# A Conserved Glutamate Residue in the C-terminal Deaminase Domain of Pentatricopeptide Repeat Proteins Is Required for RNA Editing Activity[*]

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**Background:** Pentatricopeptide repeat (PPR) proteins that are required for RNA editing frequently include a C-terminal DYW deaminase domain.
**Results:** Mutagenesis of a glutamate residue in the conserved deaminase H$X$E motif results in loss of editing activity.
**Conclusion:** The glutamate residue is required for editing.
**Significance:** The DYW deaminase domain of PPR proteins has the molecular characteristics of a deaminase.

Many transcripts expressed from plant organelle genomes are modified by C-to-U RNA editing. Nuclear encoded pentatricopeptide repeat (PPR) proteins include an RNA binding domain that provides site specificity. In addition, many PPR proteins include a C-terminal DYW deaminase domain with characteristic zinc binding motifs (C$XX$C, H$X$E) and has recently been shown to bind zinc ions. The glutamate residue of the H$X$E motif is catalytically required in the reaction catalyzed by cytidine deaminase. In this work, we examine the activity of the DYW deaminase domain through truncation or mutagenesis of the H$X$E motif. OTP84 is required for editing three chloroplast sites, and transgenes expressing OTP84 with C-terminal truncations were capable of editing only one of the three cognate sites at high efficiency. These results suggest that the deaminase domain of OTP84 is required for editing two of the sites, but another deaminase is able to supply the deamination activity for the third site. *OTP84* and *CREF7* transgenes were mutagenized to replace the glutamate residue of the H$X$E motif, and transgenic plants expressing *OTP84-E824A* and *CREF7-E554A* were unable to efficiently edit the cognate editing sites for these genes. In addition, plants expressing *CREF7-E554A* exhibited substantially reduced capacity to edit a non-cognate site, *rpoA* C200. These results indicate that the DYW deaminase domains of PPR proteins are involved in editing their cognate editing sites, and in some cases may participate in editing additional sites in the chloroplast.

RNA editing takes place in most land plant chloroplasts and mitochondria (1, 2). In flowering plants, the transcripts of chloroplasts and mitochondria are modified post-transcriptionally by C-to-U editing with about 35 C-to-U editing events in chloroplasts and hundreds of editing sites in the mitochondria (3).

Editing in higher plants and in *Physcomitrella patens* is known to require nuclear proteins (4–8).

Pentatricopeptide repeat (PPR)[2] genes have been shown to be required for RNA editing (9), and form a large family of protein-coding genes in higher plants with over 400 members in *Arabidopsis* (10). The known editing factors are members of the PLS subfamily of PPR proteins, which are composed of characteristic P, L (long), and S (short) repeats (11). Amino acid residues located in specific locations within the repeats have been shown to specify the base recognized in the *cis*-element (12–14), and the PLS repeat domain interacts with specific nucleotides within the *cis*-element to provide site specificity for RNA editing (12, 15).

The PLS subfamily of PPR proteins also includes characteristic C-terminal domains known as the E, E+, and DYW domains (10). Bioinformatics analysis identified characteristic structural motifs present in part of the E domain, the entire E+ domain, and most of the DYW domain that place the protein in the deaminase superfamily (16). This region has been identified as the "DYW family of nucleic acid deaminases" (Pfam 14432) (17) and is referred to as the "DYW deaminase domain" in this work.

There is mounting evidence that supports the role of the DYW deaminase domain as the catalytic component of the editing reaction. This region has canonical zinc binding motifs (H$X$E, C$XX$C) (18–20), which are conserved in deaminases that act on nucleotides, RNA, and DNA (16, 21–26). The DYW deaminase domain has recently been shown to bind zinc ions (19, 20). In addition, mutagenesis of the zinc binding motifs has been shown to interfere with editing in transgenic plants (20) and through transient expression in protoplasts (27). The glutamate residue of the H$X$E motif has been shown to be directly involved in the *E. coli* cytidine deaminase mechanism through deprotonation of the substrate water molecule and transfer of the proton to the product ammonia (21). Thus, the requirement of this conserved glutamate residue is a key characteristic expected for an editing deaminase.

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[2] The abbreviation used is: PPR, pentatricopeptide repeat.

The editing of the *ndhD* C2 site in *Arabidopsis* chloroplasts requires two PPR proteins, CRR4 and DYW1 (28). CRR4 lacks the entire DYW deaminase domain but has a canonical PLS repeat region and is apparently required for editing as a site specificity factor (29). DYW1 has an intact DYW deaminase domain; however, the N-terminal PLS-like region is small and composed of degenerate repeats. Furthermore, DYW1 has canonical zinc binding motifs and has been shown to bind zinc ions (19, 20). Mutagenesis of the zinc binding motifs and the catalytic glutamate residue of DYW1 ablated that ability to edit the *ndhD* site (20). Thus, the editing of the *ndhD* site requires both CRR4 and DYW1, and the functions of site recognition and deamination appear to be separated into two proteins in this case.

Many PPR proteins that are required for editing lack a DYW deaminase domain. In addition, there are several examples in which the DYW deaminase domain is present in a PPR, but may be eliminated by truncation and still support editing of the cognate sites (19, 30, 31). Finally, PPR genes have been shown to undergo truncation in evolution (19), indicating that the DYW deaminase domain is dispensable in an evolutionary context. Thus, the role of the DYW deaminase domain in these editing reactions has remained elusive.

In this work, we have investigated the role of the DYW deaminase domain of two PPR proteins, OTP84 and CREF7. In contrast to DYW1 and CRR4, both of these PPR proteins have canonical PLS repeat regions and an intact DYW deaminase domain. In this work, we examine the function of the DYW deaminase domain by gene truncation and mutagenesis of the catalytic glutamate residue and show that the DYW deaminase domain is required for editing the cognate editing sites.

## EXPERIMENTAL PROCEDURES

*Plant Materials*—*Arabidopsis* T-DNA lines SALK_078415C (*cref7-1*), SALK_120902C (*otp84-2*), and SALK_142061C (*otp84-3*) were obtained from the *Arabidopsis* Biological Resource Center. Seeds for *Arabidopsis* ecotype Columbia (Col-0) were purchased from Lehle Seeds (Round Rock, TX) and were used for the wild type line. Transgenic plant lines were produced by introducing the following genes: *OTP84 (*At3g57430); *OTP84-trcDYW* (*OTP84* truncated after residue Phe-770), *OTP84trcPG* (*OTP84* truncated before the PG box after residue Lys-754); *OTP84-E824A* (Glu-to-Ala substitution at residue 824); and *CREF7 (*At5g66520) and *CREF7-E554A* (Glu to Ala substitution at residue 554).

*Gene Cloning and Plant Transformation*—Gene sequences from *OTP84*, *OTP84trcPG* (Met-1–Lys-754), and *OTP84trcDYW* (Met-1–Phe-770) were amplified by PCR to introduce 5′ BamHI and 3′ SalI restriction sites. Sequences for *CREF7* were amplified with flanking BglII and SalI restriction sites. Mutations were introduced by amplification with primers that altered codon Glu-824 of *OTP84* and Glu-554 of *CREF7* to alanine codons. All gene fragments were cloned into pCHF1 using BamHI and SalI restriction sites (32). Binary vectors were electroporated into *Agrobacterium* strain ASE (33), and plants were transformed by floral dip (34). Seedlings were selected using 100 mg/liter gentamicin.

*RNA Editing Analysis through Bulk Sequencing*—Total RNA was isolated from green leaves using RiboZol from AMRESCO (Solon, OH). The GoScript reverse transcription system (Madison, WI) was used with random hexamers from Thermo Fisher to create a cDNA pool. Gene-specific primers were used to amplify sequences including editing sites from the cDNA templates with DreamTaq Green DNA polymerase from Thermo Fisher. Bulk sequencing of RT-PCR products was carried out at the University of California, Berkeley DNA Sequencing Facility. Peak heights were measured from electropherograms using BioEdit V7.0.9.0 to estimate the extent of RNA editing in RNA templates isolated from leaves.

*Poisoned Primer Extension Editing Analysis*—Poisoned primer extension assays were performed as described previously (35) with one modification. Purified PCR products were treated with FastAP from Thermo Scientific and incubated for 10 min at 37 °C followed by 5 min at 75 °C. This is similar to the Exo-SAP-IT step of the poisoned primer extension assay from the Hanson laboratory (36). The addition of the FastAP step consistently reduced read-through to less than 3%. Oligonucleotide primers contained a 5′-hexachlorofluorescein tag from Eurofins (Ebersberg, Germany). Vent DNA polymerase and acyNTPs from New England Biolabs (Ipswich, MA) were utilized for chain termination reactions. Primer extension products were separated by electrophoresis on 12% polyacrylamide gels with 6 M urea. Gels were covered in plastic wrap and immediately scanned using a Typhoon Trio imager from GE Healthcare (Little Chalfont, UK). The percentage of conversion was calculated from the intensity of bands measured from a gel image using GELQUANT.NET V1.8.2.

*Mass Spectrometry*—The DYW deaminase domain from *ELI1* (At4g37380: Asp-478–Trp-632) was amplified by PCR to add flanking BamHI and SalI restriction sites. A single nucleotide mutation (GAG to GCG) was introduced using a PCR primer to create the E566A mutation. The *ELI1-E566A* gene was cloned into pET28a using BamHI and SalI restriction sites and introduced into *E. coli* strain Rosetta 2 (DE3) pLysS from Novozymes (Bagsvaerd, Denmark). Recombinant ELI1-E566A was expressed as described previously (19) and purified. Native protein samples were dialyzed in 20 mM ammonium acetate prior to mass spectrometry. Denatured protein samples were treated with 50% acetonitrile and 1% formic acid prior to mass spectrometry. Protein samples were ionized with an electrospray ionization voltage of 3.6 kV, a cone voltage of 40 V, and a desolvation temperature of 120 °C. Mass determinations were made with a Waters QTOF2 mass spectrometer.

## RESULTS

*Plants Expressing Truncated OTP84 Edit One of Three Cognate Sites*—Previous studies have examined the role of the DYW deaminase domain by expression of truncated variants, and in several examples, these experiments have demonstrated that the DYW deaminase domain was not required for editing of the cognate sites (19, 30, 37, 38). Because OTP84 has three editing site targets, the role of the DYW deaminase domain was examined on these editing sites.

The *OTP84* gene has an intact DYW deaminase domain (Fig. 1) and is required for editing of *ndhF* C290, *psbZ* C50, and *ndhB*

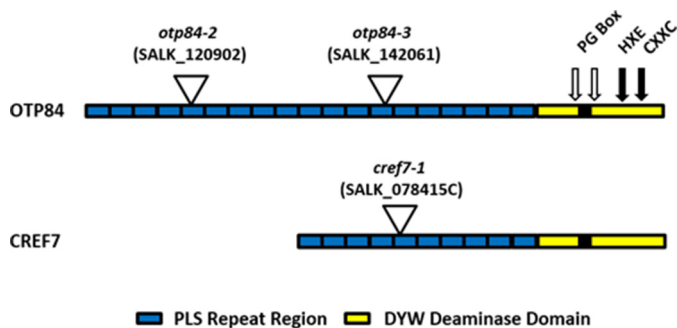# A Glutamate Residue in PPR Proteins Is Essential for RNA Editing



FIGURE 1. **PPR domain architecture for OTP84 and CREF7.** Repeats in the PLS region are indicated by *rectangles*. Features in the DYW deaminase domain include the PG box, which is indicated as the *solid black rectangle*, and the *open arrows* indicate the position of truncations in OTP84. The positions of the zinc binding and catalytic glutamate residues (H*X*E, C*XX*C) are indicated with *solid arrows*. The location of T-DNA insertions is shown at the point of the *triangle*.

C1481 (39). Two truncated variants of OTP84 were each prepared and tested for editing activity in three independent transgenic plants (Fig. 1). Truncation immediately C-terminal to the PG box eliminated most of the DYW deaminase domain (*OTP84trcDYW*), and truncation immediately N-terminal of the PG box eliminated all of the PG box and downstream DYW deaminase domain (*OTP84trcPG*). The truncated variants were introduced into an *OTP84* knock-out background (39). The effects of OTP84 truncation are shown in Fig. 2. Expression of the *OTP84trcDYW* transgene did not restore editing of *ndhF* and *psbZ* transcripts to wild type levels (Fig. 2, *A* and *B*, respectively), suggesting that the DYW deaminase domain is required for editing these two cognate editing sites. In contrast, the editing of *ndhB* C1481 remained at wild type levels in transgenic plants expressing *OTP84trcDYW* (Fig. 2*C*), which suggests that editing of the *ndhB* site does not require the OTP84 DYW deaminase domain. Truncations that eliminate the PG box (*OTP84trcPG*) resulted in highly reduced editing for all three sites. This result is consistent with earlier studies performed with ELI1 that indicated that the PG box is critical for editing a site in *ndhB* transcripts (19). Although OTP84 is required for editing three sites, transgenes lacking a large portion of the DYW deaminase domain were capable of restoring editing to wild type levels in *ndhB* transcripts, suggesting the participation of an unknown DYW deaminase(s) for that site.

*Mutagenesis of the HXE Motif of OTP84 Decreases Editing of the Three Cognate Sites*—The H*X*E motif of *OTP84* was mutagenized to H*X*A to produce *OTP84-E824A*, which was introduced into *OTP84* knock-out line (*otp84-3*) and into the wild type Col-0 background. The conversion of the *ndhF*, *psbZ*, and *ndhB* editing sites is represented in Fig. 3, *A*, *B*, and *C*, respectively. Each editing site was converted at 90% or greater in the wild type plants and remained unedited in *otp84-3*, the *OTP84* knock-out line. Complementation of *otp84-3* with a wild type transgene restored editing to ∼90% or greater. In contrast, the *OTP84-E824A* transgene was unable to restore editing of the three editing sites to wild type levels (Fig. 3). Plants expressing *OTP84-E824A* did not edit the *ndhF* and *psbZ* sites. In contrast, 23% of transcripts were edited at *ndhB* C1481 in plants expressing *OTP84-E824A*.

Because the *OTP84-E824A* transgene was unable to restore editing to the cognate sites, the mutant transgene might act in a dominant negative manner to suppress editing in wild type plants by formation of inactive editing complexes. Therefore, we tested whether expression of the *OTP84-E824A* transgene could disrupt editing in wild type plants. In three independent transgenic lines, the mutant transgene did not reduce editing levels in the wild type background and therefore did not act in a dominant negative manner over the native gene (Fig. 3).

*Mutagenesis of the HXE Motif of CREF7 Decreases Editing of the Cognate Site*—The *CREF7* gene has an intact DYW deaminase domain (Fig. 1) and a single cognate editing site, *ndhB* C1255 (40). Transgenes for wild type *CREF7* and a mutant variant with the H*X*E motif changed to H*X*A (*CREF7-E554A*) were introduced into a *CREF7* knock-out line (*cref7-1*); in addition, the *CREF7-E554A* transgene was introduced into wild type plants. Transgenic plants were analyzed for editing of the cognate site (Fig. 4). The *ndhB* C1255 site was 100% edited in wild type plants, but remained unedited in the *CREF7* knock-out plants. Editing of *ndhB* C1255 in *CREF7* knock-out plants expressing the *CREF7-E554A* transgene was ∼10% based on Sanger sequencing. In contrast, plants with wild type background expressing *CREF7-E554A* edited *ndhB* transcripts at wild type levels, and the transgene did not behave in a dominant negative manner. These results demonstrate that the Glu-554 plays a crucial role in the activity of CREF7 in editing *ndhB* transcripts.

*Expression of CREF7-E554A Reduces Editing of rpoA C200, a Non-cognate Site*—Editing site conversion was determined for the 34 major chloroplast editing sites in plants expressing *OPT84-E824A* and *CREF7-E554A* in their respective gene knock-out backgrounds (data not shown). In several instances, small changes in editing site conversion were detected at noncognate editing sites.

The strongest effect of the mutant transgene was observed on *rpoA* C200 editing in plants expressing *CREF7-E554A* (Fig. 5). The *rpoA* editing site is about 70% converted in wild type Col-0 plants, in *cref7-1* knock-out plants, and in *cref7-1* plants complemented with the wild type *CREF7* gene. The *CREF7-E554A* transgene had no detectable effect when expressed in wild type plants; however, in *cref7-1* knock-out plants, the E554A substitution caused a 25% reduction of *rpoA* editing to about 45%. Thus, expression of *CREF7-E554A* caused a significant reduction of *rpoA* editing, suggesting that CREF7 may participate in editing *rpoA* transcripts.

*The HXA Mutation of the DYW Deaminase Domain Maintains Zinc Binding*—Mutagenesis may cause a protein to lose function because of improper folding, or through a direct effect on catalysis. Previous investigations of the structure and function of *E. coli* cytidine deaminase used circular dichroism and zinc stoichiometry to demonstrate that the polypeptide with the Glu-to-Ala substitution retained native structure (41). The ELI1 DYW deaminase domain was selected for zinc analysis to determine whether the DYW deaminase domain was able to fold into a native structure. A Glu-to-Ala mutation was introduced at position 566 in the ELI1 DYW deaminase domain (19). The recombinant protein was expressed in *E. coli*, purified, and analyzed by mass spectrometry as described previously (19).
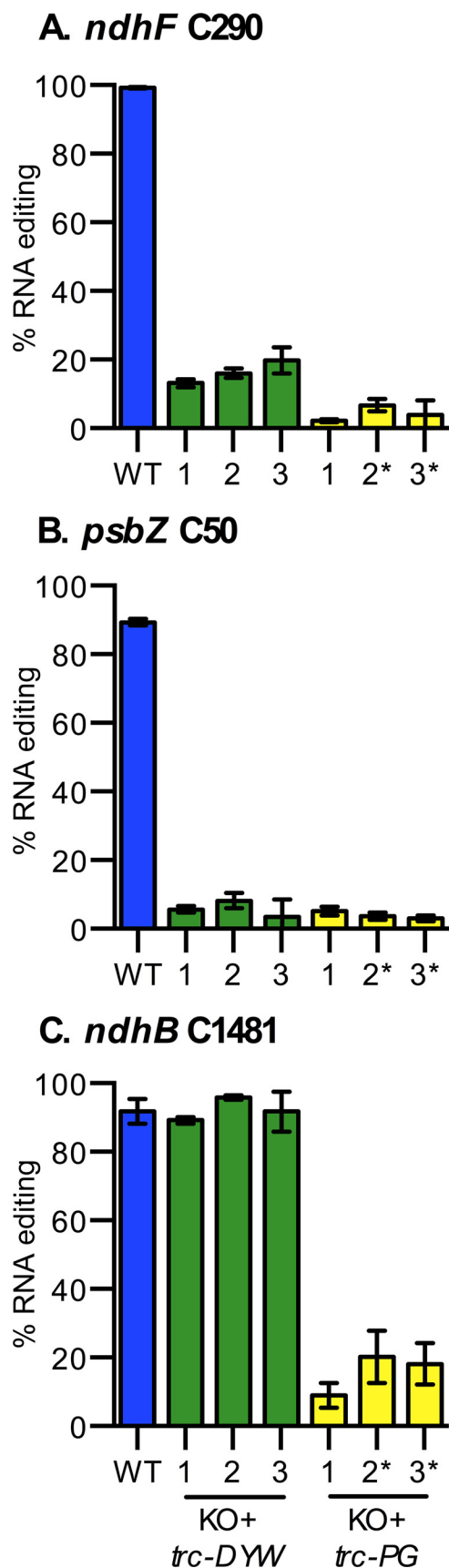
FIGURE 2. **OTP84 truncation shows differential effects on the editing of three cognate sites.** *A–C* show the response of editing *ndhF* C290, *psbZ* C50, and *ndhB* C1481, respectively. Truncation of the DYW domain (*OTP84trcDYW*) resulted in a large decrease in editing of the *ndhF* and *psbZ* sites, but the *ndhB*

The purified recombinant ELI1-E566A protein had a native mass of 19,519 atomic mass units, and the denatured protein had a mass of 19,395 atomic mass units. The mass difference of 124 atomic mass unit is consistent with the native DYW deaminase domain binding two zinc molecules of 65.4 atomic mass unit and the loss of several protons from the cysteine ligands that are predicted to coordinate the zinc atoms (19). This result demonstrates that the ELI1-E566A mutant DYW deaminase domain binds two zinc ions and retains native structure.
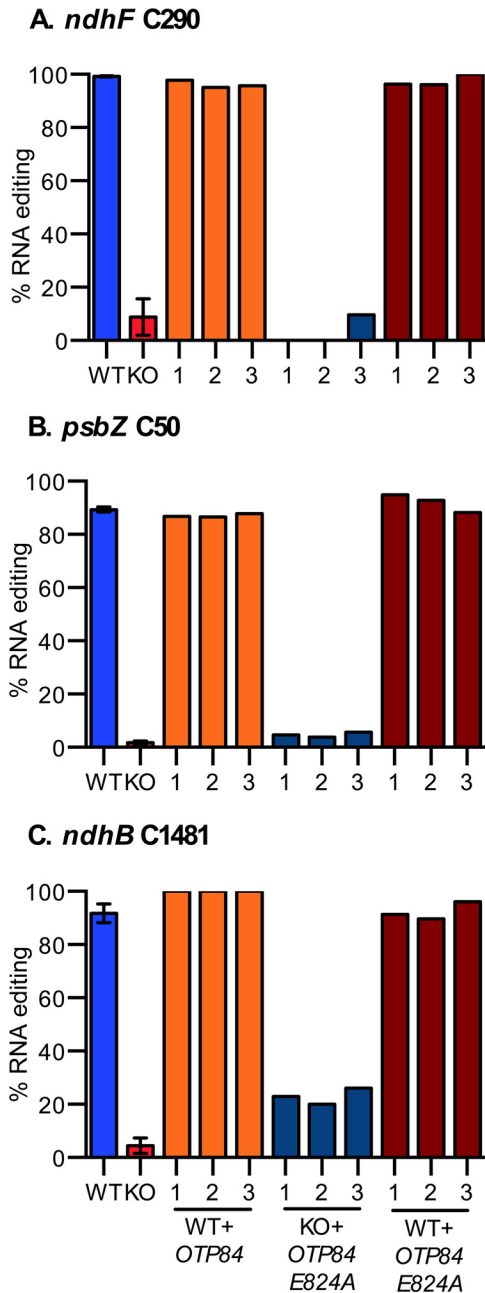
## DISCUSSION

The enzyme responsible for RNA editing in plants has been controversial. Early biochemical analyses indicated that the enzymatic reaction was probably a deamination reaction (42, 43), and *in vitro* editing assays established that the reaction occurred through a cytidine deamination mechanism (44–46). After discovery of the role of PPR proteins in RNA processing reactions in plant organelles (10), deaminase-like zinc binding motifs were recognized in the DYW domain (18). More recently, the DYW deaminase domain was shown to bind zinc as a prosthetic group (19, 20), further supporting the hypothesis that the DYW deaminase functions as the deaminase in plant editing. Mutagenesis of amino acid residues in the zinc binding motifs and of the catalytic glutamate (H*X*E, C*XX*C) of DYW1 resulted in the loss of the ability to edit *ndhD* C2 in transgenic plants (20). Thus, the DYW deaminase domain possesses several features that would be expected for an editing deaminase.

In this work, we explored the role of the glutamate residue of the H*X*E motif in full-length PPR proteins that have a canonical PLS repeat region and an intact DYW deaminase domain. Mutation of the putative catalytic glutamate residue caused a dramatic decrease in the editing of the cognate sites for OTP84 and CREF7. The requirement of the glutamate residue of the H*X*E domain and zinc binding by the DYW deaminase domain are two key features that are expected for an editing deaminase. They strongly support the hypothesis that the DYW deaminase domain provides the catalytic activity for the editing reaction.

There are several examples in which the DYW deaminase is dispensable for complementation of a knock-out mutant phenotype (19, 30, 31, 47). The truncation of OTP84 had markedly different effects on the three editing site targets. The *OTP84trcDYW* transgene restored editing of *ndhB* C1481 to wild type levels, and this site responded similarly to previously characterized editing sites for *ELI1, CRR22, CRR28, OTP82,* and *MEF11* (19, 30, 31, 47). Editing of the *psbZ* and *ndhF* sites was highly reduced in the presence of the *OTP84trcDYW* transgene, although the *ndhF* site may have been somewhat higher than gene knock-out levels. The *ndhF* C290 editing site is also affected by disruption of the PPR gene, *VAC1*, which results in a partial loss of editing of the *ndhF* site (48). The DYW deami-

# A Glutamate Residue in PPR Proteins Is Essential for RNA Editing

## A. *ndhF* C290



## B. *psbZ* C50



## C. *ndhB* C1481



FIGURE 3. **OTP84-E824A does not restore editing to wild type levels in *OTP84* knock-out plants.** *A–C* show the response of editing *ndhF* C290, *psbZ* C50, and *ndhB* C1481, respectively. Wild type *OTP84* complemented the knock-out phenotype in the *OTP84* knock-out line; however, *OTP84-E824A* did not increase editing of the *ndhF* or *psbZ* editing sites. Editing of the *ndhB* editing site was significantly greater (23%) over the level observed in the knock-out line (5%). A single leaf sample was analyzed from three independent wild type (*WT*) and *otp84-3* knock-out (*KO*) plants. A single leaf sample was analyzed from each of three independent integration events for these backgrounds and transgenes: wild type plants with *OTP84* transgene (WT + *OTP84*); *otp84-3* with *OTP84E824A* (KO + *OTP84 E824A*); and wild type expressing *OTP84E824A* (WT + *OTP84 E824A*). *Error bars* show the S.D. of the three leaf samples from three independent plants in the case of WT and KO.

nase domain of VAC1 might participate with OTP84trcDYW to incompletely edit the *ndhF* C290 site. These results suggest that a set of editing sites can share a single specificity factor, but might rely on different deaminases in *trans* to complete the editing reaction.
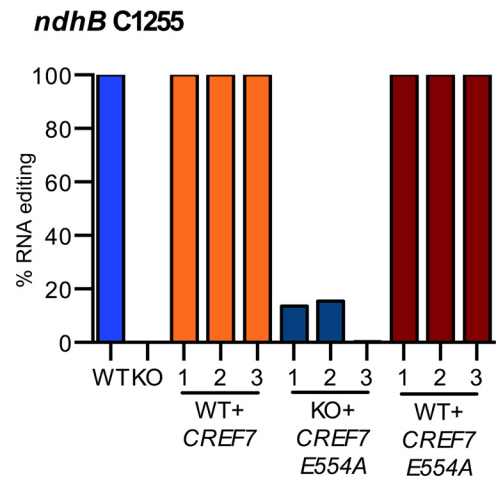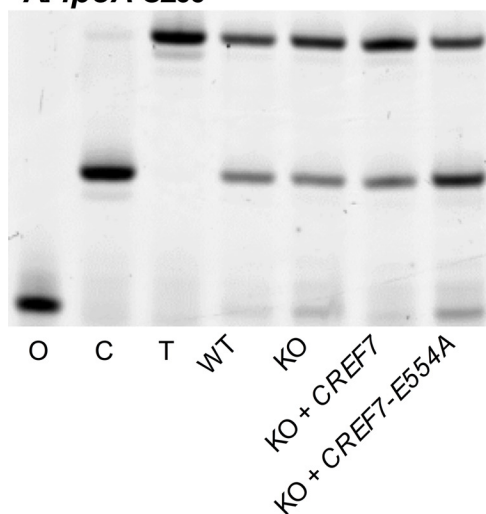
## *ndhB* C1255



FIGURE 4. **CREF7-E554A does not restore editing in *CREF7* knock-out plants.** Wild type *CREF7* complemented the knock-out phenotype when expressed in the *CREF7* knock-out line; however, *CREF7-E554A* only increased editing of *ndhB* C1255 to about 10%. A single leaf sample was analyzed from three independent wild type (*WT*) and *cref7-1* knock-out (*KO*) plants. A single leaf sample was analyzed from each of three independent integration events for these backgrounds and transgenes: wild type plants with *CREF7* transgene (WT + *CREF7*); *cref7* with *CREF7-E554A* (KO + *CREF7-E554A*); and wild type expressing *CREF7-E554A* (WT + *CREF7-E554A*).

Editing was highly reduced at the three cognate sites of OTP84 in plants expressing the *OTP84-E824A* transgene. The editing of the *psbZ* and *ndhF* sites was near gene knock-out levels, and the OTP84-E554A protein may form non-functional editing complexes for these sites. The editing of the *ndhB* C1481 site responded somewhat differently, and editing of the *ndhB* site increased well above knock-out levels to about 23%. The *ndhB* site also responded differently from the other two cognate sites in plants expressing *OTP84trcDYW*, and the increase in *ndhB* editing might result from the participation of other deaminases in the editing of this site.
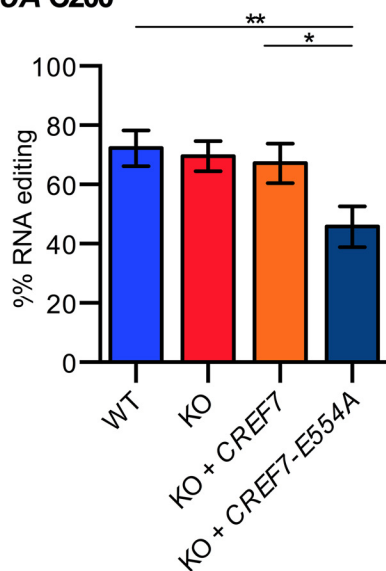
Expression of *CREF7-E554A* substantially decreased editing of *rpoA* C200, a cognate editing site for CLB19 (49). Because CLB19 is truncated at the end of the E domain and lacks an intact DYW deaminase domain (49), it could recruit a deaminase domain from another PPR protein. The reduction in editing of a non-cognate site suggests that CREF7 may participate as a deaminase in *rpoA* C200 editing. Because editing of *rpoA* C200 is not reduced in the *CREF7* knock-out line (Fig. 5), there may be other DYW deaminases that participate in *rpoA* editing.

Nineteen PPR proteins have been shown to be required for editing in *Arabidopsis* chloroplasts. Four of these PPRs (CLB19, CRR4, CRR21, and OTP80) have truncated DYW deaminase domains (37, 39, 49) and could not function in C deamination. A complex network of PPR proteins could be involved in RNA editing. The PLS repeat domain may be sufficient for specifying an editing site; however, one or more deaminases could be recruited in *trans* as enzymatic components. Table 1 summarizes the relationships between several chloroplast editing sites, site specificity factors, and possible PPR proteins involved in the deamination reaction. PPR proteins that lack complete DYW deaminase domains may acquire the activity from other proteins as in the case of CRR4 and DYW1. Based on these results, OTP84 acts as a site specificity factor for three cognate sites and participates as a deaminase along with other PPR pro-

## A. *rpoA* C200



O  C  T  WT  KO  KO + CREF7  KO + CREF7-E554A

**TABLE 1**

**Possible interactions of PPR proteins in editing site recognition and deamination reactions**

| Editing site | Site specificity factor | Putative deaminase |
|---|---|---|
| *ndhD* C2 | CRR4[a] (29) | DYW1[a] (28), DYW1[b] (20) |
| *ndhB* C1481 | OTP84[a] (39) | OTP84[b] (unknown) |
| *psbZ* C50 | OTP84[a] (39) | OTP84[b,c] |
| *ndhF* C290 | OTP84[a] (39) | OTP84[b,c], VAC1[a] (48) |
| *accD* C794 | RARE1[a] (50) | VAC1[a] (48) |
| *ndhB* C1255 | CREF7[a] (40) | CREF7[b] |
| *rpoA* C200 | CLB19[a] (49) | CREF7[b] (unknown) |

[a] Gene knockout exhibits reduced editing.
[b] Glu-to-Ala mutant transgene incapable of restoring editing in knockout line.
[c] Transgene with truncation removing DYW deaminase incapable of restoring editing in knockout line.

editing reactions, these proteins have partially redundancy. The partial reduction in editing of *rpoA* C200 by the CREF7-E554A mutant suggests that CREF7 is partially redundant with other deaminases in *rpoA* editing.

Expression of catalytically deficient PPR transgenes by mutation of the H*X*E motif has proven to be a powerful way to distinguish the DYW deaminase function for a PPR protein from the site specificity role. Our results provide direct support for the model that PPR proteins have dual and separable functions as site specificity factors and as deaminases (13, 19, 47).

## B. *rpoA* C200



**FIGURE 5. The *CREF7-E554A* mutation decreases editing of *rpoA*, a noncognate editing site.** *A*, poisoned primer extension assay was used to determine *rpoA* editing site conversion. Control lanes show oligonucleotide (*O*), unedited template (*C*), and edited template (*T*). *B*, editing of *rpoA* was about 70% in wild type and knock-out plants. Expression of *CREF7-E554A* in the CREF7 knock-out line resulted in a decrease in *rpoA* editing to about 45%. Three leaf samples were analyzed from wild type plants (*WT*) and *cref7-1* knock-out (*KO*) plants. A single leaf sample was analyzed from each of three independent integration events for these backgrounds and transgenes: wild type plants with *CREF7* transgene (WT + *CREF7*); and *cref7* with *CREF7-E554A* (KO + *CREF7-E554A*). For statistical analysis, the Student's *t* test was performed with significance indicated as * and ** for >0.05 and >0.005 at the 0.05 level, respectively. *Error bars* show the S.D. of the three leaf samples from three independent plants.

teins in the editing of these sites. CREF7 appears to function as both a specificity factor and a deaminase in the editing of its cognate site, but also contributes to *rpoA* editing with the CLB19 as a specificity factor. VAC1 is a PPR protein with a DYW domain, and the gene knock-out results in a strong lethal phenotype and partial editing of *ndhF* C290 (a cognate site of OTP84) and *accD* C794 (a cognate site of RARE1) (48). Because the *VAC1* knock-out exhibits partial editing, VAC1 is not essential for each site. If other PPR proteins participate in these

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