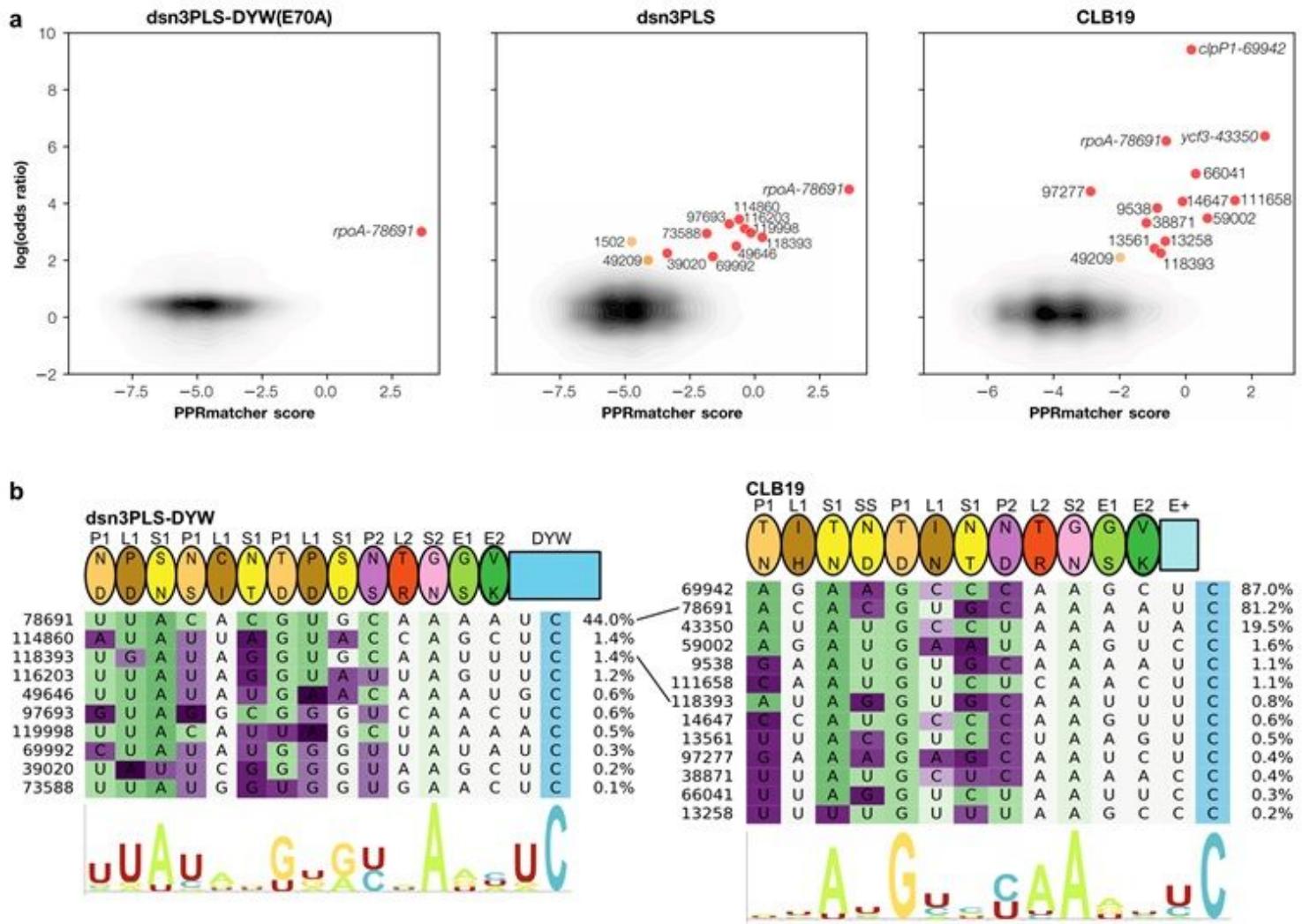


Figure 4

Putative off-target editing by dsn3PLS-DYW and CLB19. a: Potential editing sites plotted by their predicted binding to the expressed editing factor (x-axis) against the difference in the rate of editing (expressed as the log of the odds ratio) compared to a negative control. For dsn3PLS(E70A) the negative control was clb19, for dsn3PLS-DYW and CLB19, the negative control was dsn3PLS(E70A). All sites where the difference in editing is statistically significant (Fisher exact test corrected for multiple testing) and the log(odds ratio) is greater than 2 are highlighted in red (or orange for two potential ‘false positives’ discussed in the text). The ~32000 sites below this threshold are indicated by the density contours (grey-black). b: Alignment of the highlighted sites from a with the corresponding editing factor. The target sites are coloured according to the predicted favourability of the alignment at each position, from favoured (green) to neutral (white) to disfavoured (purple). These values are taken from 26. The percentage of edited transcripts at each site is indicated on the right. The sequence logos (constructed by Skylign, 49) indicate the nucleotide biases at each position.

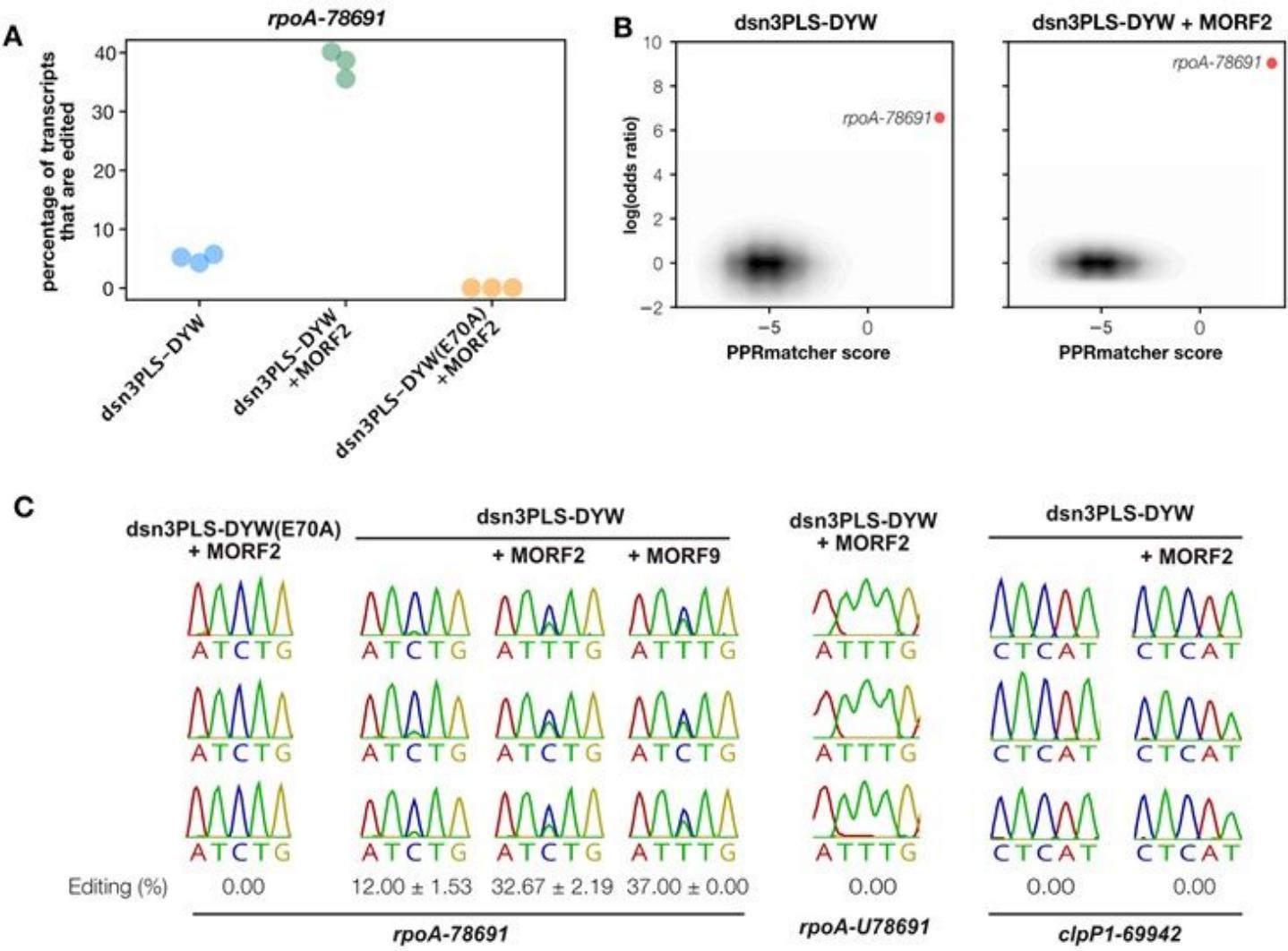


Figure 5

RNA editing in bacteria. a: RNA editing quantified by RNAseq at the intended target site in 3 independent samples of bacteria expressing dsn3PLS-DYW, dsn3PLS-DYW together with MORF2 or dsn3PLS-DYW(E70A) together with MORF2. b: Potential editing sites plotted by their predicted binding to dsn3PLS-DYW (x-axis) against the difference in the rate of editing (expressed as the log of the odds ratio) compared to the negative control (dsn3PLS-DYW(E70A) together with MORF2). The only significant site where the log(odds ratio) exceeds 2 is the intended target site, *rpoA-78691*, highlighted in red. The ~492000 sites below this threshold are indicated by the density contours (grey-black). c: Additional tests of editing in *E. coli*. From left to right: dsn3PLS-DYW(E70A) + MORF2 as a negative control; MORF2 and MORF9 enhance editing by dsn3PLS-DYW by similar amounts; a U at the *rpoA-78691* editing position is not detectably reverse-edited to C; the *cipP1-69942* site is not detectably edited with or without MORF2. All experiments were repeated in triplicate, as shown.

Supplementary Files

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