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Plant organelle C-to-U RNA editing factors can operate successfully in yeast (*Saccharomyces cerevisiae*) as an easily amenable eukaryotic system for their functional analysis

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Site-specific cytidine-to-uridine (C-to-U) RNA editing is essential for gene expression in plant mitochondria and chloroplasts. Specific RNA-binding pentatricopeptide repeat (PPR) proteins equipped with a DYW-type cytidine deaminase have been identified as the core RNA editing factors. Multiple circumstances impede their analysis *in planta* and call for experimental approaches in easily amenable heterologous setups for functional analyses of these intriguing proteins. Here, we report the establishment of the yeast *Saccharomyces cerevisiae* as such a system. Three plant RNA editing factors from the model moss *Physcomitrium patens*, namely PPR56 (PpPPR_56), PPR65 (PpPPR_65), and PPR78 (PpPPR_78), were successfully expressed in the yeast cytosol and faithfully edit not only their respective co-delivered targets but also variable ranges of off-targets with similar recognition sequences in the yeast background transcriptome. Convincingly, we found that the different editing factors behaved in the new system very much like previously found in bacteria or human cells, not only with respect to off-target conservation profiles but also with surprising differences in off-target numbers, championed by more than 125 off-targets for PPR56 with editing efficiencies of up to 41% in the *S. cerevisiae* transcriptome. We conclude that *S. cerevisiae* will be a helpful heterologous system for studying the intricacies of plant RNA editing.

Introduction

Plant-type RNA editing is an intriguing molecular phenomenon that is essential for functional gene expression in the two important energy-converting organelles in plant cells. Numerous cytidine-to-uridine (C-to-U) conversions at specific sites in the transcriptomes of chloroplasts and mitochondria correct at the RNA level what appear to be errors at the DNA level in the endosymbiotic genomes to warrant successful protein biosynthesis. Although a convincing evolutionary theory explaining why plant RNA editing has come into existence in the first place is still missing, a reasonable understanding of the underlying molecular machinery has emerged over recent years [1,2]. Arrays of RNA-binding pentatricopeptide repeats (PPRs) are central to the sequence-specific recognition of target sites [3] and a unique cytidine deaminase domain

Abbreviations
ADH, alcohol dehydrogenase; CYC, cytochrome C; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol; EFYP, enhanced yellow fluorescent protein; GAL, galactokinase; Leu, leucine; MBP, maltose-binding protein; MCS, multiple cloning site; NGS, next generation sequencing; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEG, polyethylene glycol; PPR, pentatricopeptide repeat; SC, synthetic complete; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism; TrxA, thioredoxin A; YEp, yeast episomal plasmid.

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named after its conserved carboxyterminal DYW tripeptide (aspartate-tyrosine-tryptophane) is responsible for the biochemical conversion of cytidine to uridine by deamination [4,5].

The elements of RNA target recognition and biochemical pyrimidine conversion are consistently combined in single large proteins with upstream PPR arrays and a downstream DYW cytidine deaminase in what seems to be an evolutionary ancestral state as represented by the model moss *Physcomitrium patens* [6–10]. However, a multitude of protein co-factors have led to much more complex editosomes in flowering plants, where the upstream PPR arrays have frequently been separated from a downstream DYW domain and need re-assembly *in trans* at their targets, most often with the involvement of different additional factors [11–15]. Nevertheless, editing factors in flowering and nonflowering plants alike feature characteristic PLS-type PPR arrays where canonical P-type PPRs present also elsewhere in the living world alternate with 'long' (L) and 'short' (S) PPR variants, each with deviating consensus profiles [16]. Very much like the DYW domain, the PLS-type PPRs also seem to emerge mysteriously in evolution with the origin of land plants some 500 million years ago.

Importantly, a PPR-RNA recognition code has been established matching the P-type and the S-type PPRs to target nucleobases in a 1 : 1 manner [3,17–18]. Crucial positions are the fifth and the last (L) amino acids in those PPRs to prefer fitting ribonucleotides in the following (5 + L) manner: T + N: A, T + D: G, N + D: U, N + S: C, and N + N: C or U. Hence, this code is near binary with PPR Position 5 occupied with T (threonine) selecting for purines vs. N (asparagine) selecting for pyrimidines and PPR position 'last (L)' with D (aspartate) selecting for keto bases (G or U) and N or S (serine) preferring amino bases (A or C). However, surprising exceptions exist, as we will also discuss in the context of this work, and the role of the L-type PPRs is by no means clear [19].

Investigating RNA editing *in vitro* or *in planta* is hindered by several experimental factors, including troublesome expression or purification of editing factors in sufficient amounts under native conditions (likely owing to their repeat structure), long plant generation times in reverse genetic approaches, occasional lethality of plant lines upon knockouts and genetic redundancies or overlapping functionalities of RNA editing factors. Accordingly, the *in vivo* study in heterologous systems devoid of the intriguing plant-type RNA editing is a highly welcome alternative for functional analyses to understand the details of RNA target recognition and biochemical pyrimidine base conversion. A first step in that direction has been the successful functional expression of two *Physcomitrium patens* RNA editing factors in *Escherichia coli* [4]. This bacterial setup allowed for the first time to test numerous mutant variants both on the RNA target side as well as for the successfully expressed editing factors in a short time.

Whereas their successful expression in *E. coli* largely proved that these RNA editing proteins did not rely on co-factors exclusively present in the plant organelles, it could still be prone to artifacts exerted by a different RNA turnover in the bacterium vs. the plant organelles. Regarding this issue, the successful functional expression of the plant editing factors in human cell lines came as a welcome support [20]. However, the expertise and materials required for the investigation of RNA editing in human cell lines are not easily available for many laboratories. Hence, we aimed to establish yet another heterologous system to ideally combine the advantages of the above approaches in a eukaryotic model system that can be easily established in every molecular biology lab to allow for straightforward and comparably fast experimentation to cross-check for comparability of results.

Here, we report on successful experimentation along the above lines in the brewer's (or baker's) yeast *Saccharomyces cerevisiae*. Toward this end, we tested different promoters, solubility tags and RNA editing factor variants. The model organism *S. cerevisiae* naturally contains 15 PPR proteins, exclusively of the 'classic' type and none of which are involved in RNA editing of any sort [21]. Most of these PPR proteins are involved in RNA stability, and all of them localize to mitochondria.

As in the model systems mentioned earlier, yeast proved straightforward to functionally express *Physcomitrium* editing factors PPR56, PPR65, and PPR78. Notably, RNA editing was observed not only at their co-delivered native targets but also at several sites in the yeast background transcriptome allowing for an improved understanding of RNA target recognition. Intriguingly, the off-target profiles not only matched excellently with those observed in the previously established heterologous setups but also confirmed a much larger flexibility of PPR56 to address off-targets than PPR78. We assume that the new yeast *in vivo* assay system reported here will be very helpful for future studies of plant RNA editing not only given its easy establishment and straightforward experimentation but importantly also to complement experimentation in the prokaryotic *E. coli* setup.

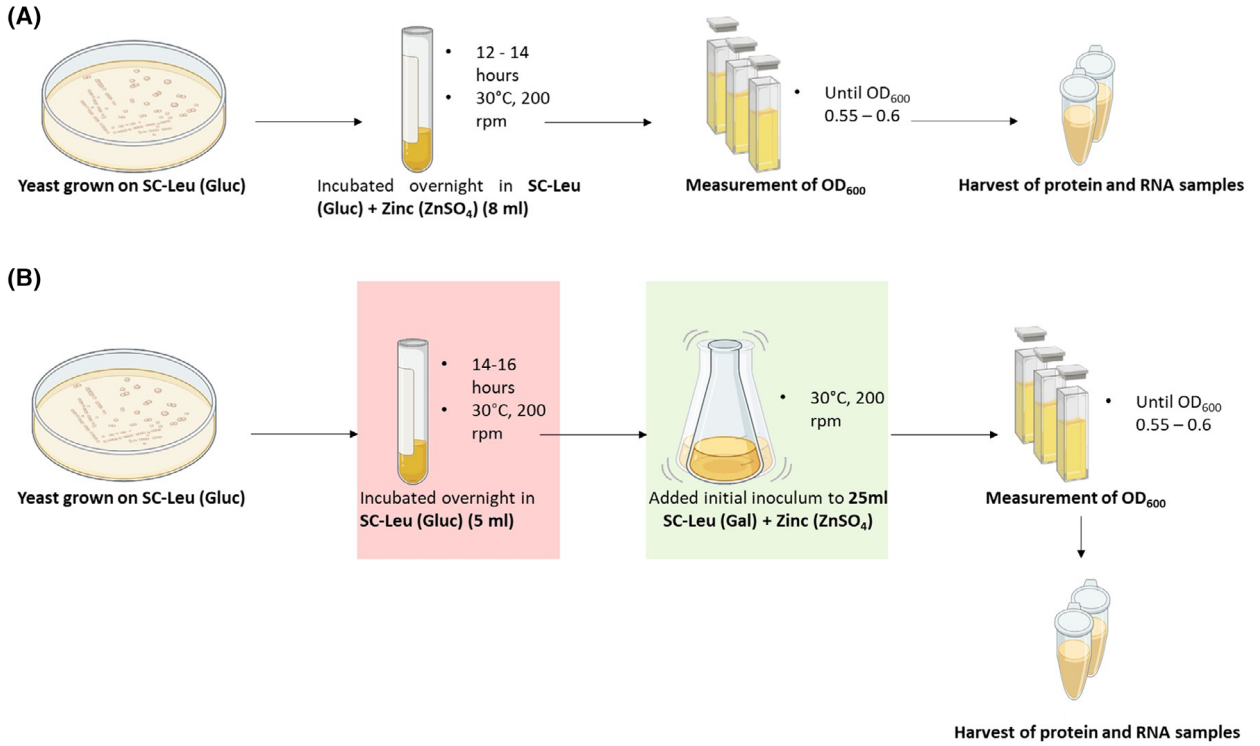


Fig. 1. Expression setups using either the constitutive alcohol dehydrogenase 1 (ADH1) promoter or the inducible galactokinase (GAL) promoter to express plant RNA editing factors in the yeast cytosol. Yeast cells (strain THY.AP5) were transformed and plated onto selective media supplemented with all amino acids except leucine (SC-Leu) and with glucose (2%) as the main carbon source. (A) Colonies transformed with the vector YEp351ADH1 with the constitutive ADH1 promoter were first selected via a colony PCR, following which they were incubated overnight in selective media containing SC-Leu + Zinc (supplemented as 0.4 mM $ZnSO_4$). Following overnight incubation of 12–14 h at 200 rpm and 30 °C the OD_{600} value of the cultures was measured. Samples at an OD_{600} of 0.55–0.6 were harvested, spun down, and stored for RNA/protein extraction. (B) Colonies transformed with the vector YEp351GAL1, containing the inducible GAL1 (galactokinase) promoter were likewise selected via a colony PCR and incubated overnight in selective media containing SC-Leu and glucose as the initial carbon source. Red shading indicates that the GAL1 promoter is repressed at that stage owing to the presence of glucose. Following the overnight incubation period, 500 µL of the overnight cultures were transferred into selective media (SC-Leu + Zinc) but now with galactose (2%) as the main carbon source to counteract repression of the GAL1 promoter and induce protein as indicated by the green shading. OD_{600} values were periodically measured during incubation and sample volumes were harvested at a range of 0.55–0.6, and stored for RNA and protein extraction. The figure was partially designed using BioRender (https://www.biorender.com/).

Results

The yeast RNA editing factor expression setup

Toward their successful functional expression, we tested different approaches in parallel, including different media, different RNA editing factors, and different promoters driving their transcription. A Kozak sequence was used to potentially enhance translation [22,23]. As promoters we chose the constitutive ADH1 (alcohol dehydrogenase 1) vs. the galactose-inducible GAL1 promoter as an alternative. The experimental procedure for yeast growth and harvest with the two different approaches is displayed in Fig. 1. All experimentation was performed in the arbitrarily chosen and leucine-autotroph yeast strain THY.AP5 available in our lab owing to previous mbSUS (mating-based split ubiquitin system) experimentation [24]. All molecular cloning was done using the modified yeast expression vectors YEp351ADH1 or YEP351GAL1, respectively.

We started our experimentation to establish a yeast RNA editing assay in the *S. cerevisiae* background with RNA editing factor PPR56, including the 6xHis and the maltose-binding protein (MBP) as upstream protein tags (Fig. 2). This approach included 14 amino acids upstream of the most N-terminal PPR L-14 of PPR56 that had previously proven successful for heterologous expression in *Escherichia coli* [4]. Using the constitutive ADH1 promoter, we were able to observe an RNA editing efficiency of *ca.* 80% on target nad4eU272SL, which was co-delivered on the same

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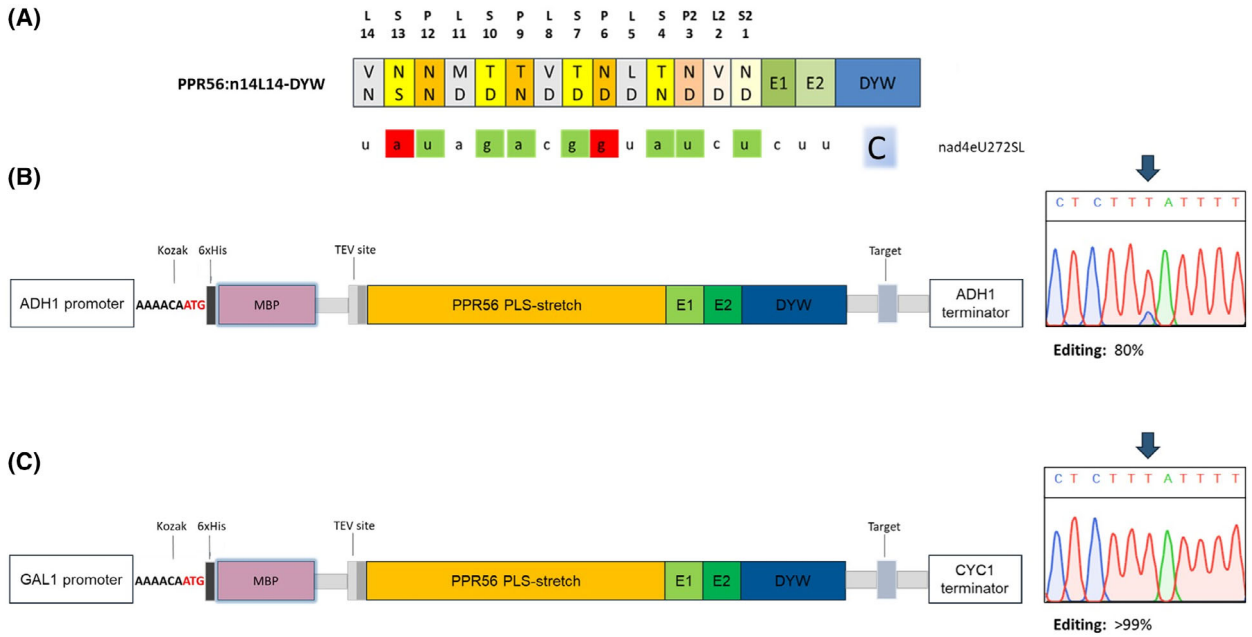


Fig. 2. Schematic representation of the *P. patens* editing factor PPR56 expressed in the *S. cerevisiae* background under two different promoters. (A) The protein coding sequence containing the PLS-stretch, E1 and E2 domains, and DYW domain was cloned directly upstream of the co-expressed target sequence (nad4eU272SL). The binding of the individual pentatricopeptide repeat (PPR) motifs to their corresponding target nucleotides is displayed on top of the figure with green indicating a perfect match, yellow shading indicating an imperfect match and red shading indicating a mismatch. A Kozak sequence downstream of the promoter (containing a start codon at its terminal end) was included to function as a translation enhancer. PPR56 was cloned with the N-terminal 6xHis (histidine) tag upstream of an MBP tag encoding for the maltose-binding protein, which was included to enhance the solubility of the construct in the yeast background. A tobacco etch virus (TEV) protease cleavage site (light gray rectangle) was included upstream of a short stretch of nucleotide sequence from the native editing factor, found upstream of the first identifiable PPR motif. (B) The editing factor PPR56 expressed under the constitutive ADH1 promoter resulted in RNA editing efficiencies of around 80% at the editing target site nad4eU272SL indicated by the arrow above the sequencing chromatogram. (C) PPR56 was alternatively expressed under the inducible GAL1 promoter, for which editing efficiencies of > 99% were observed for the co-expressed nad4eU272SL target. The data provided here were sourced across three independent biological replicates, and the individual editing efficiencies per replicate with standard deviation can be found in Table S1.

plasmid construct in the 3′-UTR of the editing factor (Fig. 2). As an example, the editing site nomenclature here indicates Position 272 in the *nad4* gene CDS of *Physcomitrium patens* resulting in a serine-to-leucine codon change. The editing efficiency remained unchanged after a test-wise switch from selective (SC-Leu) to nonselective media (YPD) in the interest of costs and simplicity.

Using the inducible GAL1 promoter as an alternative to the constitutive ADH1 promoter (Fig. 2), we observed > 99% of RNA editing efficiency. We conclude that late induction of gene expression is experimentally helpful, very much as recently observed for expression of RNA editing factors in the *Physcomitrium patens* cytosol [25]. Moreover, the GAL1 promoter is one of the strongest inducible yeast promoters on galactose and this may explain the disparity in the observed editing efficiency [26,27]. Again, a test-wise switch to nonselective media for the preculture (YPD) and for induction (YPGal), led to no change in the observed editing efficiencies. The higher editing observed for the inducible GAL1 promoter system convinced us to continue with further experimentation involving the vector YEp351GAL1.

Different N-terminal protein tags impact the RNA editing ability of PPR56

To test the impact of alternatives to the MBP as solubility tags for functional expression, we also tested alternative tags and upstream extensions of the PPR56 CDS. Neither a 6xHis-EYFP tag (enhanced yellow fluorescent protein), a 6xHis-HA tag (hemagglutinin) or the same PPR56 variant, now labeled PPR56:n14L14-DYW following a recent nomenclature proposal [28], without an added protein tag showed detectable RNA editing (Fig. 3A), although protein expression levels were comparable to the experiments

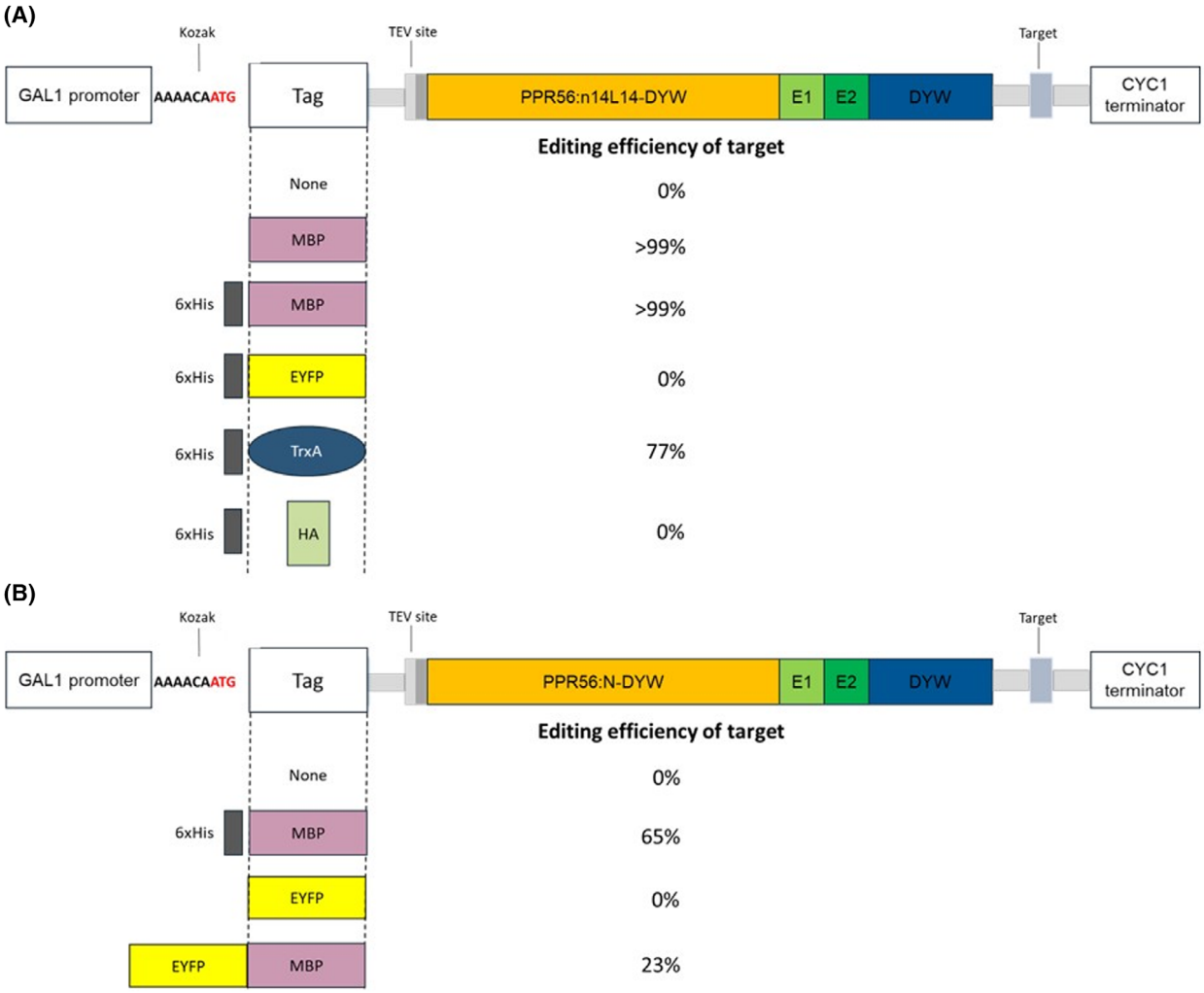


Fig. 3. Testing different protein tags upstream of RNA editing factor PPR56 co-expressed with its *nad4* target in the yeast cytosol. (A) As alternatives to the 6xHis-MBP tag (maltose-binding protein), PPR56 (as the PPR56:n14L14-DYW construct) was expressed without an upstream tag or in upstream fusion with EYFP (yellow fluorescent protein), TrxA (thioredoxin) or HA (hemagglutinin) as upstream tags. (B) Alternatively, tags were tested as additions to the full-length CDS (PPR56:N-DYW) of PPR56. The editing efficiencies at the target nad4eU272SL is indicated for the various PPR56-tag arrangements tested. The editing efficiency for functional constructs has been given as the mean of three biologically independent replicates, whereas the efficiency of nonfunctional constructs (0% editing) was derived from two replicates. The individual editing efficiencies with standard deviation can be found in Table S1.

with the 6xHis-MBP version resulting in high editing (see below). However, reasonable RNA editing of 77% of the nad4eU272SL target was found upon inclusion of a 6xHis-TrxA tag (thioredoxin) that was alternatively used in earlier PPR-based experimentation [29].

We also tested a PPR56 variant including the full native upstream coding sequence (PPR56:N-DYW) without any N-terminal protein tag using the vector YEp351GAL1 (Fig. 3B). This variant likewise showed no RNA editing at the co-expressed nad4eU272SL target. The upstream addition of a 6xHis-MBP tag to this full-length PPR56 construct recovered RNA editing to 65%, which, however, is still less than the near full editing of > 99% observed for the 6xHis-MBP-PPR56:n14L14-DYW variant. Exchanging the MBP tag for an EYFP tag abolished editing, but combining the EYFP with a downstream MBP tag combination rescued editing somewhat, but only to *ca.* 23%. All proteins were expressed in moderate levels excluding expression issues as a reason for the different editing efficiencies (see below). Accordingly, we ultimately decided to continue our experimentation with constructs using the inducible GAL1 promoter and the 6xHis-MBP combination as protein tags added to

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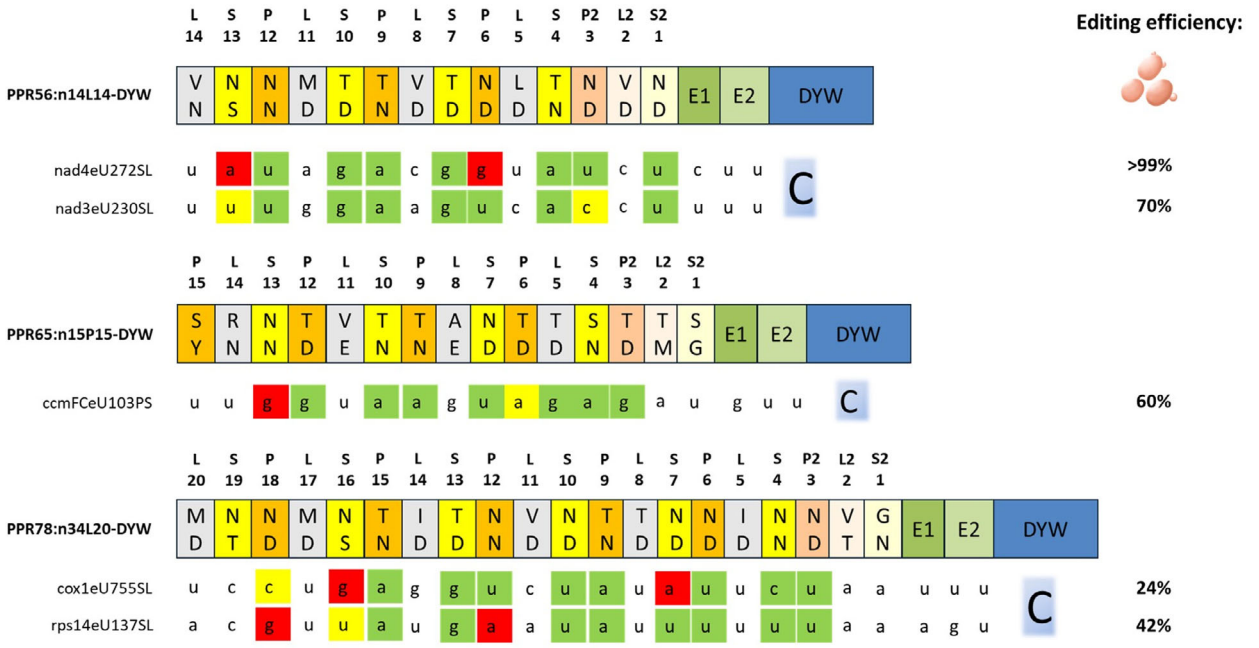


Fig. 4. Different *P. patens* RNA editing factor constructs expressed in the *S. cerevisiae* background. Like PPR56, also PPR65 and PPR78 are nuclear-encoded mitochondrial RNA editing factors in the model moss *Physcomitrium patens* and display analogous structures consisting of PLS-type pentatricopeptide repeat (PPR) arrays, terminal E1 and E2 domains and their catalytic DYW cytidine deaminase domains. The makeup of their PLS-type PPR arrays allows the editing factors to address their respective mitochondrial targets. PPR56 targets *nad3* and *nad4* transcripts with edits nad3eU230SL and nad4eU272SL, respectively. PPR65 targets the mitochondrial *ccmFC* transcript, with the editing target ccmFCeU103PS. Like PPR56, PPR78 also has two editing targets: rps14eU137SL and cox1eU755SL. Editing site designations account for the target gene transcript (*nad*: NADH ubiquinone oxidoreductase, *ccmFC*: cytochrome c maturation factor FC, *rps*: ribosomal protein small subunit, *cox*: cytochrome C oxidoreductase), the position of the editing site and the resulting amino acid change following the edit. The binding of the individual PLS-motifs to the editing sites has been shown using differently colored boxes, with green indicating a perfect match, yellow indicating an imperfect match and red indicating a mismatch. Non-shaded positions indicate that this is not likely to be a binding position. Labels behind the colons after each PPR indicate behind the '*n*' the numbers of amino acids of native CDS upstream of the respective N-terminal PPR included for recombinant cloning. The editing efficiencies of the constructs shown here are the mean of three independent biological replicates and the editing of individual replicates with standard deviation can be found in Table S1.

RNA editing factor constructs retaining only some of their native coding sequence upstream of the PPR arrays (Fig. 4).

Testing different plant RNA editing factors

For comparison with the previously obtained data in other heterologous systems, we extended our experimentation with RNA editing factors PPR65 and PPR78 and with additional native targets like nad3eU230SL, the second and less efficiently edited target of PPR56 in the mitochondria of *Physcomitrium patens* (Fig. 4). The editing efficiencies of PPR56 in yeast perfectly reflect the *in planta* situation with the nad3eU230SL target only moderately efficiently edited, to *ca.* 70%. Editing efficiencies in yeast proved somewhat less efficient for PPR65 with only 60% at its only known target (ccmFCeU103PS) and for PPR78 at its two known targets cox1eU755SL (24%) and rps14eU137SL (42%). Especially, the editing efficiencies of PPR78 in yeast are significantly lower in comparison with the *in planta* editing efficiencies of > 99% for cox1eU755SL and ~70–80% for rps14eU137SL. Intriguingly though, it is notable that the inversion of editing efficiencies at its two targets with cox1eU755SL likely being the efficiently edited prime target of PPR78 in plants has similarly been observed in the heterologous *E. coli* setup [30].

We aimed to extend the series of functionally expressed RNA editing factors with experimentation along the above lines also with *Physcomitrium patens* RNA editing factors PPR71, PPR79, PPR91, and PPR98. These RNA editing factors were initially tested in the *Escherichia coli* expression system. Different

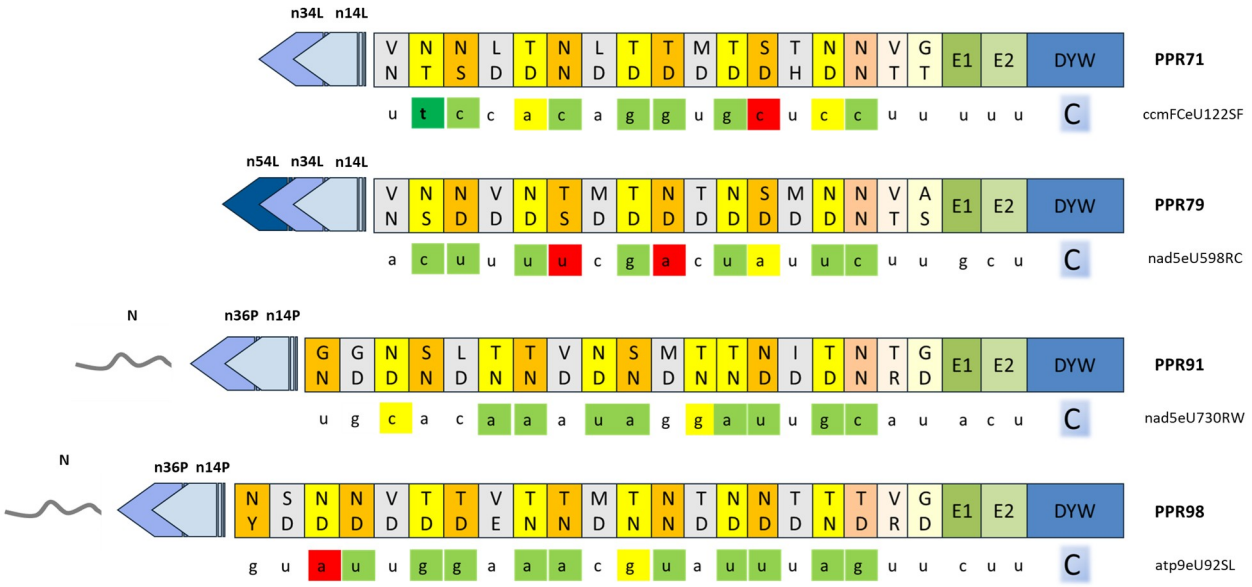


Fig. 5. Testing PPR71, PPR79, PPR91, and PPR98 for functional expression in yeast. Other than PPR56, PPR65, and PPR78, further RNA editing factors PPR71, PPR79, PPR91, and PPR98 addressing targets in the *ccmFC, nad5*, and *atp9* mRNAs of *Physcomitrium patens* failed to show RNA editing activity in both yeast or in the *Escherichia coli* setup. Respective targets and makeup of editing factors are displayed as in Fig. 4. Up to three different CDS extensions, upstream of the most amino-terminal pentatricopeptide repeat (PPR) have been tested as indicated in *E. coli*, for example, of 54, 34, or 14 amino acids upstream the N-terminal PPR L-17VN in the case of PPR79 or even, including full-length constructs for PPR91 and PPR98. The ccmFCeU122SF target (in the mRNA for cytochrome c maturation factor FC) has been modified to replace the edited cytidine Position 103 of PPR65 with uridine (or thymidine at DNA level as highlighted in bold and dark green shading), given that a dependence of PPR71 editing on the upstream editing has been documented [8]. Selected constructs have also been tested in yeast (n34L-PPR71, n34L-PPR79, n54L-PPR79, n36P-PPR91, and n-36P-PPR98). Background shading of the target sequences indicates matches (green), transition-mismatches (yellow) and transversion mismatches (red) according to the PPR-RNA code concept [3,17]. For documentation of protein expression and a detailed list of RNA editing assays, see Fig. 6 and Table S1, respectively. The editing efficiency was calculated as the mean of two independent biological replicates, and further information can be found in Table S1.

N-terminal variations of these editing factors were utilized to fully test their ability to edit co-expressed targets with different lengths (41–46 bp and 200 bp) (Fig. 5, Table S1). These attempts, however, remained unsuccessful both in *E. coli* and in yeast (Fig. 5), despite successful detection of protein expression via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and/or western blot analysis, for all protein versions (Fig. 6) with the exception of PPR98 in yeast. We conclude that success for functional expression of plant RNA editing factors will less likely depend on the system chosen for heterologous expression but rather on the respective cloning strategy with a serendipitous choice of extended CDS beyond the upstream PPR array or alternatively a widely extended RNA target sequence as exemplified by recent experimentation on PPR78 [30].

Testing RNA editing factor protein chimeras

Some RNA editing factor protein chimeras having their carboxyterminal domains replaced with those of heterologous editing factors had proven to be functional *in planta* [31–33] and very recently also proved successful in the bacterial expression system [28,34]. We wished to check whether this also holds true for the newly established yeast setup reported here. To this end we cloned various chimeras replacing the DYW domain of PPR56 and PPR78 with those of other RNA editing factors (Fig. 7), including the DYW domains of *Physcomitrium* mitochondrial editing factor PPR65, chloroplast RNA editing factor PPR45 as well as the DYW domain of angiosperm RNA editing factor OTP86 from *Arabidopsis thaliana*. PPR56 combined with the DYW domains of PPR45 and OTP86 showed almost complete editing of > 99% for the co-expressed nad4eU272SL target (Fig. 7). Interestingly, the chimeric construct with the DYW domain of PPR65 showed somewhat reduced editing of 73% (Fig. 7), fully in line with reduced RNA editing efficiency previously observed in the heterologous *Escherichia coli* system [35].

Given that PPR56 chimeras have now been shown to function in diverse heterologous systems when

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combined with downstream domains of other RNA editing factors, we decided to also test PPR78 as a longer PPR protein, also given that it has been used earlier in chimeric studies in moss knockout lines *in planta* [31]. Accordingly, we fused PPR78 with the DYW domain of PPR79, which had previously been shown to complement PPR78 functionally [31,33]. This chimera showed a low 21% editing for the co-expressed

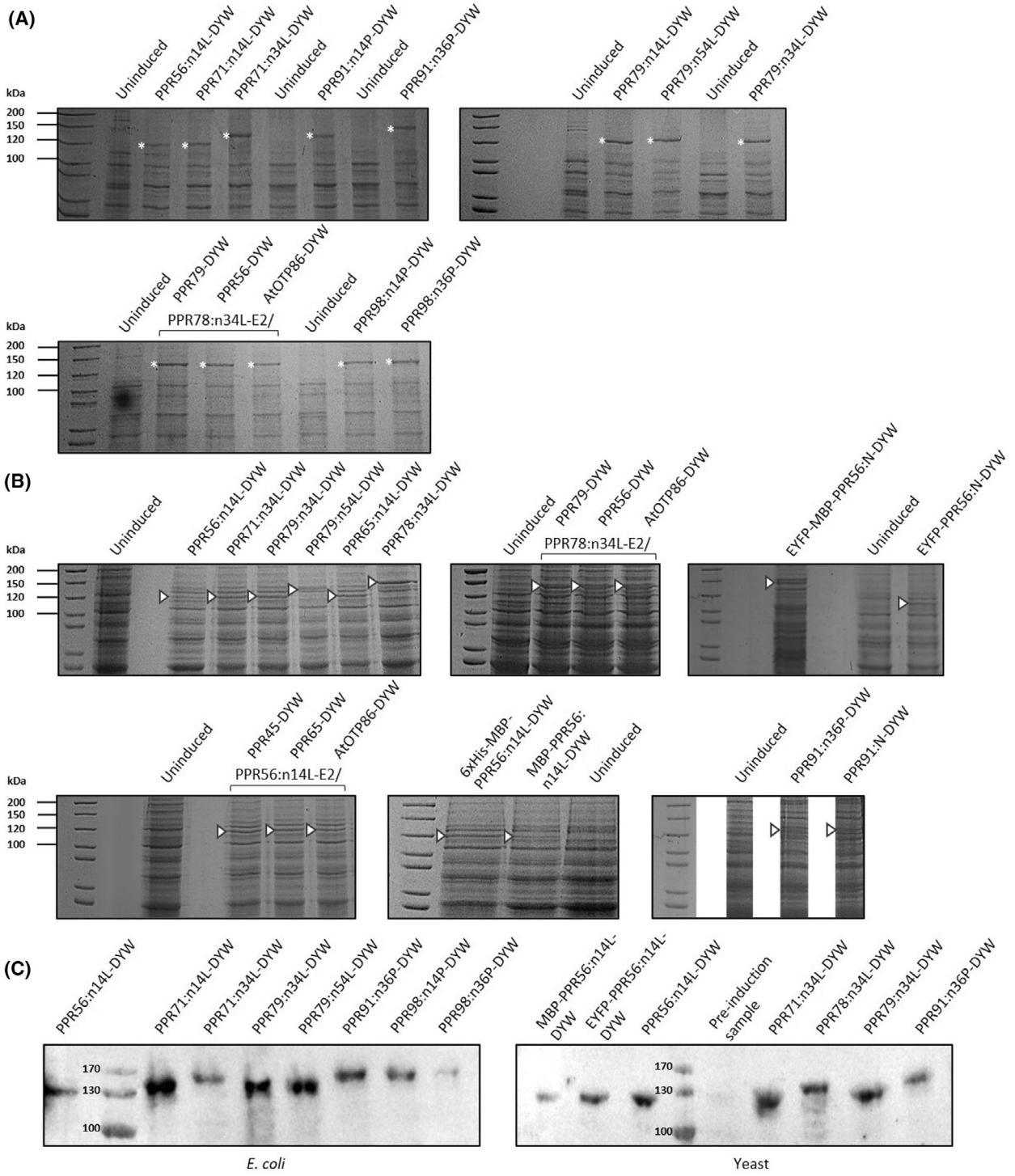


Fig. 6. Exemplary documentation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and western blots confirming protein expression. Protein expression was checked by SDS/PAGE analysis, both in the heterologous *Escherichia coli* setup (A) and in the new yeast assay system (B). Uninduced samples harvested before induction were run alongside induced samples harvested following an overnight incubation. The Pageruler unstained protein ladder was used as a protein size reference and the presence of the protein bands of expected molecular weights are indicated by white asterisks (for *E. coli* samples) and by an arrow (for yeast samples). These constructs were cloned behind the upstream 6xHis-MBP tag and include those for which RNA editing activity was successfully detected in the cases of PPR56, PPR65, and PPR78 as well as those for which we failed to find RNA editing activity in the cases of PPR71, PPR79, PPR91, and PPR98. Western blot analysis (C) was also performed for select samples of *E. coli* and yeast constructs. The Pageruler prestained protein ladder was used as a size reference, and the presence of the His-tagged protein bands at the expected heights was detected via chemiluminescence. All constructs expressed with tags other than 6xHis-MBP have been highlighted as such.

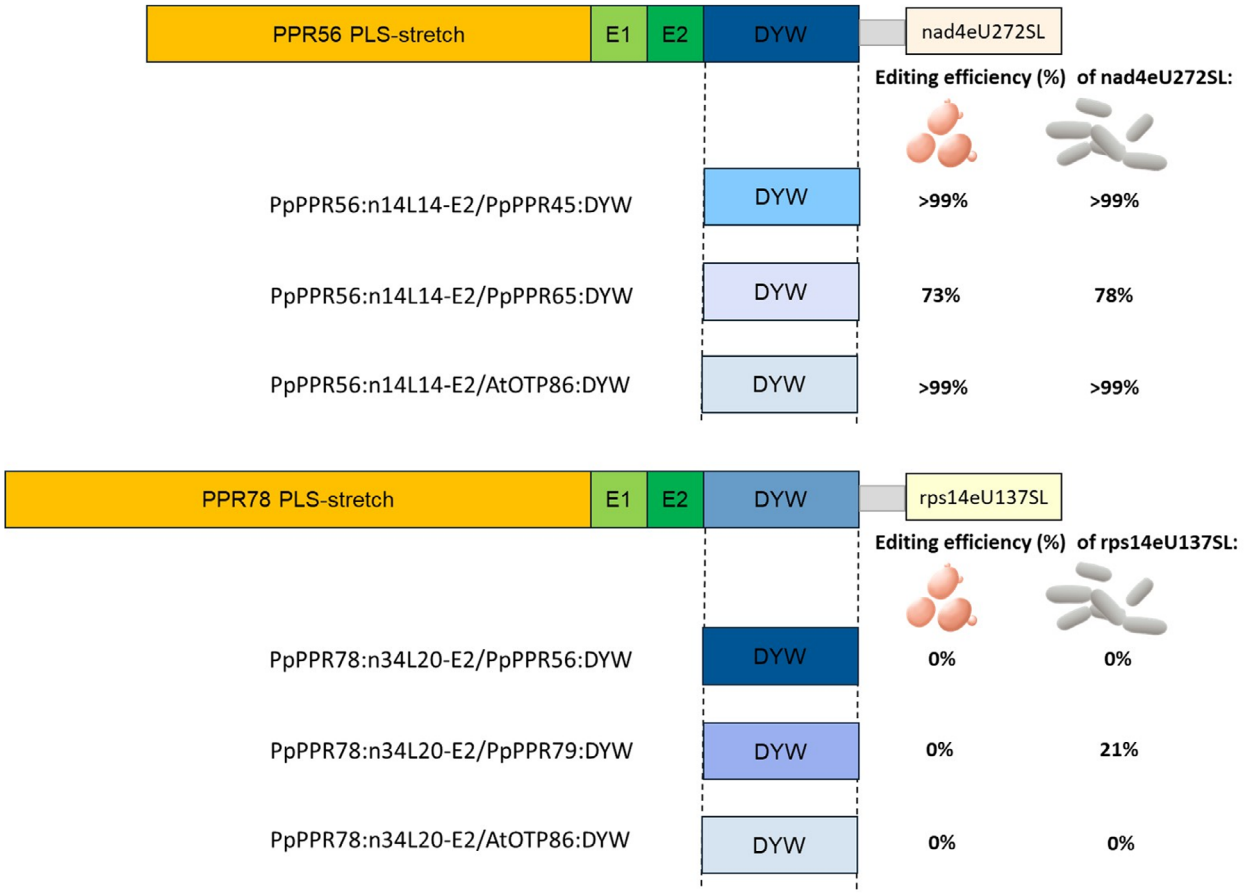


Fig. 7. Chimeric constructs of PPR56 and PPR78 with the DYW domains of other RNA editing factors tested in the yeast RNA editing assay. The DYW domain of RNA editing factor PPR56 (construct 6xHis-MBP-PPR56:n14L14-E2) was replaced with the DYW domains of *P. patens* mitochondrial editing factor PPR65, chloroplast RNA editing factor PPR45, or the DYW domain of the flowering plant *A. thaliana* OTP86 editing factor, respectively. The observed RNA editing efficiencies at the nad4eU272SL target in yeast (left column) are shown along with the results obtained previously in the *E. coli* system [28]. Similarly, the DYW domain of PPR78 (construct 6xHis-MBP-PPR78:n34L20-E2) was replaced with those of *P. patens* mitochondrial editing factors PPR56 or PPR79 or with the DYW domain of the *A. thaliana* OTP86 editing factor. The editing efficiency for functional chimeras has been presented as the mean of three biologically independent replicates, whereas the efficiency of nonfunctional chimeras (0% editing) was derived from two replicates. The individual editing efficiencies with standard deviation can be found in Table S1.

rps14eU137SL target in *E. coli* (Fig. 7), but no editing could be detected in the yeast setup. Chimeric PPR78 constructs including the DYW domains of PPR56 or the thale cress editing factor OTP86 failed to edit the co-expressed rps14eU137SL target in the heterologous yeast and *E. coli* setups alike (Fig. 7).

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The RNA editing off-target spectra in yeast

A particularly interesting benefit of the heterologous expression of RNA editing factors is their addressing of additional 'off-targets' in the respective background transcriptomes of their new artificial host environments. The conservation profiles of these off-targets allow for interesting conclusions on target selection and the respective relevance of nucleotide positions surrounding an edited cytidine. To address this point for the new heterologous yeast system, we sent out respective RNA samples for NGS transcriptome sequencing to detect C-to-U RNA editing off-targets (Table S2).

A particularly large data set of 125 off-targets could be detected for PPR56. Their conservation profile excellently confirms the dominant function of PPRs selecting for purines in their juxtaposed positions of the RNA target (Fig. 8): S-10TD with G-13, P-9TN with A-12, S-7TD with G-10, and S-4 TN with A-7. To a somewhat lesser extent this is also valid for PPRs selecting for pyrimidines: P2-3ND and S2-1ND selecting for uridines in Positions −6 and −4, respectively. Much more intriguing, however, is the unexpected lack of selectivity of P-6ND for uridine, which is even surpassed by the dominating presence of G in the conservation profile and at the same time excellently fitting the guanidine at this position in the efficiently edited nad4eU272SL target and in five of the eleven top-edited off-targets (Fig. 8). Along similar lines, and aside from the as yet unexplained preference for pyrimidines in Positions −3, −2, and −1, there is a surprising selectivity for pyrimidines in Position −8, supposedly exerted by PPR L-5LD and excellently fitting the presence of U or C in Position −8 of the two native targets of PPR56 and in nine of the top 11 off-targets alike (Fig. 8). As with the observations above, this excellently matches experimental results from the other heterologous expression setups and further emphasizes the fact that plant RNA editing factors largely operate independent of their cellular environment. Only very minor differences are identified in the PPR56 off-target consensus sequences above a 40% threshold in yeast vs. *E. coli* or human cells (Fig. 8). PPR P-12NN seems to have a higher specificity for A in Position −15 in the yeast background when compared to the other heterologous systems. Yet, another difference between the setups is the slightly higher preference for uridines at Positions −8 and −5 in the yeast system opposite PPR L-5LD and L2VD, respectively, as compared to the slightly higher preference for cytidines found in the *E. coli* and human setups. Finally, the two eukaryotic setups feature a preference for adenosines in Positions −14 and +1 that is not reflected in the *E. coli* off-target set (Fig. 8). This once again highlights the fact that the target recognition capability of PPR56 at least seems to be unhindered by a change of editing environment—proving that while flexible, the PPR protein still strives to bind to transcripts with similar profiles.

Intriguingly, the top-edited (41%) off-target rmt2eU1037SL in yeast matches both the top-conserved positions and the native targets of PPR56 in positions where the mechanisms for preferential nucleotide selection remain presently unexplained (Fig. 8): the UCU triplet directly upstream of the editing site, the pyrimidines in Positions −5 (U) and −8 (C), and the adenosines in Positions −11 and −14 opposite of L-type PPRs as well as the counterintuitive selection for G in Position −9 opposite of PPR P-6ND. The only example to the contrary is guanosine instead of the expected adenosine in Position −7 deviating both from the consensus profile and the native targets. Surprisingly, the two top-edited off-targets feature better fits than the two native targets in Position −16 with the expected C opposite of PPR S-13NS. Experimentation with mutations of key binding positions of the *nad4* and *nad3* targets of PPR56 revealed that changing the conceptual mismatching A to C at Position −16 had surprisingly reduced RNA editing to 70% [35]. Moreover, the urb2eU830TI (Unhealthy Ribosome Biogenesis 2) off-target of PPR56 showed lower editing (31%) when compared to the rmteU1037SL off-target (41%) despite overall better matches to the expected binding code for PPR56.

Except for matching off-target profiles in the different heterologous systems, similarities also extend to the overall numbers of off-targets identified for different editing factors which are strikingly lower for PPR78 than for PPR56. Notably, the same observation has been made earlier for heterologous expression of PPR78 in *E. coli* and in the plant cytosol [25,30], but it remains presently unexplained why PPR56 is so much more promiscuous in editing off-targets than PPR78 across all heterologous expression systems now tested. In fact, we could identify only five off-targets in the yeast transcriptome for PPR78 (Fig. 9). Very much like in the previous case, the top-edited off-target of PPR78 in yeast, ebs1eU2459SL, however, matches the native targets of PPR78 in Positions −5 to −1 directly upstream of the editing site (AAAGU) excellently where mechanisms for nucleotide selection are not yet understood, which emphasizes the importance of these positions behind those addressed following the PPR-RNA code mechanisms. In fact, it may

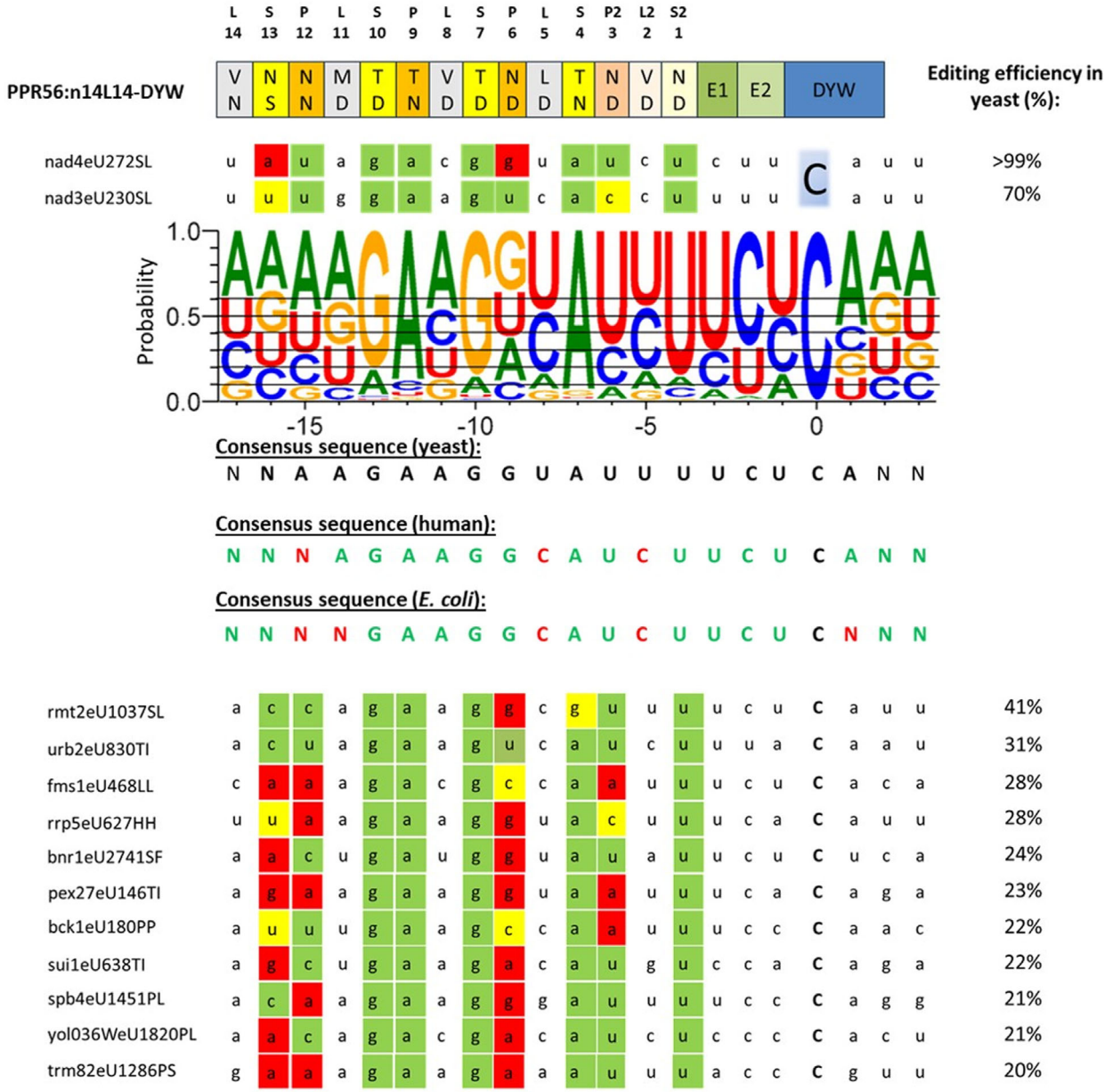


Fig. 8. Off-target weblogo profile for RNA editing factor PPR56 upon its expression in yeast. RNA samples of yeast with PPR56 expressed with the 6xHis-MBP tags were sent out for Next Generation Illumina Sequencing. The weblogo [36] reflects the conservation profile of 125 identified off-targets. The pentatricopeptide repeat (PPRs) of PPR56 with their respective amino acid identities at Positions 5 (above) and last (below) that mediate binding, and the RNA sequences of the native nad4eU272SL and nad3eU230SL targets of PPR56 are juxtaposed on top, aligning target Position −4 with PPR motif S2-1ND and with the DYW domain positioned over the editing site (Position 0). The horizontal lines signify cutoffs in increments of 10%, starting from 40% for a nucleotide in any given position. The 11 top-edited off-targets of PPR56 with > 20% editing are shown below, adapting the nomenclature suggested earlier [37,38], for example, rmt2eU1037SL for a serine-to-leucine exchange in Position 1037 of the arginine-N5-methyltransferase (rmt2) coding sequence. Background shading indicates matches (green), transition-mismatches (yellow) and transversion mismatches (red) according to the PPR-RNA code concept. The consensus profiles of yeast in comparison with the human and *E. coli* off-targets is depicted below the weblogo. Green font indicates a shared consensus with the yeast profile for the given position, while red font indicates a divergence between the systems when compared to yeast. The complete list of PPR56 off-targets, along with the RNA editing efficiencies for individual replicates with standard deviation, is given in Table S1.

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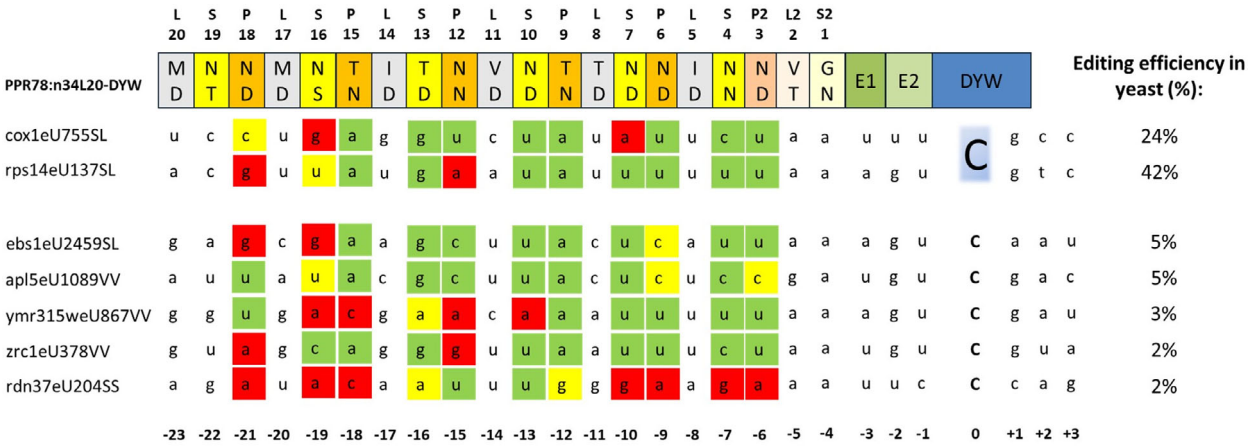


Fig. 9. Off-targets identified for PPR78 in the yeast RNA editing assay setup. Similar to PPR56, RNA samples of yeast with PPR78 expressed with the 6xHis-MBP tags were sent out for Next Generation Illumina Sequencing. The PPR motifs of PPR78 with their respective amino acid identities at Position 5 (above) and last (below) that mediate binding, and the RNA sequences of the native cox1eU755SL and rps14eU137SL are juxtaposed on top, aligning target Position −4 with pentatricopeptide repeat (PPR) motif S2-1GN, with the DYW domain positioned over the editing site (designated as Position 0). Only five off-targets could be identified for PPR78 in the yeast background transcriptome, as shown in the figure. The highest edited off-target identified for PPR78 was ebs1eU2459SL, with an editing efficiency of 5%. The complete list of PPR78 off-targets, along with the RNA editing efficiency for individual replicates (with standard deviation) has been provided in Table S1.

even be matches with the native targets in Positions −5 to −2 that result in rdn37eU204SS among the off-targets given its otherwise very inferior matches of positions opposite of the upstream PPRs. As in the case of PPR56 (Fig. 8), we otherwise observe mostly expected nucleotides opposite of purine-selecting PPRs, A-18 juxtaposed with P-15TN, G-16 with S-13TD, and A-12 with P-9TN. Again, the pyrimidine-selecting PPRs seem to have an inferior role and those placed at the amino-terminal end of the PPR array even more so.

The TargetScan module of the PREPACT software [39] was used to identify candidate off-target sites in the yeast transcriptome, most of which were ultimately not found to be edited (Fig. 10). Upon selecting for the top 40 hits for the provided weight matrices for both PPR56 and PPR78, the resulting data were compared with the set of confirmed off-targets in our transcriptome analyses (Table S4). Only eight detected off-targets featured in this list of 40 best off-target candidates for PPR56, with the target rho3eU126PP (editing of 4.81%) featuring as the top-scoring hit. The highest edited PPR56 off-target predicted by the scanning tool was urb2eU830TI (editing 30.51%), which placed at Position 6 of the TargetScan ranking table (Fig. 10). The highest edited yeast PPR56 off-target (rmt2eU1037SL, 41% editing) did not feature among the top 40 hits, surprisingly. All the more flagrant was the conspicuous lack of confirmed PPR78 off-targets altogether among the top 40 predicted candidates (Table S4), again confirming the impact of numerous factors beyond summing up individual PPR-RNA interactions for predicting RNA editing targets.

Discussion

RNA editing is present in fungi, but this is exclusively of the adenosine-to-inosine (A-to-I) type [40–43], whereas no evidence exists for plant-type cytidine-to-uridine editing. Likewise, 'classic' P-type PPR proteins do exist in yeast [21], but plant-like PPR proteins of the PLS-type characteristic of plant C-to-U RNA editing factors are absent in fungi, including yeast.

The new yeast-based assay system to investigate plant RNA editing reported here lends strong support to the idea that the core plant RNA editing factors act independently from their cellular environment, at least in their likely ancestral form of PLS-type PPR proteins equipped with a terminal DYW-type cytidine deaminase domain. We here arbitrarily chose yeast strain THY.AP5 and expression vector YEp351 for setting up the yeast assay system, simply based on the fact that these were successfully employed earlier in unrelated scientific work in our group [24,44]. We assume, however, that analogous setups can easily be established using other yeast strains or expression vectors.

On the contrary, details of construct cloning and experimentation do evidently matter. The notable



Fig. 10. PPR56 weight matrix and candidate off-targets identified by the TargetScan module of PREPACT. Each column of the weight matrix corresponds to the associated pentatricopeptide repeat (PPR) motif and is labeled as follows: (1) type of motif, (2) position, counting backward from the S2 motif, (3) fifth and last amino acids of the PPR or (4) as EdS, for Editing Site. The given nucleotide weightages have been set in accordance with the PPR-RNA binding code [3,17]. The weightage for each possible nucleotide for the given amino acid combination is also depicted in the weight matrix. The top 10 candidate off-targets identified by the TargetScan module have been shown below and have been labeled as follows: name of the transcript, eU to indicate editing C-to-U and position of the putative edit followed by a * to indicate that the target in question is simply a candidate off-target, and not an actual off-target. Two of the identified off-targets of PPR56, rho3eU126PP, and urb2eU830TI, with editing efficiencies of 5 and 31%, are among the top 10 and highlighted in green. Background shading for the targets indicates matches (green), transition-mismatches (yellow) and transversion mismatches (red, none present for the predicted targets) according to the PPR-RNA code concept [3,17].

examples are the superiority of an inducible setup over constitutive gene expression as here reflected by the inducible GAL1 over the constitutive ADH1 promoter (Fig. 2). This could be caused by the off-target editing or RNA binding resulting from the expression of RNA editing factors that may cause impeded protein functions or RNA turnover in some cases. Another contributing factor could be the strength of the inducible GAL1 promoter, considered to be one of the strongest inducible yeast promoters, vs. that of the

constitutive ADH1 promoter [26,27]. Notably, while constitutive expression was at least principally possible in yeast (Fig. 2), no stable transgenic plant lines expressing editing factors in the cytosol of *Physcomitrium patens* could be raised at all [25]. In that context, it should be remembered that RNA editing factors are very lowly expressed in plants with *ca*. only one protein per organelle [45] and that heterologous systems for *in vivo* analysis of RNA editing should accordingly not necessarily aim for high expression in the first place.

Somewhat surprisingly, we found that the MBP proved very much superior as an N-terminal protein tag for yeast expression (Fig. 3). This observation is slightly different from our findings for expression in human cell lines and in the cytosol of *P. patens* where the effects of different protein tags were less dramatic [20,25]. In the cytosol of *P. patens*, even the tag-free version of PPR56 fully edited its *nad4* target [25].

Equally interesting is the question, which molecular features favor the expression of PPR56, PPR65, and PPR78 in the new yeast system (Fig. 4) and the prokaryotic setup in *E. coli* alike while we failed to functionally express other *Physcomitrium patens* editing factors despite their analogous makeup (Fig. 5). It can now be safely assumed that the results are unlikely caused by features of the prokaryotic or eukaryotic hosts for heterologous expression but more likely in the details of providing proteins and targets in molecular cloning. With respect to the latter, it now becomes ever more likely that RNA sequences far beyond the region ultimately targeted by the PPR array can matter significantly [30,35]. For example, it now seems likely that the high efficiency of RNA editing of PPR78 at the cox1eU755SL site *in planta* could result from the complex turnover of the long native *cox1* transcript with processing of its multiple introns in mitochondria [46,47], whereas rps14eU137SL as the second and 'simpler' target of PPR78 is favored in the heterologous setups providing only shorter target environments (Fig. 4).

Moreover, on the PPR side, numerous studies and experimentations have shown that both the E domains connecting the PPR array and the catalytic DYW domain [48] and the DYW domain itself contribute toward discriminating between nucleotides, thus influencing PPR-RNA binding, and by extension, editing [33,48–49]. For instance, the E domains of the angiosperm DYW-type PPR protein CRR2 were found to play a significant role in RNA binding. The protein in question, however, is not an RNA editing factor but is involved in the stabilization of the transcripts of the chloroplastic *rps7* and *ndhB* genes [50]. The role of the E1 and E2 domains in contributing to the PPR-RNA binding code is as yet unclear—but PPR protein chimeric studies have shown their importance, along with the DYW domain, in discriminating between different RNA targets [28,34]. A recent study with synthetic PPR proteins transiently expressed in *Nicotiana* again confirmed the importance of the binding preferences of the DYW domains as well [51]. Therefore, when it comes to expressing PPR proteins in heterologous systems, not just the PPR-RNA binding code needs to be considered—and until we have a clear understanding of the intricacies of the interaction between protein and RNA, the reason for differing editing efficiencies that are observed between heterologous expression and *in planta* needs to be further evaluated.

Notably though, the new yeast expression system revealed extraordinarily high RNA editing efficiencies for artificial editing factor chimeras having the DYW cytidine deaminase domain of PPR56 replaced with those of others (Fig. 7). Altogether, the new experimentation in yeast reported here is largely congruent with earlier findings in *E. coli* and strongly suggests that plant RNA editing factors, at least in their likely evolutionary ancient state reflected in *Physcomitrium patens*, act independent of their *in vivo* environment and that success for functional heterologous expression depends much more on details of recombinant cloning. A majority of the PPR proteins expressed in this study involved truncations of the N terminus or variable modifications to their native sequence. The functionality of these variants in both yeast and *E. coli* varies, with certain variants ultimately functioning better than others [35]. Thus, the details of recombinant cloning involving not just the choice of vector but also the length of the protein, the combination and choice of the domains and its associated RNA target and position [25,28] seem to be more relevant than the heterologous system chosen.

On the contrary, the establishment of RNA editing in the yeast cytosol as yet another domain of the living world shows that plant RNA editing factors can principally easily move to and become functional in a new cellular environment. This observation fits to explain that certain protists have likely acquired PPR-type RNA editing factors by horizontal gene transfer from plant sources [52–55].

The new yeast system can also be utilized to craft custom-made synthetic PPR proteins, a field which has seen significant advances in recent years [25,51,56]. First speculations regarding the general potential role of PPR proteins in biotechnology arose in 2013 [57,58], and these were expanded upon by Rose

McDowell, Farley Kwok van der Giezen et al. [59,60]. Designed or modified PPR proteins could be used for their RNA-binding properties to help with RNA stabilization, editing, and potentially gene therapy in the fields of agriculture and medicine. While this seems an attractive prospect, doubts revolving at least around the specificity of RNA editing PPR proteins remain, especially given the large off-target datasets unearthed not just in this study, but in preceding studies as well [4,20,25]. The first successful studies of synthetic RNA editing factors that modified specific new positions therefore targeted mitochondrial and chloroplast transcripts, rather than nucleo-cytosolic ones [51]. A recent study, however, reported a successful medically relevant application of a synthetic PPR protein acting on nucleo-cytosolic RNAs. A synthetic RNA-binding PPR protein was engineered to selectively bind toxic CUG-expanded RNAs that cause myotonic dystrophy type 1 (DM1). Delivery in a DM1 mouse model restored normal splicing patterns and produced long-term therapeutic effects on myotonia [61]. Yeast, as an easily accessible alternative eukaryotic model, could play a role in testing synthetic PPR proteins in the future, prior to their application in other model systems like mouse or human cells. Even more attractive, however, might be the design of PPR proteins, that can be used in yeast itself to optimize the production of heterologous proteins for applications in pharmacology, medicine, and the food industry [62,63]. With the ability to produce transcript mixtures through partial editing by PPR proteins, the simultaneous generation of a variety of protein variants might be an interesting future application in yeast for example for antibody or vaccine production.

Materials and methods

Preparation of vectors for PPR protein expression in the selected yeast strain THY.AP5

The *Saccharomyces cerevisiae* strain THY.AP5 is an alpha mating type strain that is associated with the mating-based split ubiquitin system [24,64–65]. The strain carries the auxotrophic selection markers *ade2*-, *his3*-, *leu2*-, and *trp1*-. To take advantage of these markers, specifically the *leu2*-marker, the yeast episomal plasmid YEp351 containing a LEU2 gene, was modified in two ways. First, a GAL1 promoter sequence, a multiple cloning site and a CYC1 terminator sequence were inserted between the *SacI* and *SalI* recognition sites. This modification allowed for the inclusion of the PPR CDS, along with the target by classic cloning. This modified vector was named YEp351GAL1. Second, a similar modification was performed to alternatively insert the ADH1 promoter, a multiple cloning site and an ADH1 terminator to create the vector YEp351ADH1. This allowed for the parallel comparative investigation of an inducible (GAL1) and constitutive (ADH1) expression setup in yeast. The new vector sequences were deposited in the nucleotide sequence database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) under accession numbers PV346738 and PV417277, respectively.

Media used for growth of the yeast culture, as well as for the induction of protein expression in the case of YEp351GAL1, was predominantly selective media, synthetic complete without leucine (SC-Leu)—although a budget approach involving nonselective media in the form of yeast extract peptone dextrose (YPD) and yeast extract peptone galactose (YPGal) was also attempted.

Molecular cloning of tagged PPR proteins and their respective targets

Molecular cloning constructs utilized in this study were based on amplification by a proof-reading Q5 polymerase-based (New England Biolabs GmbH, Frankfurt am Main, Germany) PCR reaction, from plasmids of the *E. coli* expression vector pETG_41k_MCS containing coding sequences of *P. patens* RNA editing factors. These include constructs that have been utilized in previous studies, such as PPR56, PPR65, PPR78, and their corresponding chimeras along with their respective targets [4,20,28,30], and further constructs newly created for the purpose of this study but also tested in the *E. coli* system in parallel, such as PPR71, PPR79, PPR91, and PPR98 [9]. A complete list of oligonucleotide primers is given in Table S5. The ultimate constructs contained a Kozak sequence leading into a start codon, an N-terminal protein tag like MBP, EYFP or TrxA [66–68], most often with an upstream 6xHis tag, the coding sequence of the PPR protein and a small cognate RNA target of 46 or 200 bp in length (Table S1). They were amplified with oligonucleotide primers (Integrated DNA Technologies Europe, BVBA, Leuven, Belgium) containing a 5′-*PmeI* and a 3′-*NotI* overhang (Table S5) for insertion into the yeast episomal plasmids YEp351ADH1 or YEP351GAL1, respectively. To generate further PPR protein chimeric constructs, overlap extension PCRs, with primers complementary to the respective fragments of the PPR proteins to be combined (Table S5), were performed to combine the different segments of the PPR proteins.

Transformation of plasmids containing PPR protein constructs into yeast cells

Transformation of the expression vectors YEp351ADH1 or YEp351GAL1 containing the respective constructs into yeast was performed via the high-efficiency lithium acetate

transformation protocol [69]. *S. cerevisiae* THY.AP5 cells were freshly plated onto YPD medium (1% yeast extract, 2% tryptone, 2% agar, and 2% glucose) and incubated at 30 °C for 3 days. Yeast colonies were used to prepare chemically competent cells, which were either stored using a frozen competent cell (FCC) solution (5% v/v glycerol, 10% v/v DMSO) or used immediately for transformation by addition of a transformation mix of 260 µL 50% (w/v) PEG 3350, 36 µL LiOAc (1 M), 50 µL salmon sperm carrier ssDNA (2 mg·mL^{-1}), 14 µL plasmid DNA (600 ng). The transformed cells were plated on selection plates comprised of media supplemented with all amino acids except leucine (1% yeast nitrogen base with ammonium sulfate, 2% glucose, 2% agar, and 0.2% leucine-deficient amino acid drop-out media) to utilize the *leu2* gene present in the transformed plasmids. The plates were placed at 30 °C for 3 days, following which they were checked for the presence of colonies.

Expressing plant RNA editing factors in *S. cerevisiae*

A colony PCR, using GoTaq DNA polymerase (Promega, Madison, WI, USA), was performed on the yeast colonies obtained via transformation, to confirm the presence of the construct. A minimum of three correct independent colonies (three replicates) per construct were incubated overnight for 14–16 h at 30 °C, 200 rpm in selective SC-Leu media supplemented with 2% glucose. Five hundred microliters of the overnight cultures were then transferred to culture flasks containing 25 mL of SC-Leu + Galactose + Zinc for constructs inserted into the YEp351GAL1 vector, to induce expression (Fig. 1). The flasks were then incubated at 30 °C and 200 rpm until an OD_{600} value of 0.55–0.6 was reached, and used for harvest thereafter. In the case of the YEp351ADH1 constructs, three independent colonies were incubated in 8 mL of culture media consisting of SC-Leu + Glucose + Zinc, for 12–14 h at 30 °C, 200 rpm. Following measurement of OD_{600} to ensure values between 0.55 and 0.6, samples were then harvested (Fig. 1). Yeast culture samples were harvested in volumes of 1.5 mL for RNA extraction and 2 mL for protein extraction, respectively, and pelleted by centrifugation at 10 000 ***g*** for 5 min and yeast pellets were stored at −70 °C.

Determining C-to-U RNA editing efficiencies

To check for RNA editing of the co-expressed target, a total RNA extraction kit (BLIRT; Biolab Innovative Research Technologies, Gdansk, Poland) was used to obtain RNA from the harvested cell pellets. Before proceeding with the kit-based extraction, a modified RNA preparation protocol for yeast cells, including a short 'heat-shock' based cell rupture strategy, was started by placing the frozen cell pellets at 37 °C for 20 min. Subsequently, 20 µL of the enzyme Zymolyase [70] was used to rupture the yeast cell wall via a short incubation at 30 °C for 25 min, followed by brief vortexing.

Following the RNA extraction, a DNaseI (Thermo Fisher Scientific GmbH, Dreieich, Germany) treatment was performed to eliminate the possibility of potential DNA contamination. Reverse transcription was performed using oligo-dT or random-hexamer primers (Carl Roth GmbH, Karlsruhe, Germany), followed by an RT-PCR with a forward primer binding in the PPR protein coding sequence, a reverse primer binding downstream of the target sequence (Table S5) and the synthesized cDNA as template.

The cDNA-based RT-PCR approach was performed with an amount of 55 ng of cDNA, along with 0.2 µM of a forward and reverse primer flanking the target sequence in question. The assay also contained the recommended amount of PCR buffer, double-distilled water, dNTPs (0.2 mM), and GoTaq DNA polymerase (1 U, Promega) to make up a final volume of 25 µL. The samples were loaded into a thermocycler, with a base amplification program that included an initial denaturation step at 94 °C, followed by a cyclical repetition of 30 s of denaturation at 94 °C, 30 s of annealing at the determined annealing temperature and an elongation step at 72 °C whose duration depended on the construct in question. The number of cycles was set at 35 for all the PCRs for all the constructs utilized in this study, and was followed by a final step of synthesis at 72 °C for 5 min. The product of the PCR reaction was then evaluated via an agarose gel electrophoresis.

After gel-purification (Macherey Nagel GmbH & Co. KG, Düren, Germany) and subsequent Sanger sequencing (Macrogen Europe, Amsterdam, Netherlands), the RNA editing efficiency of each construct was assessed as the ratio of the height of the thymidine peak to the sum of the thymidine and cytidine peaks found at the respective editing positions using BioEdit 7.2.5 [71]. Editing efficiencies were represented in percent with standard deviations calculated for each construct from at least three experimental replicates (Table S1).

Confirmation of protein expression in yeast and *E. Coli*

Protein expression was assayed by SDS/PAGE. The harvested *E. coli* culture pellets were first dissolved in 2× Laemmli buffer (4% SDS, 20% glycerol, 0.005% bromophenol blue, 0.125 M Tris-Cl, pH 6.8 supplemented with 10% DTT), vortexed briefly, and placed at 98 °C for 10 min. Following this, the samples were centrifuged at 11 000 ***g*** for 5 min. In the case of yeast cells, the harvested pellets were initially subjected to an alkaline lysis pretreatment, which involved the addition of 100 µL of 0.2 M NaOH [72] following which the cells were resuspended via vortexing and incubated at room temperature for 10 min. Subsequently, the samples were centrifuged at 11 000 ***g*** for

5 min. The pellets thus obtained were dissolved in yeast sample buffer [2% SDS, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.06 M Tris–HCL, pH 6.8, 0.0025% bromophenol blue], vortexed briefly, and placed at 98 °C for 10 min [73]. The samples were then centrifuged at 11 000 ***g*** for 5 min. Standardized amounts were calculated for loading both uninduced and induced samples onto SDS/PAGE gels. The resulting gels were then stained using Coomassie blue and protein expression was confirmed via the observation of a protein band at the desired height.

Western blot analysis

Harvested *E. coli* and yeast cell culture pellets were used to run an SDS/PAGE as detailed above. A higher amount of cell culture, corresponding to 3.6×10^7 cells, was loaded for the yeast samples as opposed to the 1.2×10^7 cells used for the SDS/PAGE. A volume corresponding to 4.5×10^7 cells was loaded for the *E. coli* western blot, similar to the amount used for the Coomassie stained SDS-PAGE, detailed above. Following SDS/PAGE, the proteins were transferred to a nitrocellulose membrane by semi-dry electroblotting using the Trans-Blot SD Semi-dry transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Test staining with Ponceau S solution (0.1% w/v Ponceau S, 5% v/v Acetic acid) was used to confirm protein transfer, after which the membrane was transferred to a blocking solution (1× TBS—50 mM Tris, 150 mM NaCl, 4% milk powder) for 1.5 h. The blocked membrane was washed with TBS-T (1× TBS, 0.1% v/v Tween 20) three times for 10 min each and then incubated with the respective anti-His peroxidase conjugated antibody (His Antibody, HRP—Miltenyi Biotec, Bergisch Gladbach, Germany), diluted 1 : 2000 in TBS, for 60 min with regular shaking at room temperature. Washing with TBS-T was repeated to remove excess antibody, and the membrane was incubated with a 1 : 1 Luminol mixture (Clarity Western ECL substrate; Bio-Rad Laboratories) for 5–15 min. The chemiluminescence was imaged using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories).

RNA sequencing and off-target identification

Total RNA sequencing was performed for 6xHis-MBP-PPR56 and 6xHis-MBP-PPR78 to detect off-targets in the yeast transcriptome. Culture pellets obtained from experimentation conducted in selective media and stored at −70 °C were utilized for RNA preparation (BLIRT, Biolab Innovative Research Technologies). A DNase I treatment was performed to remove any potential DNA contamination. The samples were outsourced to Novogene (Cambridge, UK) for library preparation (via PolyA enrichment) and RNA sequencing (Illumina sequencing) for a minimum of three replicates (Table S2).

RNAseq raw data in the FastQ file format were subjected to quality checks to evaluate the presence of low-quality sequences using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adaptors and low-quality sequences were removed via a twofold method, first using Trimmomatic v.0.39 [74] with the options ILLUMINACLIP:2:30:10, LEADING:20, TRAILING:20, SLIDINGWINDOW:4:24, and MINLEN:100. Further adaptor sequences were removed using BBDuk of the BBtools suite (BBMap - Bushnell B. - sourceforge.net/projects/bbmap/) with default settings.

The samples sent for sequencing included three RNA replicates each from experiments involving constructs containing the *P. patens* editing factors PPR56 and PPR78, expressed under the inducible GAL1 promoter along with 6xHis and MBP protein tags. The transcriptomic data for the yeast strain S288C were used as the reference for mapping (*Saccharomyces cerevisiae* Genome assembly R64, GCA_000146045.2). Furthermore, two replicates of the vector YEp351GAL1, containing only a 6xHis-MBP sequence, were used to eliminate false positives. The trimmed reads were mapped individually to the reference transcriptome using bbmap.sh, with the default options outu/outm, maxindel = 200 k, mdtag = true, sam = 1.4 and pairedonly = true. The samtools view option of the samtools suite [75] was used to convert the SAM output files to BAM files for use in SNP calling. The mapped reads were also deduplicated (samtools fixmate and samtools markdup) and indexed (samtools index). SNPs identified across the three replicates and between the provided reference transcriptome were called via JACUSA v2.0.2, using the call-2 option (Table S3) [76]. The call-2 option was used to input the reference and sample reads in simultaneously (Options: -m 3; -q 25; -T 1.56; -a H:condition = 1,M,B,Y and -f V). Only SNPs that were present in all three replicates, but not in the two reference 6xHis-MBP vector replicates or in the three replicates of the respective other PPR protein, were considered as definitive off-target candidates. Furthermore, only reads with a coverage of at least 20 reads in both the RNA replicate and the reference sample were considered. Only C to T transitions in SNPs having a clean cytidine background of > 90% were further evaluated for RNA editing. An RNA editing efficiency of 1.5% was determined as the minimum value for filtering the remaining SNPs. The identified off-target sequences were then evaluated manually via a combination of MySQL and Microsoft Excel. Off-target sequences with 17 nucleotides upstream of the target editing site, and 3 nucleotides downstream were then manually extracted (Table S2). Labelling of off-targets was based on the editing site nomenclature established earlier [37,38]. Total RNA editing for the selected off-target sequences was presented as the ratio of edited reads to the total RNA reads obtained for the given editing position.

Detection and identification of candidate off-targets

The reference yeast S288C transcriptome was scanned using the TargetScan tool of PREPACT [38] to identify putative candidate RNA editing off-targets for both PPR56 and PPR78 by applying weight matrices based on the PPR-RNA binding code [3,17]. The respective 40 top-scoring candidate RNA editing sites were evaluated against the experimentally confirmed off-targets.

Use of BioRender

Assets from the illustration tool BioRender (https://www.biorender.com/) were used to partly create the graphical abstract [figure partially created in BioRender (2025). https://BioRender.com/67g34zp] as well as Fig. 1 [figure partially created in BioRender (2025). https://BioRender.com/jiydotb], and publication licenses have been obtained for the same.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SR, VK, and MSR initially conceptualized the study. SR did most of the experimentation with significant contributions by AH and JX. SR created figures and VK wrote the manuscript, which was edited and approved by all co-authors.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/febs.70352.

Data availability statement

The data that support the findings of this study are available in the Tables S1–S5. Transcriptome-wide RNA-seq data have been uploaded and made openly available at the Sequence Read Archive (SRA) under BioProject accession number PRJNA1346093. The modified episomal plasmid vectors used in this study can be found under accession numbers PV346738 and PV417277.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Detailed list of constructs investigated for RNA editing in the new yeast model system and in the previously established *E. coli* system.

Table S2. List of identified off-targets for PPR56 and PPR78 in yeast RNAseq transcriptome datasets.

Table S3. Summary of RNA-seq datasets (JACUSA outputs) used for off-target analyses.

Table S4. Output of TargetScan analyses used to predict candidate off-targets present in the yeast transcriptome for PPR56 and PPR78.

Table S5. Oligonucleotide primers.